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# Multifaceted role of SUMOylation in maintaining centromere biology and regulation of replication fork restart in *Schizosaccharomyces pombe*

Shrena Chakraborty

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Multifaceted role of SUMOylation in  
maintaining centromere biology and  
regulation of replication fork restart in  
*Schizosaccharomyces pombe*

Rôle multifacette de la SUMOylation dans le maintien de la biologie du  
centromère et la régulation du redémarrage des fourches de réplication  
chez *Schizosaccharomyces pombe*

**Thèse de doctorat de l'Université Paris-Saclay**

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Directrice de recherche

**Thèse soutenue à Paris-Saclay, le 21 Octobre 2024, par**

**Shrena CHAKRABORTY**

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**Titre:** Rôle multifacette de la SUMOylation dans le maintien de la biologie du centromère et la régulation du redémarrage des fourches de réplication chez *Schizosaccharomyces pombe*

**Mots clés:** SUMO Targeted Ubiquitin Ligase, SUMOylation, centromère, recombinaison homologue, redémarrage de fourches, positionnement nucléaire,

**Résumé:** Les défauts dans le processus de réplication de l'ADN, connus sous le nom de stress de réplication, sont une source majeure d'instabilité du génome qui favorise le développement du cancer. La résolution du stress de réplication se produit dans un noyau compartimenté qui présente des capacités distinctes de réparation de l'ADN. Les fourches de réplication stressées présentent une mobilité accrue et se déplacent vers la périphérie du noyau pour s'ancrer aux complexes du pore nucléaire, une structure hautement conservée de l'enveloppe nucléaire qui agit comme un site d'amarrage pour permettre à d'autres voies de réparation de l'ADN de se mettre en place. Ces changements dans le positionnement nucléaire sont régulés par le métabolisme des petits modificateurs de type ubiquitine (SUMO), qui jouent un rôle essentiel dans la ségrégation spatiale des activités de la voie de la recombinaison homologue (RH). Nos travaux antérieurs chez la levure de fission ont établi qu'une fourche de réplication bloquée par une protéine liée à l'ADN se relocalise et s'ancre au NPC d'une manière SUMO-dépendante. Les chaînes SUMO déclenchent la relocalisation des fourches arrêtées à la périphérie du noyau pour s'ancrer au NPC. Cet ancrage nécessite les chaînes SUMO et la voie de l'ubiquitine ligase ciblée par les SUMO (STUbL) Slx8. Cependant, les chaînes SUMO limitent également la voie de redémarrage de la fourche. Ces conjugués SUMO peuvent être éliminés par la protéase SENP Ulp1 et le protéasome, dont les activités sont enrichies à la périphérie nucléaire. Ainsi, une relocalisation vers les NPCs permet un redémarrage de la réplication dépendant de la RH en contrecarrant la toxicité des chaînes SUMO. La formation de chaînes SUMO et la voie Slx8 étant cruciales pour la relocalisation des fourches de réplifications bloquées au NPC.

Mon projet s'est d'abord attaché à déterminer si Slx8 STUbL pouvait être exploitée en tant que marqueur des chaînes SUMO induites par des dommages à l'ADN.

Pour ce faire, j'ai marqué Slx8 avec une étiquette GFP et j'ai suivi le marquage GFP par microscopie à fluorescence. De manière inattendue, je n'ai pas pu détecter de foyers Slx8 spécifiquement induits par le stress de réplication. Cependant, j'ai découvert que Slx8 forme un foyer nucléaire unique, enrichi à la périphérie nucléaire, qui marque à la fois les centromères groupés au niveau du centre organisateur des microtubules et la région silencieuse du *mating type*. La formation de ce foyer unique de Slx8 nécessite la ligase E3 SUMO Pli1, la poly-SUMOylation et l'histone méthyl transférase Clr4 qui est responsable de la méthylation de l'histone H3K9 qui marque l'hétérochromatine. Enfin, j'ai établi que Slx8 favorise le regroupement des centromères et le *silencing* des gènes dans les domaines de l'hétérochromatine. Dans l'ensemble, mes données mettent en évidence des relations fonctionnelles et conservées au cours de l'évolution entre STUbL et les domaines de l'hétérochromatine pour promouvoir le *silencing* des gènes et l'organisation nucléaire. En outre, j'ai mieux caractérisé les voies de redémarrage des fourches bloquées dans l'espace nucléaire. L'équipe a précédemment établi que les fourches arrêtées nécessitent l'activité d'échange de brins de Rad51 pour être acheminées vers le NPC en vue d'un redémarrage. Dans ce contexte, j'ai dévoilé l'existence d'une voie alternative de redémarrage qui implique la mono-SUMOylation, dans le nucléoplasme en absence de relocalisation au NPC. Ici, je révèle le nouveau rôle de Rad52 dans l'orchestration du redémarrage de la fourche dans le nucléoplasme, un rôle qui implique son activité SSA (single strand annealing).

Pris ensemble, mes résultats suggèrent deux aspects. Une partie souligne comment la SUMOylation régulée par Slx8 STUbL favorise la maintenance du centromère. L'autre partie élucide le "contrôle SUMO" des voies alternatives de redémarrage de fourches résolues dans l'espace nucléaire.

**Title:** Multifaceted role of SUMOylation in maintaining centromere biology and regulation of replication fork restart in *Schizosaccharomyces pombe*

**Keywords :** SUMO Targeted Ubiquitin Ligase, SUMOylation, centromere, homologous recombination, fork restart, nuclear positioning

**Abstract:** Flaws in the DNA replication process, known as replication stress, is a major source of genome instability that fuels cancer development. Resolution of replication stress occurs within a compartmentalized nucleus that exhibits distinct DNA repair capacities. In different eukaryotic organisms, stressed replication forks (RFs) shift to the nuclear periphery for anchorage to the nuclear pore complexes (NPCs), a highly conserved structure in the nuclear envelope that act as docking sites to allow alternative DNA repair pathways to occur. These changes in nuclear positioning is regulated by the small ubiquitin-like modifier (SUMO) metabolism, which is pivotal to spatially segregate the activities of the homologous recombination (HR) pathway. Our previous work in the fission yeast *Schizosaccharomyces pombe*, has established that a replication fork blocked by a DNA-bound protein relocates and anchors to NPC in a SUMO-dependent manner. SUMO chains trigger the relocation of single arrested forks to the nuclear periphery to anchor to the NPC. This anchorage requires the SUMO chains and the SUMO-targeted ubiquitin ligase (STUbL), Slx8 pathway. However, SUMO chains also limit the Recombination-Dependent Replication (RDR) pathway, necessary to promote fork restart. These SUMO conjugates can be cleared off by the SENP protease Ulp1 and the proteasome, whose activities are enriched at the nuclear periphery. Thus, a routing towards NPCs allows HR-dependent replication restart by counteracting the toxicity of SUMO chains.

Since, both SUMO chain formation and the Slx8 STUbL pathway were crucial for NPC routing of arrested replication forks. My thesis project initially focused on unraveling if the Slx8 STUbL can be exploited as a readout of damage-induced SUMO chains. To do so, I tagged Slx8 with a GFP tag and monitored them using the fluorescence microscopy technique. Unexpectedly, I was unable to detect

replication stress-induced Slx8 foci. However, I discovered that Slx8 forms a single nuclear focus, enriched at the nuclear periphery, which marks both clustered centromeres at the spindle pole body and the silent mating type region. The formation of this single Slx8 focus requires the E3 SUMO ligase Pli1, poly-SUMOylation and the histone methyl transferase Clr4 that is responsible for the heterochromatin histone mark H3-K9 methylation. Finally, it was established that Slx8 promotes centromere clustering and gene silencing at heterochromatin domains. Altogether, my data highlight evolutionarily conserved and functional relationships between STUbL and heterochromatin domains to promote gene silencing and nuclear organization.

Additionally, I have better characterized pathways of fork restart within the nuclear space. The team previously established that arrested RFs require SUMO chains and the strand exchange activity of Rad51 for routing to the NPC for subsequent fork restart. In this context, I unveiled the existence of an alternate fork restart pathway that occurs by mono-SUMOylation, in the nucleoplasm when forks do not shift to the NPC, as SUMO chains are not formed. Here, I revealed that fork restart within the nucleoplasm still depends on the strand exchange activity of Rad51 largely, while the single strand annealing (SSA) activity of Rad52 plays an important role in mediating error-prone fork progression in the absence of SUMO chains.

Taken together, my results suggest two different ideas about SUMOylation. One part underscores how Slx8 STUbL-regulated SUMOylation promotes centromere clustering and gene silencing at heterochromatin domains. Whereas, the other section elucidates the "SUMO control" on the spatially segregated, alternative pathways of fork restart within the nuclear space. Therefore highlighting the importance of maintaining SUMO balance for preserving genome integrity.

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# Abbreviations

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<b>Aos1</b>	Activation of Smt3p
<b>APH</b>	Aphidicolin
<b>ATM</b>	Ataxia-Telangiectasia Mutated
<b>ATO</b>	arsenic trioxide
<b>ATPase</b>	Adenosine Triphosphate
<b>ATR</b>	Ataxia Telangiectasia and Rad3-related
<b>ATRIP</b>	ATR-interacting protein
<b>BIR</b>	Break Induced Repair
<b>BLM</b>	Bloom Syndrome RecQ Like Helicase
<b>Bp</b>	base pair
<b>BPDE</b>	benzo(a)pyrene diol epoxide
<b>BRCA (1 and 2)</b>	Breast Cancer
<b>C</b>	cytosine
<b>Cas9</b>	<i>CRISPR</i> associated protein 9
<b>Cc (1, 2 and 3)</b>	Central core
<b>Cdt1</b>	Chromatin Licensing and DNA replication factor 1
<b>Cen</b>	Centromere
<b>CDC (6, 25, 45...)</b>	Cell Division Cycle
<b>CDE</b>	Centromere DNA element
<b>CDK (1 and 2)</b>	Cyclin-dependent kinase
<b>CFS</b>	chromosomal fragile sites
<b>ChIP</b>	Chromatin Immunoprecipitation
<b>CHK (1 and 2)</b>	Checkpoint Kinase
<b>CMG</b>	Cdc45-MCM-GINS complex
<b>CPT</b>	<i>camptothecin</i>
<b>CRISPR</b>	Clustered Regularly Interspaced Short Palindromic Repeats
<b>CtIP</b>	CtBP-interacting protein

<b>Dbf4</b>	<i>Dumbbell former 4 protein</i>
<b>DDK</b>	DBF4-Dependent <i>Kinase</i>
<b>DDR</b>	DNA damage response
<b>DDT</b>	DNA Damage Tolerance pathways
<b>dHJ</b>	double Holliday Junction
<b>D-loop</b>	Displacement loop
<b>dNTPs</b>	deoxyribonucleotides
<b>DNA</b>	deoxyribonucleic acid
<b>DPCs</b>	DNA–protein Cross-link
<b>DRC</b>	DNA Repair Checkpoint
<b>DSB</b>	Double-Strand Break
<b>dsDNA</b>	double-stranded DNA
<b>DSBR</b>	Double-Strand Break Repair
<b>DUB</b>	deubiquitinase
<b>Ebp2</b>	EBNA1-binding protein
<b><i>E.coli</i></b>	<i>Escherichia coli</i>
<b>EM</b>	Electron Microscopy
<b>EME1</b>	Essential Meiotic Structure-Specific Endonuclease 1
<b>ERCC1</b>	Excision Repair Cross-Complementation group 1
<b>ETAA1</b>	Ewing Tumor-Associated Antigen 1
<b>EXO1</b>	Exonuclease 1
<b>FACS</b>	Fluorescence Activated Cell Sorting
<b>Fbh1</b>	F-Box DNA Helicase 1
<b>G</b>	guanine
<b>GEN1</b>	Gen Endonuclease Homolog 1
<b>GFP</b>	Green Fluorescent Protein
<b>GINS</b>	go-ichi-ni-san complex
<b>HDAC</b>	histone deacetylase
<b>HECT</b>	Homologous to the E6AP Carboxyl Terminus

<b>HJ</b>	Holliday Junction
<b>HLTF</b>	Helicase Like Transcription Factor
<b>HR</b>	Homologous Recombination
<b>Hs</b>	Homo sapiens
<b>HU</b>	hydroxyurea
<b>ICLs</b>	Interstrand Crosslinks
<b>IKK</b>	phosphoinositide-3-like kinase
<b>Inr</b>	inner repeats
<b>IR</b>	Ionizing Radiation
<b>Kap (60 and 121)</b>	<i>karyopherins</i>
<b>Kb, Mb</b>	kilo- and mega- base
<b>Ku</b>	Ku70-Ku80 heterodimer
<b>MCM</b>	Minichromosome Maintenance Complex
<b>Mec1</b>	Mitosis Entry Checkpoint 1
<b>MiDAS</b>	Mitotic DNA synthesis
<b>Mlp (1 and 2)</b>	<i>Myosin-Like Proteins</i>
<b>MMS</b>	Methyl-Methanesulfonate
<b>Mms21</b>	Methyl Methanesulfonate sensitivity 21
<b>Mot1</b>	Modifier of Transcription
<b>MRE11</b>	Meiotic Recombination 11
<b>MRN/MRX</b>	Mre11-Rad50-Nbs1/Xrs2
<b>MSD</b>	Mean-Square Displacement
<b>Msh (2, 3, 6)</b>	Mitochondrial Targeting Sequence
<b>MTOC</b>	microtubule-organising centre
<b>MUS81</b>	MMS and UV Sensitive 81
<b>NBS1</b>	Nijmegen Breakage Syndrome 1
<b>NER</b>	nucleotide excision repair
<b>NHEJ</b>	Non-Homologous End Joining
<b>NP</b>	Nuclear Periphery

<b>NPC</b>	Nuclear Pore Complex
<b>Nup (84, 132...)</b>	nucleoporins
<b>ORC</b>	Origin Recognition Complex
<b>Otr</b>	outer repeats
<b>PARP1</b>	Poly (ADP-ribose) polymerase 1
<b>PCNA</b>	Proliferating Cell Nuclear Antigen
<b>Pc2</b>	<i>Polycomb 2</i>
<b>PCM</b>	pericentriolar material
<b>Phase G1</b>	Phase Gap1
<b>Phase G2</b>	Phase Gap2
<b>Phase M</b>	Phase of mitosis
<b>Phase S</b>	Phase of synthesis
<b>PIAS</b>	Protein Inhibitor of Activated STAT
<b>Pif1</b>	Petite Integration Frequency 1
<b>PIP</b>	PCNA-interacting protein
<b>PML</b>	promyelocytic leukemia
<b>Pol (<math>\delta</math>, <math>\eta</math>...)</b>	DNA polymerase
<b>POT1</b>	Protection of <i>Telomeres</i> 1
<b>preRC</b>	pre-Replication Complex
<b>pre-mRNA</b>	precursor messenger RNA
<b>PRIMPOL</b>	human <i>DNA</i> polymerase-primase
<b>PRR</b>	post-replication repair
<b>PTM</b>	post-translational modification
<b>Pu-seq</b>	Polymerase Usage Sequencing
<b>Rad (50, 51 ...)</b>	radiation sensitive
<b>RANBP2</b>	RAN Binding Protein 2
<b>Rap1</b>	Ras-associated protein 1
<b>rDNA</b>	ribosomal DNA
<b>RDR</b>	Recombination-Dependent Replication

<b>RecA</b>	Recombinase A
<b>RecQ</b>	RecQ-like helicase
<b>RFB</b>	Replication Fork Barrier
<b>RF-C</b>	Replication Factor C
<b>RIF1</b>	Replication Timing Regulatory Factor 1
<b>RING</b>	<i>Really Interesting New Gene</i>
<b>RITS</b>	RNA-induced transcriptional silencing
<b>RNA</b>	ribonucleic acid
<b>RNAi</b>	RNA interference
<b>RNase</b>	Ribonuclease
<b>RNF (4 and 111)</b>	Ring Finger Protein 4
<b>RNR</b>	Ribonucleotide Reductase
<b>rNTP</b>	Ribonucleotide Triphosphates
<b>RNF4</b>	RING finger protein 4
<b>ROS</b>	Reactive Oxidative Species
<b>RPA</b>	Replication Protein A
<b>RS</b>	Replication Slippage
<b>RSR</b>	Replication Stress Response
<b>Rtf (1 and 2)</b>	Replication termination factors
<b>RTS1</b>	Replication Terminator Sequence 1
<b>SAE (1 and 2)</b>	<i>SUMO</i> -Activating Enzyme Subunit 1 and 2
<b>Sc</b>	<i>Saccharomyces cerevisiae</i>
<b>SD</b>	Standard Deviation
<b>SDSA</b>	Synthesis-Dependent Strand Annealing
<b>SENP (1, 2, 3...)</b>	SUMO-specific <i>proteases</i>
<b>Sgs1</b>	Slow Growth Suppressor 1
<b>SIM</b>	SUMO Interaction Motif
<b>siRNA</b>	small interfering RNA
<b>SLX (1, 2, 5, 8)</b>	<i>Synthetic Lethal</i> of unknown (X) function



<b>SMARCAL1</b>	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily A Like 1
<b>SNF2</b>	Sucrose Non-Fermentable
<b>SNURF</b>	small nuclear RING finger protein
<b>Sp</b>	<i>Schizosaccharomyces pombe</i>
<b>SPB</b>	spindle pole body
<b>Srs2</b>	Suppressor of Rad Six 2
<b>SSA</b>	Single Strand Annealing
<b>SSB</b>	single-strand DNA break
<b>ssDNA</b>	single-stranded DNA
<b>STUbL</b>	SUMO-Targeted Ubiquitin Ligase
<b>SUMO</b>	Small Ubiquitin-Like Modifier
<b>Swi2</b>	SWItch 2
<b>T</b>	thymine
<b>TBZ</b>	thiabendazole
<b>TAD</b>	Topologically Associating Domains
<b>TLS</b>	Translesion Synthesis Polymerases
<b>TOPBP1</b>	Topoisomerase II Binding Protein 1
<b>TOPORS</b>	<i>TOP1 Binding Arginine/Serine Rich protein</i>
<b>TPR</b>	Translocated Promoter Region
<b>TRCs</b>	Transcription-Replication Conflicts
<b>TS</b>	Template Switch
<b>Ub</b>	Ubiquitin
<b>Uba2</b>	Ubiquitin-like 1-activating enzyme
<b>Ubc (2 and 9)</b>	Ubiquitin conjugating enzyme 2 and 9
<b>UBP</b>	Ubiquitin specific processing protease
<b>UCH</b>	Ubiquitin C-terminal hydrolase
<b>UFB</b>	Ultrafine Anaphase Bridge
<b>Ufd</b>	Ubiquitin fusion degradation protein 1

<b>Ulp (1 and 2)</b>	Ubiquitin-like specific protease 1 and 2
<b>Uls1</b>	Ubiquitin Ligase for SUMO conjugates 1
<b>UPS</b>	Ubiquitin proteasome system
<b>UV</b>	Ultraviolet
<b>WT</b>	Wild Type
<b>XP (C, F, G)</b>	Xeroderma pigmentosum group
<b>XRCC (1, 2, 3, 4)</b>	X-ray Repair Cross-Complementing protein
<b>Y2H</b>	Yeast two-hybrid
<b>ZNF451</b>	Zinc Finger Protein 451
<b>ZRANB3</b>	Zinc Finger RANBP2-Type Containing 3
<b>μM, nM, mM</b>	micro-, nano- and mili-meter
<b>2DGE</b>	bi-Dimensional Gel Electrophoresis
<b>53BP1</b>	p53-Binding Protein 1

# INTRODUCTION

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# I. Replication stress and DNA damage

## A: Replication stress

Every living organism depends on a web of complex molecular interactions, essential for survival. Cell division is a key process that enables the growth of new tissues, the healing of existing ones, and the continuous renewal of life. This renewal relies on the precise duplication of genetic material, known as DNA replication. Any disruption in this process leads to replication stress, which can cause genetic instability and potentially lead to cancer (Magdalou et al., 2014). The causes and resolutions of replication stress will be explored further in the subsequent sections.

### 1. Sources

DNA replication, generally regarded as a dependable process can face assaults at different levels. The various triggers range from internal to external sources that may stall, block, or terminate DNA polymerization with important consequences on genome stability. ([Figure 1](#)) (Zeman & Cimprich, 2014a).

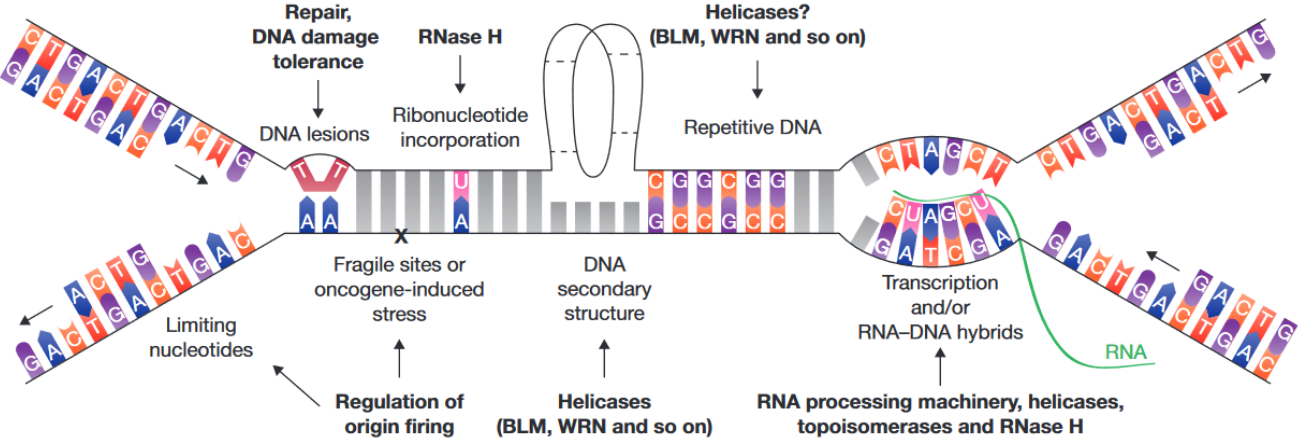
#### 1.1. Obstacles to replication fork progression and replicative DNA synthesis.

Replicative stress is characterized by the slowing or stalling of replication fork progression and/or DNA synthesis. This stress can arise from various physical or metabolic limitations, as outlined below:

**DNA lesions.** Replication stress can be triggered by DNA lesions that arise spontaneously during DNA biosynthesis or external environmental factors (Ashour & Mosammaparast, 2021; Ciccia & Elledge, 2010). Spontaneous DNA alterations, estimated to occur up to  $10^5$  lesions/cell/day, can result from the misincorporation of nucleotides, DNA base deamination, depurination, or alkylation (De Bont & van Larebeke, 2004; Lindahl, 1993). Additionally, reactive oxidative species (ROS) produced by regular cellular processes can lead to oxidation of DNA bases, strand breaks, and base removal (Hoeijmakers, 2009). Nicks and gaps may also form during various DNA repair pathways, such as the release of topological stress, contributing further to replicative stress.

Environmental sources of DNA damage include agents like ultraviolet (UV) and ionizing radiation (IR), which cause oxidation of DNA bases, leading to the formation of single-strand and double-strand DNA breaks (SSBs and DSBs, respectively) (Cadet & Wagner,

Figure 1: Sources of DNA replication stress. See text for details. (Zeman & Cimprich, 2014a)



2013; Hoeijmakers, 2009; Vallerga et al., 2015). Other mutagens, such as methylmethanesulfonate (MMS) transfer methyl groups to DNA bases, impeding replication fork progression (Beranek, 1990). Whereas, crosslinking agents such as cisplatin and mitomycin C introduce covalent intra-strand and inter-strand crosslinks, respectively (Malinge et al., 1999; Noll et al., 2006; Schärer, 2005; Volpato et al., 2005). Interstrand-crosslinks (ICLs) were considered absolute roadblocks for replication forks for very long. However, it was later described in human cells that ICLs induced global replication fork slowing, which provides time for template repair and promotes lesion bypass by DNA translocase, FANCM/MHF complex (Huang et al., 2013; Mutreja et al., 2018).

**Misincorporation of ribonucleotides.** The replicative DNA polymerases have dual functions. They ensure high-fidelity base pairing alongside differentiating between deoxyribonucleotides (dNTPs) and ribonucleotides (rNTPs). The two types of nucleotides contain a similar, but not identical sugar-phosphate backbone. DNA polymerases exploit the steric interaction between Tyr416 with the 2'OH in the phosphosugar backbone to discriminate the rNTP molecules (Yoon & Warshel, 2016). Nevertheless, the high concentration of ribonucleotides and occasional failure in nucleotide selection leads to rNTP misincorporation into DNA - a common replication error occurring at a rate of one ribonucleotide per 1.5-2 kb in human cells (J. A. Brown & Suo, 2011; Joyce, 1997; Z.-X. Zhou et al., 2021). As it occurs at a strikingly high rate, ribonucleotide misincorporation is the most common type of replication error in normal cells (Clausen et al., 2013; Nick McElhinny et al., 2010; Zong et al., 2020). The presence of ribonucleotides in DNA stalls the replicative polymerases during semi-conservative replication and the specialized enzyme RNase H2 catalyses their removal. Deficiency of RNaseH2 has been associated with replication stress, elevated mutation rate, and genome instability (Hiller et al., 2012; Lazzaro et al., 2012). RNase H2 consists of three subunits and one of it contains the PCNA-interacting box (PIP box). Interestingly, the interaction between RNase H2 and PCNA helps to localize RNase H2 at the replication fork upon any damage (Bubeck et al., 2011). Notably, mutations in RNase H2 lead to the Aicardi-Goutières syndrome, which is an autosomal recessive inflammatory brain disorder that resembles congenital viral infection (Crow et al., 2006, p. 200).

**Non-B DNA structures.** Canonical B-DNA is a right-handed double helix with two antiparallel strands that separate during replication to allow the formation of alternative DNA structures including G-quadruplexes and hairpins. They can stall replication forks, leading to chromosomal fragility, and increase genome instability (R. E. Brown & Freudenreich, 2021; J. Lopes et al., 2011; Richl et al., 2024). G-quadruplexes

are four-stranded structures formed in GC-rich regions through Hoogsteen base pairing between guanine residues and are particularly abundant in the S-phase (Biffi et al., 2013; Di Antonio et al., 2020; Mirkin, 2007; Sen & Gilbert, 1988). Whereas, hairpins arise from sequences such as inverted repeats and palindromes potentially forming cruciform structures in AT-rich sequences when two hairpins align. Specialized helicases and structure-specific nucleases are necessary to unwind, disassemble, and ensure tolerance to these structures during replication, while their dysfunction or stabilization of the secondary structures can impede replication and cause deletions (L. Guo et al., 2024; Paeschke et al., 2011; S. Sharma, 2011).

**Centromeres and Telomeres.** Genetic features of certain regions are inherently challenging to replicate due to the presence of repeated sequences forming secondary structures, compacted chromatin conformation, distribution of origins, replication timing, and transcription-mediated obstacles (Barra & Fachinetti, 2018; W. Feng & Chakraborty, 2017; Gadaleta & Noguchi, 2017; Miotto et al., 2016; Voutsinos et al., 2018). Such difficulties can cause replication stress, leading to slowed or stalled replication forks, which may lead to fork collapse or result in replication-associated double-strand breaks (DSBs) (Cortez, 2019). Maintenance of centromeric DNA is dependent upon the centromere-specific H3 histone, CENP-A. Despite being loaded onto nucleosomes in G2 and not in the S-phase, CENP-A nucleosome removal on parental DNA slows replication fork progression at centromeres. Delay in fork progression is likely due to replication-transcription conflicts that stabilise R-loops (Giunta et al., 2021). Moreover, centromeres are the hotspots of increased fragility and rearrangements (Giunta et al., 2021, p. 202). Whereas, in human telomeres, G4 accumulation causes telomere dysfunctions upon depletion of the RECQ helicase, which aids in unwinding the G-quadruplexes (T. Li et al., 2023).

**Transcription-Replication Conflicts.** Replication and transcription occur concurrently on DNA with a spatio-temporal distribution (S & Ka, 2016). Disturbance in synchronization between these central processes can lead to Transcription-Replication Conflicts (TRCs), marked by collisions between the transcription and replication machinery (Browning & Merrikh, 2024; García-Muse & Aguilera, 2016; X. Wei et al., 1998). TRCs feature the emergence of R-loops, which are three-stranded structures composed of DNA-RNA hybrids (Thomas et al., 1976). During transcription, the RNA polymerase synthesizes RNA using one strand of the DNA as a template. As the RNA is synthesized, it re-anneals back to the template by displacing the non-template, complementary strand to create the three-stranded structures. (Fielden et al., 2018; Tretyakova et al., 2015; Vaz et al., 2017). R-loops can be prevented by the action of

DNA topoisomerases, RNA-binding proteins, and the RNA exosome complex (Petermann et al., 2022). However, R-loops already formed can be processed by specific DNA-RNA nucleases. RNaseH1 and RNaseH2 degrade R-loops, whereas the SETX helicase can resolve R-loops by unwinding the RNA from the DNA (Gatti et al., 2023; Lockhart et al., 2019). In addition, other exonucleases like XRN2 also contribute to the resolution of R-loops by degrading them (Villarreal et al., 2020). R-loops can interfere with replication fork progression, causing replication stress and potentially leading to double-strand breaks (DSBs). Persistent R-loops can lead to DNA damage and genomic instability. (Gan et al., 2011; Hamperl et al., 2017; Helmrich et al., 2013; D. A. Matos et al., 2020; Sollier et al., 2014; Xu et al., 2024)

**DNA-protein crosslink.** DNA-protein crosslinks (DPCs) are another source of replication stress, arising from the covalent binding of proteins to DNA (Fielden et al., 2018; Tretyakova et al., 2015; Vaz et al., 2017). Enzymatic DPCs arise from DNA-associated enzymes, like topoisomerases, being trapped during their catalytic activities, creating not only a physical barrier to the replication machinery but also potentially leading to DNA breaks. For instance, Topoisomerase 1 compromises genome integrity by forming covalent, cleavage complexes which are associated with fork stalling, fork collapse, and generation of DSBs in both, yeast and humans (Sordet et al., 2010; Tuduri et al., 2009). Specific chemotherapeutic agents, such as Camptothecin and etoposide, trap topoisomerase I and II respectively, in these covalent cleavage complexes. Other agents like Aphidicolin, inhibit DNA polymerases, effectively halting DNA replication. Fork stalling can lead to the stabilization of the replication machinery, which in turn can cause the formation of DNA-protein crosslinks as the proteins involved in replication remain bound to the DNA (Baranovskiy et al., 2014; Krokan et al., 1981). On the other hand, Olaparib inhibits DNA polymerases and PARP1, leading to the formation of DPCs at the site of trapped PARP1 (Ide et al., 2011; Kedar et al., 2012; Murai et al., 2012; Pommier & Marchand, 2012; Vare et al., 2012). Non-enzymatic DPCs, on the other hand, involve the covalent attachment of any nearby protein to DNA following exposure to agents like UV light or aldehydes (Stingle et al., 2017).

## 1.2. Defects in the replication machinery

Efficient DNA synthesis depends on a tightly-regulated supply of deoxyribonucleotides (dNTPs), catalyzed by the ribonucleotide reductase (RNR) enzyme (Mathews, 2016; Nordlund & Reichard, 2006; Y.-W. Zhang et al., 2009). Changes in RNR expression or activity alters intracellular dNTP concentrations, influencing replication in various ways. Elevated dNTP pools disrupt replication initiation and promote mutagenesis (Chabes & Stillman, 2007; Kumar et al., 2010). Conversely, dNTP deficiency precedes slower



replication, hindering fork progression (Bester et al., 2011; Chabosseau et al., 2011; Poli et al., 2012). In addition, mutations in replisome components that affect stoichiometry, stability, and even timely removal of certain factors like PCNA can contribute to genome instability (Kang et al., 2024). These include mutants that impact the stability and catalytic efficiency of DNA polymerases, helicases, or RPA (Alvarez et al., 2015; Bellelli & Boulton, 2021; Heitzer & Tomlinson, 2014). For example, mutations in DNA polymerases like Pol  $\alpha$  lead to X-linked intellectual disability and mutation in Pol  $\delta$  are linked with the predisposition to colorectal and endometrial cancer in humans (Palles et al., 2013; Van Esch et al., 2019). Whereas, germline mutations in RPA, a key player in DNA replication and repair, have been linked to a syndrome characterized by bone marrow failure, myelodysplastic syndrome, T- and B-cell lymphopenia and pulmonary fibrosis underscoring its role in maintaining genome stability (R. Sharma et al., 2022). Additionally, mutations in genes encoding components of the pre-replication complex (ORC1, ORC4, ORC6, CDT1, and CDC6) are linked to a condition called the Meier-Gorlin syndrome (Bicknell et al., 2011; Guernsey et al., 2011). This condition is characterized by dwarfism, small ears, and other developmental abnormalities. The mutations in the pre-replication complex lead to defects in origin licensing, causing replication stress and impaired cell proliferation. Another syndrome called the Bloom syndrome is the result of mutations in the BLM helicase (N. A. Ellis et al., 1995). BLM is required to unwind DNA ahead of replication forks to facilitate DNA synthesis. Bloom syndrome is characterized by short stature, sun-sensitive skin changes, and a high risk of cancer. The BLM helicase is crucial for resolving replication stress and mutations in the BLM gene result in increased sister chromatid exchanges and genomic instability (G. Luo et al., 2000).

### 1.3. Dysregulation in origin firing

Over 50% of replication origins in yeast and around 90% in humans that are licensed during the G1 phase, are not activated during an unperturbed S phase (Blow & Ge, 2009; Dai et al., 2005; Fragkos et al., 2015). They remain dormant, serving as a backup to buffer against replication issues (Heichinger et al., 2006; McIntosh & Blow, 2012). These origins may activate under stress conditions, increasing the origin density to ensure complete and timely genome duplication (Ibarra et al., 2008; Moiseeva et al., 2019). Defective replication origin firing can result in genomic regions remaining unreplicated when cells enter mitosis. Such regions are often referred to as chromosomal fragile sites (CFS). Mice and human models show that replication stress induced by agents such as aphidicolin (APH) or hydroxyurea (HU), exacerbates the sensitivity of fragile sites. Cells with reduced loading of the replicative helicase (MCM complex) have a diminished capacity to handle this stress, leading to increased incidence of breaks

and instability at fragile sites. (Kawabata et al., 2011; Kunnev et al., 2010; Letessier et al., 2011). The inability to activate dormant origins, combined with the slowing down of fork progression, can lead to incomplete DNA replication in chromosomal regions where the converging forks do not have enough time to merge before entering mitosis. Such unresolved replication intermediates interfere later with chromosome segregation during mitosis leading to chromosome breakage and instability.

Conversely, excessive origin firing in checkpoint-defective cells, can deplete essential resources like dNTPs and histones, leading to global replication fork stalling (H. Beck et al., 2012). Uncontrolled origin activation can also overwhelm the cellular RPA pool, leaving ssDNA unprotected and causing widespread replication fork breakage called replication catastrophe (L. Toledo et al., 2017; L. I. Toledo et al., 2013). Increased origin firing can also trigger re-replication, but despite the higher number of active forks, the slow elongation rate prevents complete genome duplication (Fu et al., 2021; Fujita, 2006).

## 2. Consequences of replication stress

Replication stress disrupts normal DNA duplication by causing obstacles that interfere with the replication machinery, leading to a halt in the process of replication. A common initial effect is the accumulation of single-stranded DNA (ssDNA), often resulting from ongoing DNA unwinding by helicases while DNA polymerases stall (Zeman & Cimprich, 2014a). Additional sources of ssDNA include the degradation of newly synthesized DNA by nucleases and helicases. Crucial enzymes like MRN, CtIP, EXO1, and BLM, active in processing DNA ends at double-strand breaks (DSBs), also contribute to ssDNA buildup at stalled forks, increasing susceptibility to damage and mutations that can lead to chromosome breaks (W. Feng et al., 2011; Saini & Gordenin, 2020).

Replication stress compromises the fulfilment of chromosome duplication leading to under-replicated regions in DNA. When these loci enter mitosis, each of the intertwined DNA strands already belongs to a separate sister chromatid. During chromosome segregation in anaphase, they form ultra-fine bridges (UFBs), which are subjected to increasing mechanical tensions and may therefore break. Such breakages challenge even chromosome segregation and pose a risk that the resulting damage will be transmitted to the daughter cell (Chan & West, 2018; A. Moreno et al., 2016).

To safeguard genomic integrity, cells deploy mechanisms to prevent, manage, or halt damage caused by replication stress, essential for maintaining genome stability and preventing genetic errors.

**Table 1: Factors required in DNA damage response.** This table provides examples of key components in the DNA damage signalling pathway in human cells, alongside their yeast counterparts.

Function	Human	<i>S.cerevisiae</i>	<i>S.pombe</i>
Sensors	MRN	MRX	MRN
	RPA	RPA	RPA
	RAD9/RAD1/HUS1	Ddc1/Rad17/Mec3	Rad9/Rad1/Hus1
	RAD17	Rad24	Rad17
Kinases	ATM	Tel1	Tel1
	ATR	Mec1	Rad3
	ATRIP	Ddc2	Rad26
Mediators	MDC1	-	Mdb1
	53BP1	Rad9	Crb2
	TopBP1	Dbp11	Cut5/Rad4
	Claspin	Mrc1	Mrc1
Effectors	CHK1	Chk1	Chk1
	CHK2	Rad53	Cds1
	p53	-	-
	CDC25	Mih1	Cdc25
	CDK1	Cdc28	Cdc2
	WEE1	Swe1	Wee1/Mik1

## B: How to prevent and deal with replication stress?

### 1. Replication Stress Response

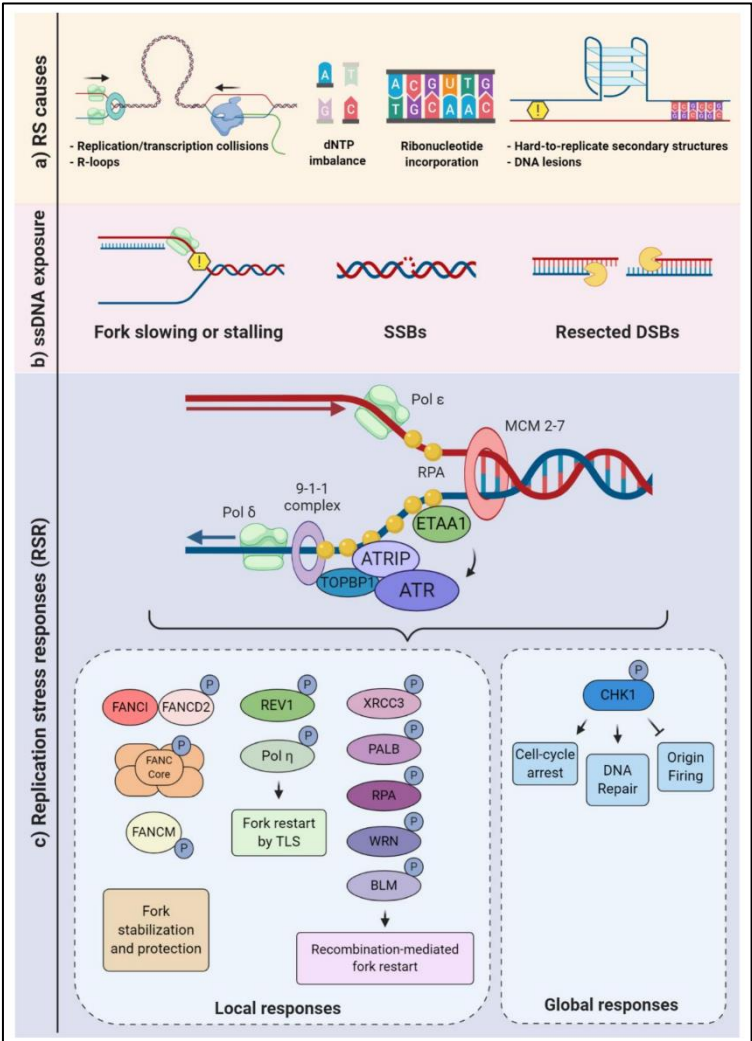
Cell division aims to precisely duplicate and segregate the genetic content into the daughter cells. The process of duplication and segregation is monitored by highly conserved molecular checkpoints, which prevent premature or irreversible cell-cycle transitions, especially when DNA damage is present.

#### 1.1. DNA replication checkpoint

Cells employ cell cycle checkpoints as quality control systems to detect and respond to DNA perturbations. The DNA replication checkpoint (DRC) particularly monitors single-stranded DNA (ssDNA) formation at the replication fork, a key indicator of replication stress that triggers its activation (Branzei & Foiani, 2008; Byun et al., 2005; Longhese et al., 1996; Pellicioli et al., 1999). To simplify, I will describe the mechanistic pathway using human protein factors. However, the corresponding homologs in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are listed in [Table 1](#).

ssDNA coated with the replication protein A (RPA) is associated with lagging strand synthesis during normal replication (Audry et al., 2015; Pike et al., 2023; Wold, 1997). An increase in the length of the RPA coated ssDNA accumulated at stalled replication forks is the hallmark of replication stress (Zou & Elledge, 2003). The ssDNA bound by replication protein A (RPA) attracts sensor proteins such as ATR-interacting protein (ATRIP), the 9-1-1 DNA clamp complex (RAD9-RAD1-HUS1), topoisomerase II binding protein 1 (TOPBP1), and Ewing tumor-associated antigen 1 (ETAA1) in humans (Bass et al., 2016; Kumagai et al., 2006). These proteins facilitate the activation of the apical protein kinase of the DNA replication checkpoint called ATR (Mec1 in budding yeast and Rad3 in fission yeast). ATR belongs to the IKK (phosphoinositide-3-like kinase) family, responsible for phosphorylating and activating downstream checkpoint kinase and DNA repair proteins (Keith & Schreiber, 1995). ATR remains active during the S phase to regulate the firing of replication origins and the repair of damaged replication forks, and to prevent the premature onset of mitosis (Nyberg et al., 2002; Shechter et al., 2004b). Upon encountering stalled forks, ATR phosphorylates targets including CHK1, which then disperses from the fork to propagate the checkpoint signal throughout the cell. The ATR-CHK1 pathway is essential for coordinating the cellular response to replication stress, both globally and locally as depicted in [Figure 2](#) (Byun et al., 2005; Haahr et al., 2016; Nam & Cortez, 2011; Saldivar et al., 2017).

**Figure 2: Replication stress response induced by ATR.** During replication stress, the replisome is prone to slowing down or stalling, which results in the exposure of ssDNA. ATR kinase detects RPA-bound ssDNA at these stressed replication forks and coordinates both global and local responses to address the DNA replication stress. (Forrer Charlier & Martins, 2020)



**Local response.** Research indicates that controlled local checkpoint activation is important in replication stress response (K et al., 2002; Tercero et al., 2003). ATR activity is not only required for stress response but is also involved in normal DNA replication (Lanz et al., 2018). ATR facilitates the progression of forks by removing the transcription machinery (Poli et al., 2016). It is also required in transcription, RNA metabolism, and chromatin remodelling during unperturbed DNA replication (Bastos de Oliveira et al., 2015). In terms of replication stress, studies revealed that in checkpoint-deficient yeast treated with hydroxyurea, dysfunctional replication forks accumulate, unable to resume DNA synthesis (al-Khodairy & Carr, 1992; Enoch & Nurse, 1990; M. Lopes et al., 2001; Sogo et al., 2002; Tercero et al., 2003; Tercero & Diffley, 2001). Further research identified three key functions of the ATR checkpoint for local fork protection:

**1. Fork Preservation:** ATR maintains the structure and functionality of stalled forks, allowing them to restart once conditions improve (Cortez, 2015; Dungrawala et al., 2015). ATR interacts directly with replication machinery, preventing disassembly and keeping the replisome ready-to-act (Cobb et al., 2003, 2005; Katou et al., 2003; Lucca et al., 2004, p. 2). Although in contrary, evidence suggests that replisome stabilization is not the main mechanism for preventing fork collapse (De Piccoli et al., 2012). Additionally, a number of proteins driving fork remodelling has been identified as ATR-Chk1 substrates. When encountering DNA lesions, forks can reverse their course by annealing the two newly synthesized strands, leading to four-way junction structures resembling Holliday junctions (M. Lopes et al., 2001; Sogo et al., 2002). This protective mechanism stabilizes the stalled replication forks and ensures their ability to continue or resume replication without chromosomal breakage (Berti et al., 2013; Neelsen et al., 2013; Zellweger et al., 2015). Fork reversal was initially seen as a pathological structure but emerging evidence suggested that it is indispensable for maintaining genome stability (Bermejo et al., 2012; Huang et al., 2013; Neelsen & Lopes, 2015; Quinet et al., 2017; Räschle et al., 2008). For instance, impairing fork reversal by PARP inhibition, which is a crucial mediator of fork reversal leads to the formation of DSBs, indicating that fork reversal prevents fork collapse at camptothecin-induced lesions (Ray Chaudhuri et al., 2012). However, reversed forks can be cleaved by nucleases like Mus81 to deal with stalled replication forks, this process generates DSBs that require repair by homologous recombination (Regairaz et al., 2011). This mechanism, while risky, is essential for managing replication stress and maintaining genomic stability.

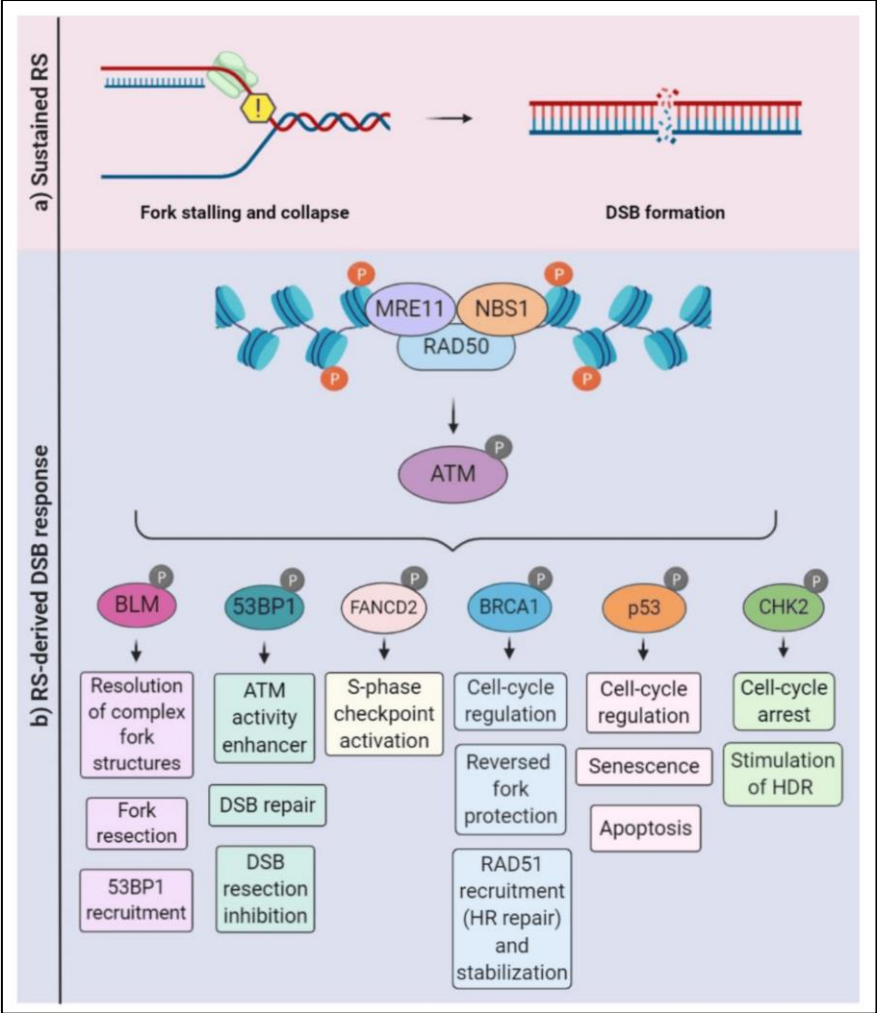
**2. Activation of Dormant Origins:** In prolonged stress, ATR may trigger dormant origins to fire near stalled forks through unknown mechanisms. It is suggested that checkpoint activation could allow a neighbouring dormant origin to fire by suppressing

CHK1 activity near the stalled polymerase, facilitating DNA synthesis completion in a compensatory process (Ge & Blow, 2010; Técher et al., 2017). This compensation for halted replication depends on their proximity to stalled forks despite a broader suppression of origin activation.

**3. DNA Damage Tolerance (DDT) Pathways:** ATR activates DDT to complete replication due to prolonged stalling. This includes translesion synthesis (TLS) by error-prone TLS polymerases and template switching (TS) using a homologous template for accurate bypass (Sale, 2012; Waters et al., 2009; Branzei, 2011; Giannattasio et al., 2014). Replisomes bypass lesions and resume downstream synthesis aided by PrimPol in vertebrates, which catalyzes repriming for replication continuation, whereas ssDNA gaps created during the process are subsequently filled by TLS or TS (Mehta et al., 2022; Mourón et al., 2013). Whereas, in yeast the polymerase  $\alpha$  (Pol $\alpha$ )/primase complex along with Ctf4, a replisome factor, bridges the replicative helicase and the Pol $\alpha$ /primase complex for repriming (Fumasoni et al., 2015).

**Global response.** DNA replication checkpoint is quantitative and the checkpoint response depends upon the extent of accumulated ssDNA at stalled forks (Bantele & Pfander, 2020). Local checkpoints are crucial to sense replication stress as they enable specific activation of the ATR kinase. However, the global effect is only triggered upon acute replication stress by the activation of the downstream signalling cascade by CHK1 and CHK2 kinases (Bastos de Oliveira et al., 2015; Lanz et al., 2018). Hence, the DNA replication checkpoint regulates cell cycle progression globally, by slowing down replication speeds, inhibition of origin firing, delayed entry into mitosis and a global slowdown of replication fork progression across the cell. (Paulovich & Hartwell, 1995; Yekezare et al., 2013). In human cells, during the S-phase, the components of the replisome complex recruits and activates the ATR kinase followed by CHK1 activation (Rankin & Rankin, 2024). CHK1 downregulates CDK2 (Cyclin-dependent kinase 2) activity by inhibiting the CDC25A phosphatase. Inhibition of CDC25A prevents necessary phosphorylation of the origin recognition complex (ORC) and the CMG replicative helicase at replication origins, which is required for the competence of replication initiation. (Bartek et al., 2004; Falck et al., 2002; Molinari et al., 2000; Shechter et al., 2004a). Additionally, CHK1 activates WEE1, a kinase that acts as a negative regulator of CDK1/2. This coordinated slowdown prevents the firing of additional origins, reducing potential replication stress and avoiding ssDNA accumulation and RPA depletion, thereby preventing replication catastrophe (RC) (Moiseeva et al., 2019; L. I. Toledo et al., 2013).

**Figure 3: Replication stress response induced by ATM.** Persistent replication stress results in fork collapse and the formation of double-strand breaks (DSBs). This triggers the recruitment of the MRN complex and ATM kinase. Once activated, ATM phosphorylates a range of substrates, coordinating various cellular responses to the damage. (Forrer Charlier & Martins, 2020)





Hence, the global control of origin firing prevents the depletion of RPA. The RPA could in turn provide essential local protection against fork breakage (Meyer et al., 1980; L. Toledo et al., 2017). This model provides an integration between the local and global checkpoint signalling events.

Additionally, replication catastrophe could be exploited for cancer therapy. For instance, CHK1 and WEE1 inhibitors show synthetic lethality when paired with depletion of the dNTP pool in pancreatic cancer and human sarcoma respectively (Azorsa et al., 2009; Krehling et al., 2013). In this context, depletion of the dNTP pool leads to uncoupling between the helicase and the polymerase, forming long tracts of ssDNA, that is bound by RPA and becomes a limiting factor. This implies that RPA exhaustion plays a role in the lethality of these kinase inhibitors when used in combination with replication inhibitors. (Koh et al., 2015, p. 201; Prevo et al., 2012; Walton et al., 2010; Y. Xiao et al., 2013). Pointing to replication catastrophe as a major contributor to the efficient killing of cancer cells following checkpoint inhibitor monotherapy.

## 1.2. DNA damage checkpoint

The other checkpoint pathway, which was initially discovered before the replication checkpoint pathway, was the DNA damage checkpoint. Prolonged stalling of replication forks or disruptions in the ATR-CHK1 pathway can lead to fork collapse and result in double-strand breaks (DSBs) (Dungrawala et al., 2015; Petermann et al., 2010). The occurrence of DSBs due to replication stress activates other signalling kinases, such as the ataxia-telangiectasia mutated (ATM) kinase ([Figure 3](#)), which, unlike ATR, is not essential for cell survival (Durocher & Jackson, 2001; Harrigan et al., 2011; Hirao et al., 2002; Jackson & Bartek, 2009).

The MRN complex, consisting of MRE11, RAD50, and NBS1, acts as the primary DSB sensor and rapidly recruits and activates ATM (Uziel, 2003). While both the exo- and endonuclease activities of MRN have been implicated in ATM activation through in vitro studies, experiments using mouse cells with a nuclease-deficient MRE11 mutant still demonstrated ATM activation (Buis et al., 2008; Jazayeri et al., 2008). ATM-mediated phosphorylation initiates specific cellular responses for handling DNA damage (Lavin, 2008).

**Cell-cycle arrest:** The activation of ATM leads to the phosphorylation and activation of CHK2. This activated CHK2 then phosphorylates targets including the Cdc25 family of phosphatases. The phosphorylation of CDC25 results in its degradation, preventing it from dephosphorylating CDK1-cyclin B, which leads to cell-cycle arrest, primarily at

**Figure 4: Human syndromes related to replication stress.** (Zeman & Cimprich, 2014a)

Human disease	Aetiology		Characteristics
	Affected pathway	Defective protein(s)	
Aicardi-Goutieres syndrome (OMIM 610333, 610181, 610329, 225750, 612952)	Removal of ribonucleotides, RNA–DNA hybrids	RNase H2, TREX1, SAMHD1	Neurological dysfunction, appearance of chilblains
Amyotrophic lateral sclerosis 4 (OMIM 602433)	Resolution of RNA–DNA hybrids, transcription termination	Senataxin	Childhood- or adolescent-onset degeneration of motor control
Ataxia-ocular apraxia 2 (OMIM 606002)			Adolescent-onset cerebellar ataxia
Ataxia-telangiectasia-like disease (OMIM 604391)	MRN complex; ATR/ATM activation	Mre11	Neurodegeneration, ataxia
Bloom syndrome (OMIM 210900)	DNA remodelling, replication fork structure resolution	BLM	Premature ageing, growth retardation, cancer predisposition
Cancer <sup>127</sup>	Many	Many	Uncontrolled cell growth, leading to organ failure
Ciliopathies <sup>138</sup>	Centrosome, primary cilia formation	CEP164, Nek8, Mre11, Znf423, Fan1	Dysfunction or degeneration of organs, particularly kidney, retina, and brain
Congenital dyserythropoetic anemia, type 1 (OMIM 224120) <sup>127</sup>	Histone deposition	CDAN1	Anaemia, skeletal abnormalities
Fanconi anaemia <sup>44</sup>	DNA inter-strand crosslink repair	FANC family of proteins	Heterogenous — bone marrow failure, skeletal defects, hypopigmentation, cancer predisposition
	Replication fork protection	FANCD2, BRCA2	
Friedreich's ataxia (OMIM 229300)	Trinucleotide repeat expansion	FXN	Neurodegeneration (ataxia, loss of coordination, loss of sensation)
Laminopathies <sup>139</sup>	Nuclear envelope structure	Lamins	Premature ageing
Meier-Gorlin syndrome (OMIM 224690)	Origin licensing, centrosome maintenance	ORC1, ORC4, ORC6, CDT1, CDC6	Growth retardation, microcephaly
Nijmegen breakage syndrome (OMIM 251260)	MRN complex; ATR/ATM activation	Nbs1	Microcephaly, growth retardation, cancer predisposition
Nijmegen breakage syndrome-like disorder (OMIM 613078)	MRN complex; ATR/ATM activation	Rad50	Microcephaly, growth retardation, mental retardation
Rothmund-Thomson syndrome (OMIM 268400)	DNA remodelling, replication fork structure resolution	RecQL4	Premature ageing, growth retardation, cancer predisposition
Schimke immuno-osseous dysplasia (OMIM 242900)	Replication fork stabilization and reversal; DNA re-annealing	SMARCAL1 / HARP	Dwarfism, skeletal abnormalities, renal failure, and immunodeficiency
Seckel syndrome (OMIM 210600)	ATR signalling	ATR, ATRIP, CENPJ, CEP152, PCNT	Growth retardation, dwarfism, microcephaly, mental retardation
Spinocerebellar ataxia type 10 (OMIM 603516)	Trinucleotide repeat expansion	ATXN10	Ataxia, seizures
Werner syndrome (OMIM 277700)	DNA remodelling, replication fork structure resolution	WRN	Premature ageing, growth retardation, cancer predisposition
Wolf-Hirschhorn syndrome (OMIM 194190) <sup>125</sup>	DNA damage response, nucleosome deposition	NELF-A (WHS2), SLBP, MMSET (WHS1)	Growth retardation, mental retardation, seizures
Xeroderma pigmentosum — variant (OMIM 278750)	Translesion synthesis	Polymerase $\eta$	Cancer predisposition (especially skin cancer)

OMIM numbers refer to the Online Mendelian Inheritance in Man database.

the G1/S and G2/M transitions. This mechanism provides the cell time to repair replication-associated DNA damage before proceeding with DNA replication and cell division (Z. Xiao et al., 2003).

**DNA repair:** After its initial activation, ATM orchestrates a series of events at the chromatin surrounding DSBs. At damaged replication forks, ATM plays a vital role in managing the repair of single-ended DSBs on multiple levels. It has been suggested that ATM regulates the speed of DNA end resection and helps counteract detrimental DNA end-joining processes (Balmus et al., 2019). Recent proteomic studies at damaged replication forks have shown that ATM promotes DNA end resection and supports homologous recombination repair (Nakamura et al., 2021). Moreover, it has been found that ATM limits the build-up of non-homologous end joining factors by inhibiting the canonical DSB-associated ubiquitin signalling (Nakamura et al., 2021).

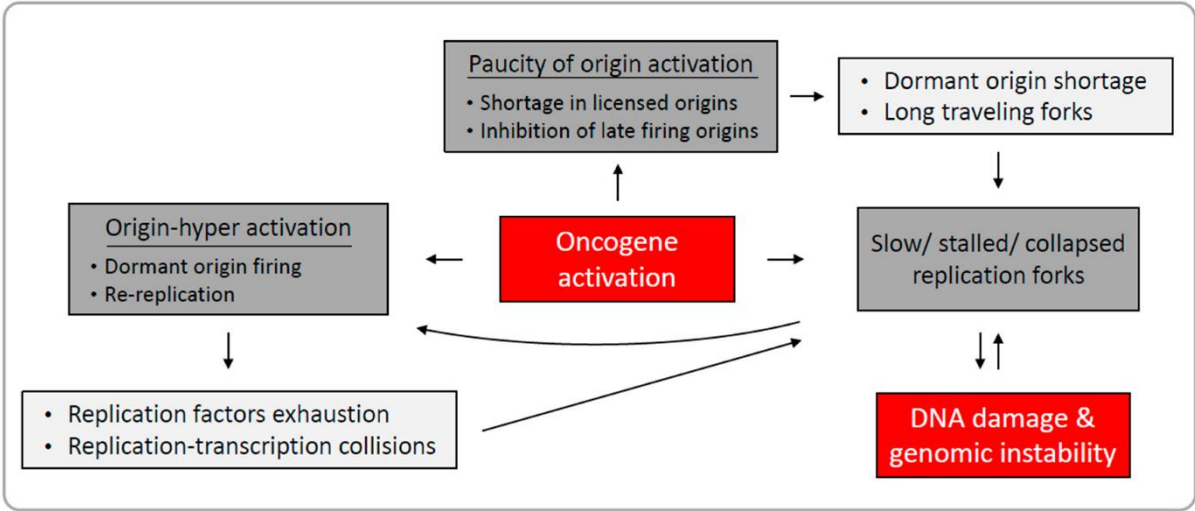
## 2. Dealing with DNA replication stress: implications in human diseases

Replication stress response (RSR) is crucial for maintaining genome stability, and its malfunction can lead to DNA damage accumulation, potentially causing congenital diseases. (Baple et al., 2014; Fenwick et al., 2016; Logan et al., 2018; Schmit & Bielinsky, 2021; Vetro et al., 2017).

Mutations in RSR-related genes are associated with developmental anomalies like growth restriction and microcephaly (Goodship et al., 2000; Katyal & McKinnon, 2008; Kerzendorfer & O'Driscoll, 2009; O'Driscoll et al., 2004). Neurodegenerative disorders could occur due to DSBs leading to ATM-dependent apoptosis in neural cells. For example, cerebellar neuron degeneration, characteristic of conditions like ataxia or apraxia, involves impaired motor coordination and eye movement defects, respectively (Katyal & McKinnon, 2008; Matsuoka et al., 2007; O'Driscoll & Jeggo, 2008; A. M. R. Taylor et al., 2004). These conditions highlight the essential role of effective RSR in cell proliferation, stem cell differentiation, and tissue regeneration. Moreover, unresolved replication stress can hinder stem cell differentiation and tissue regeneration, including implications in haematopoiesis and innate immune responses (Ragu et al., 2020). [Figure 4](#) summarizes the human diseases associated with defects in the replication stress response (Zeman & Cimprich, 2014a).

Furthermore, an enhanced RSR is linked to oncogene activation, which can disturb DNA replication by deregulating origin licensing and replication fork progression as shown in [Figure 5](#). Furthermore, a single oncogene can induce replication stress through multiple routes, highlighting the complex relationship between oncogene activity and

Figure 5: **Oncogene-induced replication stress.** (Sarni & Kerem, 2017)



replication stress (Bartkova et al., 2006; Di Micco et al., 2006; Gorgoulis et al., 2005; Halazonetis et al., 2008). In all cases, oncogene-induced disturbances in DNA replication can lead to chromosomal aberrations and genomic instability, which may accelerate the onset of cancer.

Another hallmark of RSR in cancer arises from increased conflicts between transcription and replication, driven by oncogenes like c-Myc and activated Ras. Collisions between replication machinery and transcription complexes or R-loops induce fork stalling, activating DNA damage response (DDR) and exacerbating replication stress, contributing to genomic instability (Khamidullina et al., 2024).

Collectively, these insights underscore the importance of a robust RSR and accurate DNA replication in preventing a spectrum of genetic disorders and mitigating cancer risk.

## II. Homologous recombination: the replication fork safeguard

### A: Homologous recombination – an overview

#### 1. Key steps and players

Homologous recombination (HR) is an evolutionarily conserved process vital for genetic exchange between DNA molecules sharing sequence homology crucial for evolutionary adaptability, genetic diversity, and genome stability. HR efficiently and accurately repairs DNA damage, including gaps, double-stranded breaks (DSBs), and inter-strand crosslinks (ICLs), whilst also maintaining telomeres and aiding DNA replication. (Costes & Lambert, 2013; Jasin & Rothstein, 2013).

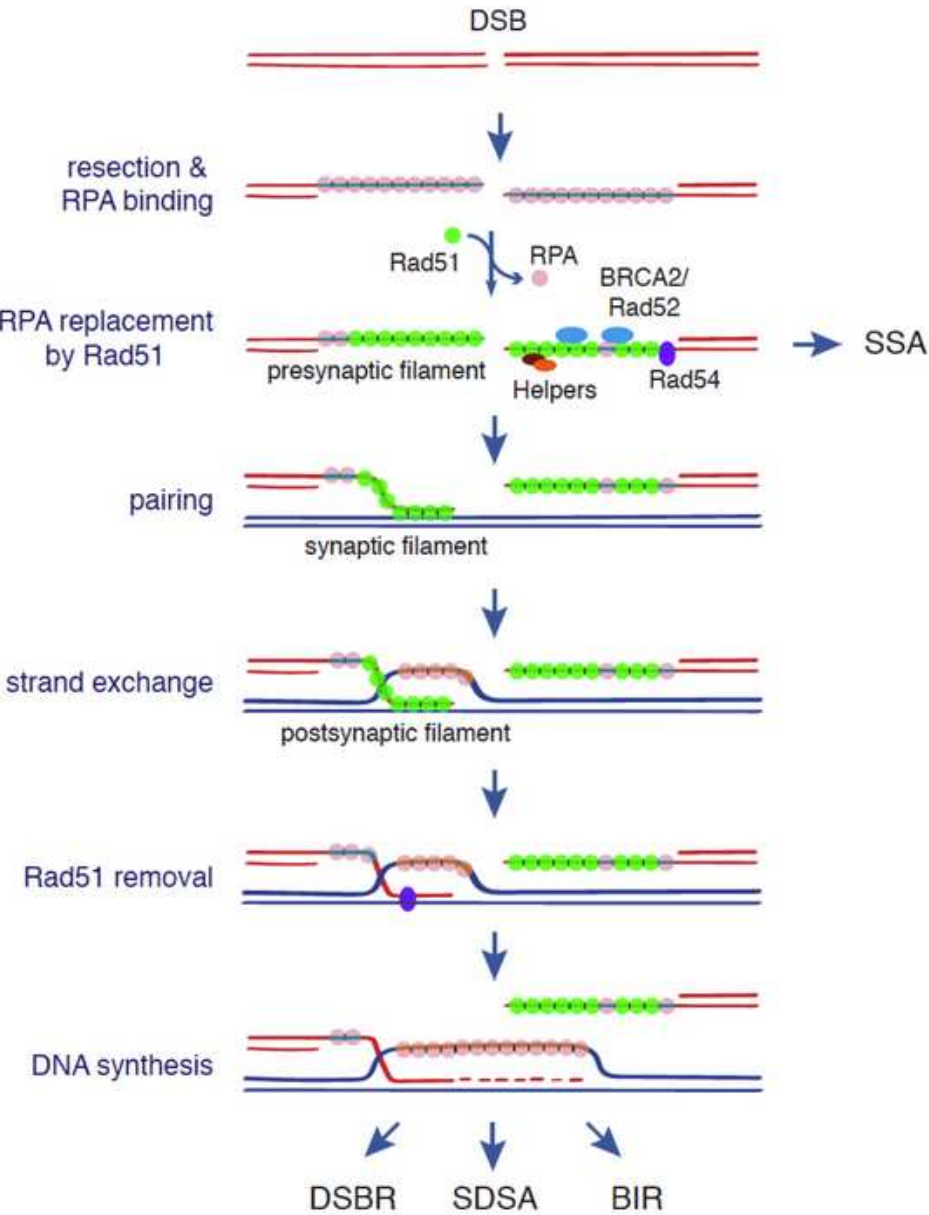
HR-mediated DNA repair employs an intact DNA template, commonly the sister chromatid, although homologous chromosomes or ectopic sites are alternatives (R. D. Johnson, 2000). This process, mediated by a core set of proteins, involves various sub-pathways, all initiated by the recombinase Rad51, a highly conserved protein across many organisms.

Rad51, akin to bacterial RecA, functions similarly in eukaryotes, with significant sequence identity between yeast (ScRad51 and SpRad51) and human (hsRAD51) core domains (Baumann & West, 1998; Shinohara et al., 1992). Its recombinase activity is essential for HR, driving processes like homology search, DNA strand invasion, and exchange, categorized into presynaptic, synaptic, and postsynaptic phases biochemically ([Figure 6](#)).

#### 1.1 Presynaptic phase

As proposed by the classical model of DSB repair by HR, DNA end resection is the key step to produce a 3' single stranded DNA end that serves as a platform for the recruitment of factors involved in the homology-directed repair (Szostak et al., 1983). DSB is recognized by the MRN complex (composed of SpMre11/SpRad50/SpNbs1 in fission yeast, ScMre11/ScRad50/ScXrs2 in budding yeast and hsMRE11/hsRAD50/hsNBS1 in human) that binds to each side of the break to initiate the repair (J.-H. Lee & Paull, 2004; Petrini & Stracker, 2003). The subsequent end-resection occurs in two steps ([Figure 7](#)), First, in a process termed the initial short-range resection, the MRN complex together with its co-factor (ScSae2/SpCtp1 in yeast and CtIP in humans) trims the broken ends to produce short (100-300 bp) 3' single-stranded overhangs (Garcia et al., 2011; Mimitou et al., 2017). Then, during the long-range resection, the short ssDNA overhangs up to several kilobases by the action of Exo1 and SpRqh1/ScSgs1/hsBLM – SpDna2/ScDna2/hsDNA2

Figure 6: Key steps of HR-mediated DSB repair. (Prado, 2021)



(Mimitou & Symington, 2008; Nimonkar et al., 2011; Zhu et al., 2008).

Given the abundance of the replication protein A (RPA) and its high affinity to ssDNA as compared to other ssDNA binding proteins (Bastin-Shanower & Brill, 2001). The first protein that coats the ssDNA overhangs at stalled replication forks is the RPA. RPA is a highly conserved heterotrimeric protein complex that varies in composition across species and coats ssDNA from structure-specific nuclease cleavage (Wold, 1997; Wold & Kelly, 1988; C. Zhou et al., 2015). Subsequently, Rad51 nucleates and oligomerizes on the ssDNA to form a helical nucleoprotein filament known as the presynaptic complex (Conway et al., 2004; Short et al., 2016). Since RPA has a stronger affinity for ssDNA than Rad51, the two compete for binding, necessitating the displacement of RPA by Rad51, a task achieved with the help of mediator proteins (Carreira et al., 2009; Jensen et al., 2010; Shinohara & Ogawa, 1998, p. 199; Sung, 1997).

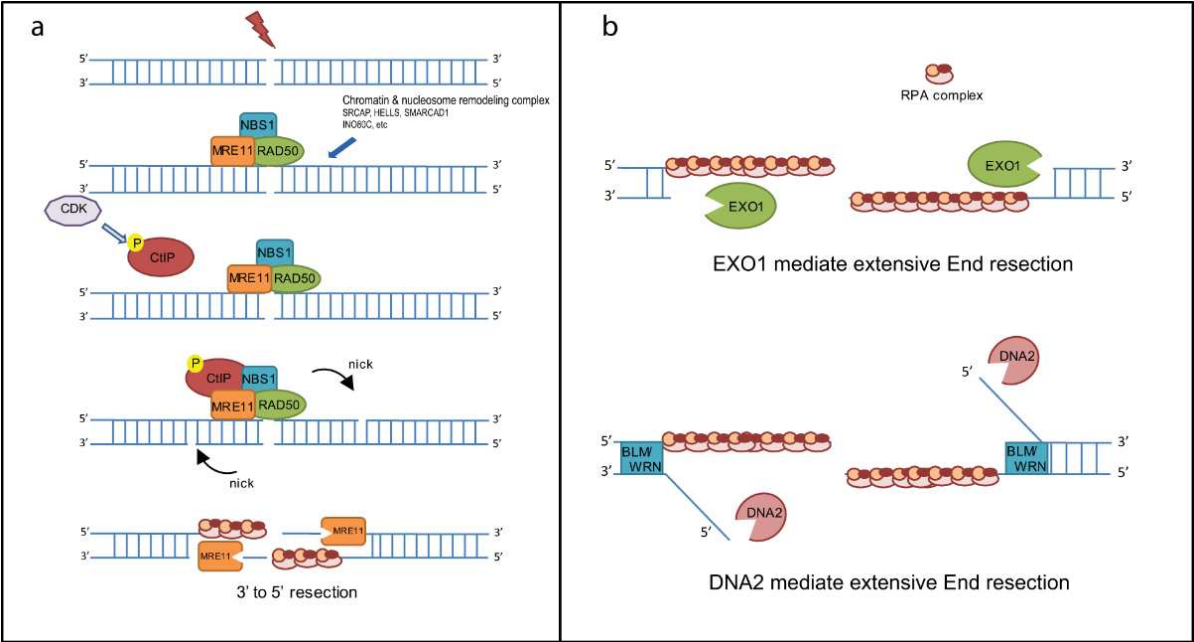
In yeast, Rad52 plays a crucial role in displacing RPA from ssDNA and loading Rad51, essential for *in vivo* filament assembly and HR-mediated DSB repair (Benson et al., 1998; Milne & Weaver, 1993; Miyazaki et al., 2004; New et al., 1998; Shinohara & Ogawa, 1998; Sugawara et al., 2003; Sung, 1997). However, in mammalian cells, the absence of RAD52 does not result in significant DNA repair defects, unlike in yeast. Instead, the tumor suppressor BRCA2 is the primary mediator, loading RAD51 at DSB sites to facilitate nucleoprotein filament formation (Carreira et al., 2009; Davies et al., 2001; Jensen et al., 2010).

Furthermore, yeast Rad51 paralogs (Rad55-Rad57 and Shu1-Psy3) and their mammalian counterparts (RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3) enhance the stability of the Rad51 filament and facilitate its nucleation (J. Liu et al., 2011; Suwaki et al., 2011). Additionally, members of the Swi2/Snf family like Sp/ScRad54 in yeast and hsRAD54 in humans promote Rad51 nucleation in an ATPase-independent manner (Ceballos & Heyer, 2011; Wolner & Peterson, 2005).

Mediators also counteract anti-recombinases like Sp/ScSrs2 and SpFbh1 in yeast, and hsFBH1 in humans, which work to dissociate Rad51 from ssDNA to recycle Rad51 molecules or remove unwanted HR intermediates (Burgess et al., 2009; Kohzaki et al., 2007; Qiu et al., 2013). This delicate balance between assembly and disassembly of Rad51 filaments regulates the initiation of HR.



**Figure 7: Model of two-steps resection of DSB.** a) A model illustrating the role of the MRN complex, CtIP, and chromatin/nucleosome remodelling proteins in the initiation of DNA end resection. b) A model depicting the involvement of EXO1, DNA2, BLM, WRN, and the RPA complex in the extension of DNA end resection. (F. Zhao et al., 2020)



## 1.2 Synaptic phase

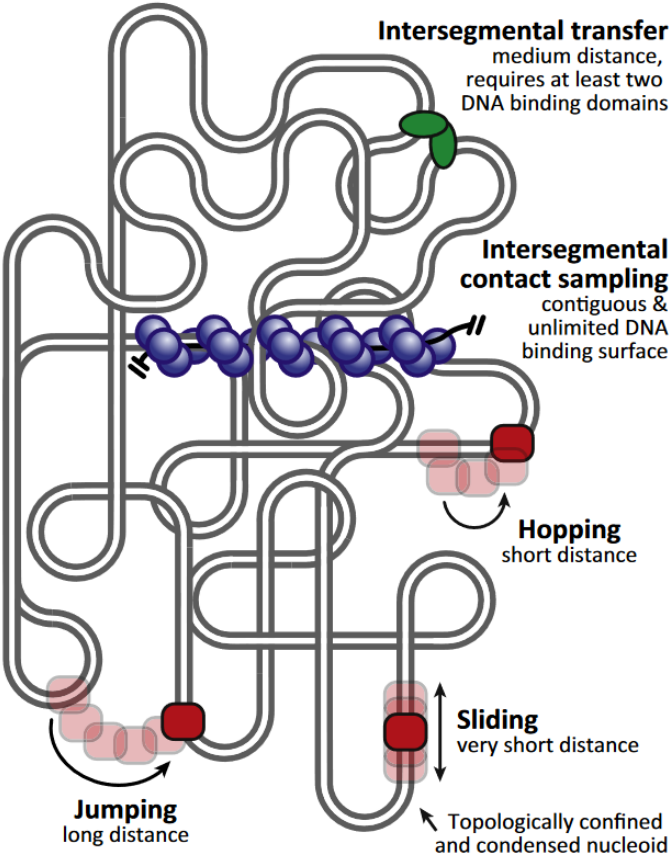
Once the Rad51-ssDNA nucleoprotein filament is formed, it initiates the search for a homologous DNA sequence within the genome, where most sequences are not related. Insights from both in vitro and in vivo single molecule studies have shed light on how this search is efficiently conducted (Figure 8). Firstly, if the DSB site and the homologous sequence are already near each other before the DSB occurs (as seen with sister chromatids held together by cohesion), the likelihood of successful recombination is greatly enhanced (Aylon & Kupiec, 2004, p. 2). Secondly, the mobility of chromosomes bearing the DSB increases, which raises the chances of encountering a suitable donor template even if it is relatively distant (Dion et al., 2012; Miné-Hattab & Rothstein, 2012; Neumann et al., 2012). Thirdly, through a process known as "intersegmental contact sampling," the RecA/Rad51-ssDNA filament scans multiple regions of dsDNA by making brief, weak contacts, allowing it to quickly identify homologous sequences throughout the genome (Forget & Kowalczykowski, 2012; Piazza et al., 2017). Moreover, Rad51 utilizes a length-based recognition strategy that only considers sequences of eight consecutive homologous nucleotides in the donor molecule, efficiently filtering out shorter, non-matching sequences to enhance the probability of finding a correct match (Qi et al., 2015).

Upon locating a homologous sequence, the 3'-end of the nucleofilament invades the intact dsDNA, initiating a strand invasion reaction. This reaction forms a Rad51-bound heteroduplex where the invading strand pairs with its complementary strand from the donor DNA, displacing the non-complementary strand and creating a three-stranded structure known as a displacement loop (D-loop). Rad54 then facilitates the removal of Rad51 from the heteroduplex, enabling the commencement of DNA repair synthesis (X. Li et al., 2007; Solinger et al., 2002; Thomä et al., 2005; W. D. Wright & Heyer, 2014).

## 1.3 Postsynaptic phase

The commencement of DNA synthesis from the invading 3'-end marks the beginning of the post-synaptic phase of repair. In yeast, this synthesis is primarily conducted by polymerase delta (Pol  $\delta$ ), aided by its processivity factor, PCNA (X. Li et al., 2009; Maloisel et al., 2008; X. Wang et al., 2004). In humans, POL $\delta$  is also supported by the translesion synthesis polymerase eta (POL $\eta$ ) (McIlwraith et al., 2005; Sneed et al., 2013). This activity involves copying the complementary sequence of the donor DNA, progressively extending both the heteroduplex and the displacement loop (D-loop), leading to the formation of a structure known as a Holliday junction (HJ) (Holliday, 1964).

**Figure 8: Single molecule methods used to measure RecA-mediated homology search.** An illustration depicting various mechanisms through which RecA locates the target, including sliding, hopping, jumping, intersegmental transfer, and intersegmental contact sampling. (Bell & Kowalczykowski, 2016)



Ultimately, it results in the recovery of the missing genetic information from the donor DNA sequence.

Once DNA synthesis is over, recombination can proceed through pathways involving or bypassing DNA strand crossover, where a crossover event entails exchanging distal arms of broken DNA with template molecule arms, facilitating reciprocal genetic exchange between donor and acceptor DNA molecules ([Figure 9](#)).

### 1.3.1 Synthesis-dependent strand annealing (SDSA)

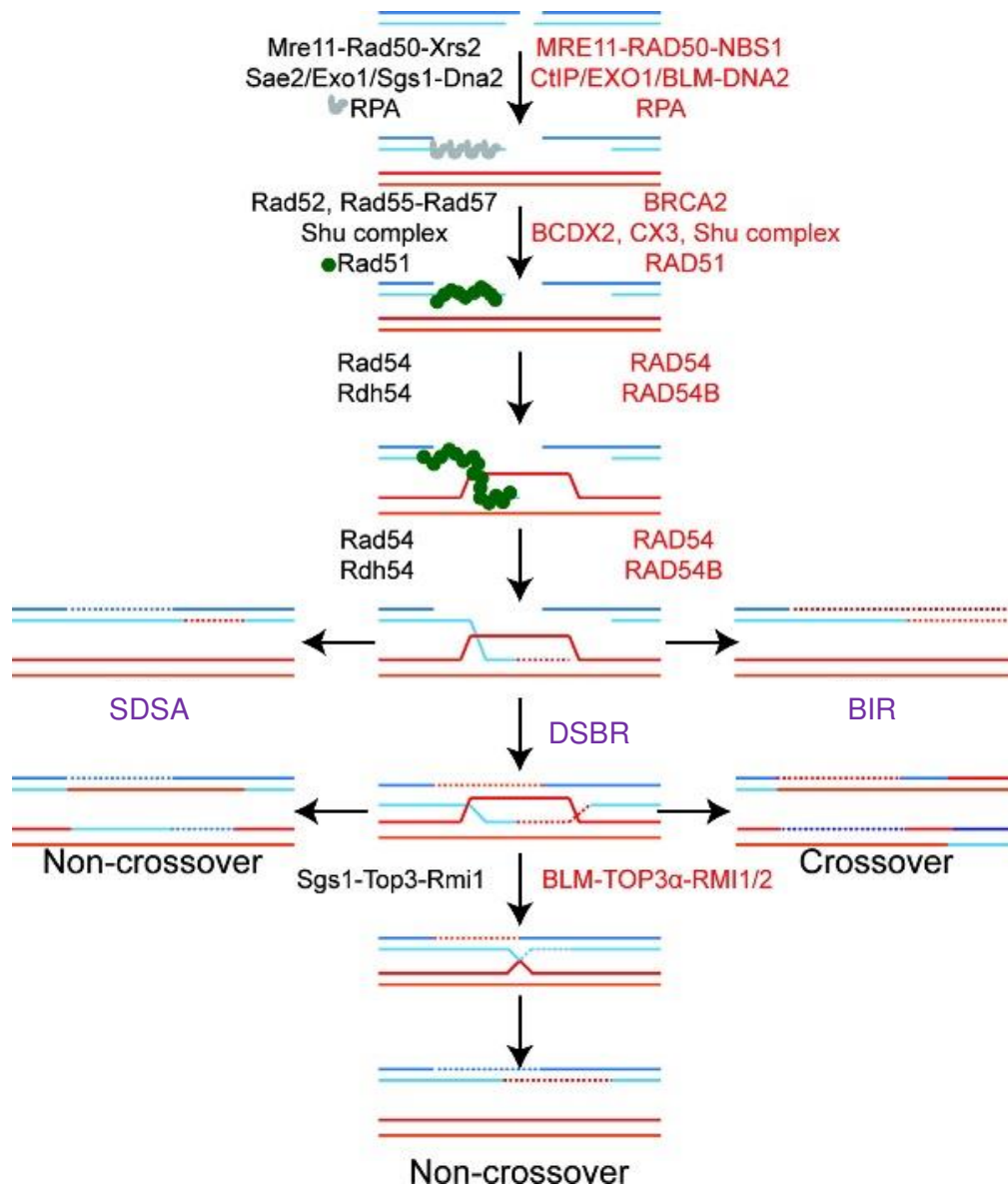
In the SDSA pathway, the invading strand extends within the displacement loop (D-loop) which is subsequently displaced—a process known as D-loop dissociation ([Figure 9](#)). The newly synthesized DNA then re-anneals with the broken molecule's single-stranded DNA (ssDNA), followed by gap-filling and ligation for DNA restoration. Several motor proteins play crucial roles in facilitating D-loop dissociation. In yeast, these include Srs2 and the Sgs1-Top3-Rmi1 (STR) complex (Chavdarova et al., 2015; Fasching et al., 2015). Srs2 is therefore considered to display anti-recombinogenic function in this case, despite dismantling Rad51-ssDNA filaments being its primary role in HR regulation (Meir et al., 2023). Recent work suggests that Rad54 rather works as pro-recombinase leading to faster invasions and non-crossover repair in SDSA (Sridalla et al., 2024). Similarly, in humans, the BLM-TOPOIII $\alpha$ -RMI1/2 complex (also known as the BTRR complex) is responsible for unwinding the D-loop intermediate (Bachrati, 2006; Bugreev et al., 2007; Harami et al., 2022). The coordinated actions of helicases and topoisomerases prevent the formation of double Holliday junctions, ensuring the exclusive production of non-crossover products. This outcome renders the SDSA pathway one of the least mutagenic sub-pathways of homologous recombination (HR), making it the preferred pathway in somatic or vegetative cells.

When reannealing is not possible due to the absence of the second broken end, another pathway known as Break-Induced Repair (BIR) becomes active (Kramara et al., 2018). This alternative mechanism, primarily involved in broken fork repair, will be discussed in more detail in Subchapter B (Section 3.1).

### 1.3.2 Double-strand break repair (DSBR)

In the DSBR pathway, stabilization of the displacement loop (D-loop) allows the displaced strand to anneal with the second resected double-stranded DNA (dsDNA) end ([Figure 9](#)), an event known as "second end capture". This leads to the formation of a structure called a double Holliday junction (dHJ) (Duckett et al., 1988). These dHJs must be processed before the physically linked DNA molecules can be separated. This

**Figure 9: Pathways of homologous recombination in DSB repair.** After strand invasion and the formation of a D-loop intermediate, three distinct pathways can be followed during the post-synaptic phase of homologous recombination. These pathways include SDSA (synthesis-dependent strand annealing), DSBR (double-strand break repair), and BIR (break-induced repair). The protein names involved in these pathways are shown in black for budding yeast HR factors and in red for human. (Adapted from Godin et al., 2016)



processing occurs through two enzymatically distinct mechanisms, each leading to different genetic outcomes ([Figure 10](#)).

In one approach, dHJs are dissolved by complexes such as the STR/BTR complexes in yeast and humans, respectively (Cejka et al., 2010; L. Wu & Hickson, 2003). Dissolution of the dHJs separates the recombining DNA molecules without exchanging the adjacent DNA sequences, thereby exclusively producing non-crossover products. Alternatively, dHJs can be resolved by specific enzymes known as resolvases. These enzymes include mammalian MUS81-EME1, SLX1-SLX4, GEN1, and their yeast counterparts: ScMus81-Mms4, ScSlx1-Slx4, Yen1 (absent in fission yeast) (Boddy et al., 2001; X.-B. Chen et al., 2001; Ciccina et al., 2003; Fricke & Brill, 2003; Rass et al., 2010; Wyatt et al., 2013). The specific cleavage of DNA strands within the dHJs by resolvases determines whether the genetic outcome will be a crossover or non-crossover product.

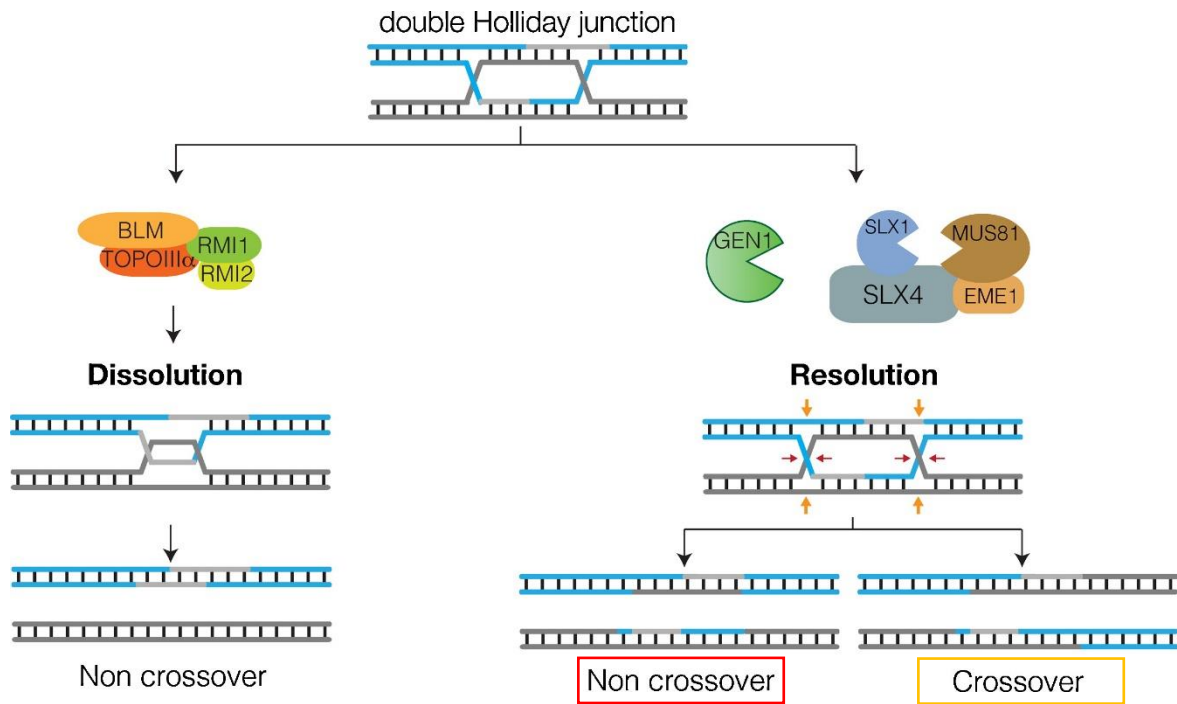
### 1.3.3 Single-strand annealing (SSA)

The SSA pathway repairs double-strand breaks (DSBs) occurring between two direct repeated sequences. While this pathway does require the recombinase Rad51, it is still often considered alongside other homologous recombination (HR) pathways. This classification stems not only from the enzymatic requirements but also because repair begins with an extensive resection—a common feature across all recombination pathways—and necessitates sequence homology (Bhargava et al., 2016).

During SSA, end-resection reveals homology within the repeats, allowing the resultant 3' overhangs to anneal with each other under the guidance of Rad52. This process depends on the single-strand annealing activity of Rad52, but notably, does not require its interaction with Rad51. Cryo-electron microscopy and biochemical studies demonstrated that human RAD52 drives single-stranded DNA (ssDNA) annealing in conjunction with replication protein-A (RPA) (C.-C. Liang et al., 2024). The N-terminal domain of RAD52 promotes ssDNA annealing while the C-terminal region regulates ring conformation and RPA interaction (C.-C. Liang et al., 2024). Subsequently, any unpaired single-strands, or flaps, are trimmed by specific endonucleases such as yeast ScRad1-Rad10 and ScMsh2-Msh3, or XPF-ERCC1 in mammals (Fishman-Lobell & Haber, 1992; Sugawara et al., 1997). DNA synthesis then fills in the gaps, and ligation follows to restore the integrity of the DNA duplex.

Although the SSA pathway is mutagenic—leading to the loss of the sequences between the repeats and one of the two copies—it serves as a critical backup mechanism in scenarios where other repair options are unavailable. Inactivating RAD52 in cells with

**Figure 10: Processing of the double Holliday junction.** Two enzymatically distinct processes result in the resolution of Holliday junctions with different genetic outcomes. Double Holliday junctions (dHJ) can be disengaged through dissolution, leading to the formation of non-crossover recombinants. Alternatively, dHJs can be resolved by endonucleolytic cleavage, which can produce either non-crossovers or crossovers, depending on which strands are cleaved (as indicated by the arrows). (J. Matos & West, 2014)



deficiency in BRCA1 or BRCA2 leads to synthetic lethality, and aberrant RAD52 expression correlates with unfavorable cancer prognosis (W. Feng et al., 2011; V. Ho et al., 2020; Lok et al., 2013; Uhlen et al., 2017).

## 2. Homologous Recombination in DSB repair: competition with Non-Homologous End Joining

DSBs prompt various repair pathways beyond HR, including SSA and NHEJ, each with distinct outcomes as shown in [Figure 11](#) (Chapman et al., 2012; R. D. Johnson, 2000; F. Liang et al., 1998; Rouet et al., 1994). Non-Homologous end joining (NHEJ) is a template-independent mechanism that swiftly joins DNA blunt ends, facilitated by the heterodimer Ku70-Ku80 complex (here-after, called Ku), safeguarding ends from excessive processing, thus maintaining genomic integrity (Bertuch & Lundblad, 2003; H. H. Y. Chang et al., 2017; Mimori & Hardin, 1986; Spagnolo et al., 2006; Walker et al., 2001).

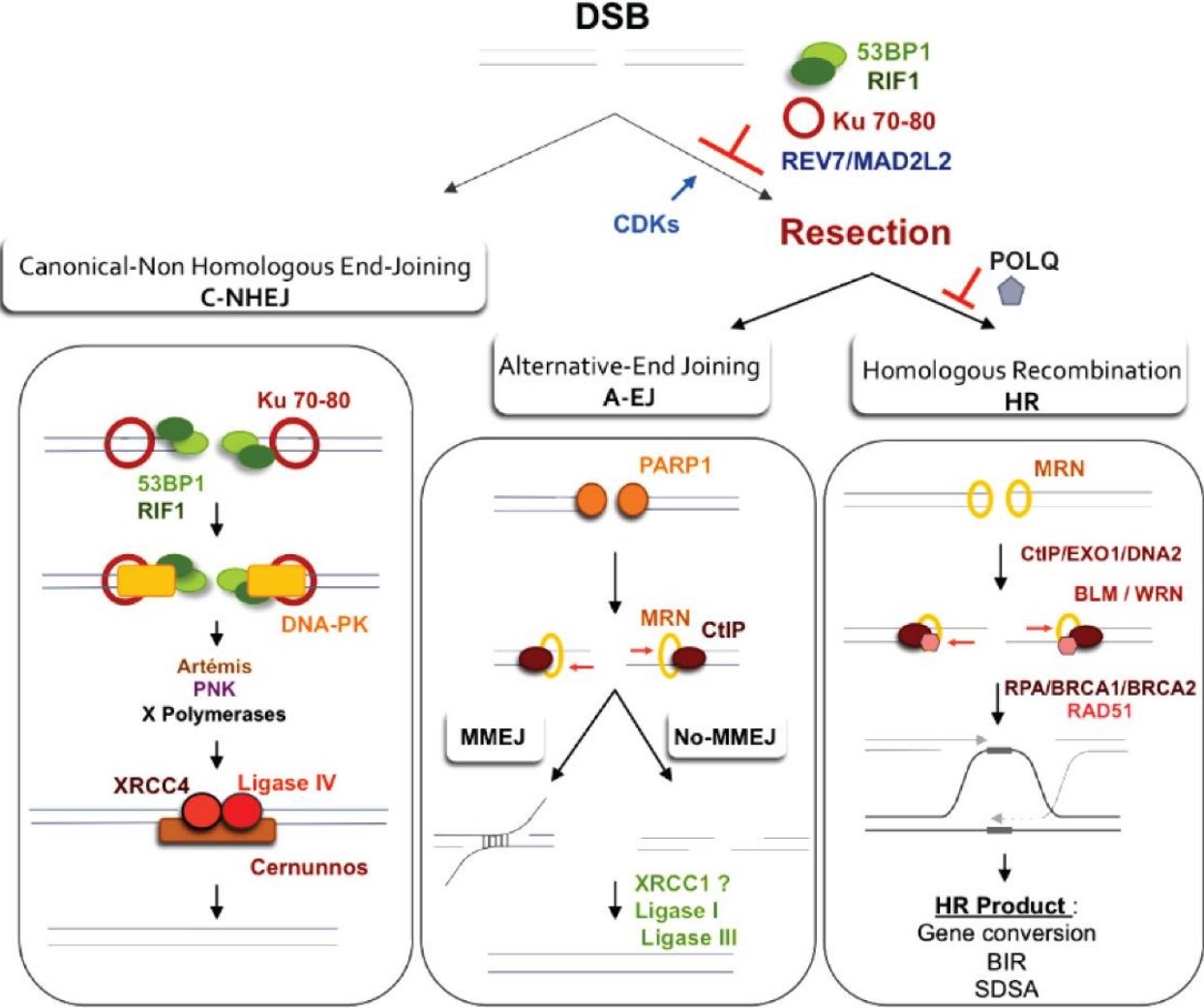
It is fast and efficient and often not mutagenic when no DNA end processing is engaged. However, the repair of dirty DSBs that require processing to make them prone to ligation, often results in limited loss of genetic information at the damage locus, accompanied by short deletions that can disrupt the reading frame of a gene, thus resulting in a loss of function. This error-prone NHEJ repair of DSBs is exploited during gene inactivation by CRISPR-Cas9-based strategy (Jayavaradhan et al., 2019).

The balance between HR and NHEJ for DSB repair, pivotal for genome stability, is governed by various mechanisms (Shrivastav et al., 2008). One significant factor influencing the choice of repair pathway is the cell cycle phase. HR requires a homologous sequence from an intact donor, typically the sister chromatid, making it predominant during the S and G2 phases of the cell cycle when these templates are available (Escribano-Díaz et al., 2013; Ira et al., 2004; Orthwein et al., 2015). In contrast, NHEJ does not require a homologous template, allowing it to function throughout the cell cycle, except during mitosis. Consequently, NHEJ is the primary repair pathway in G1, while HR becomes more active during the S phase, and both pathways are in competition during the G2 phase (Branzei & Foiani, 2008; Karanam et al., 2012; Rothkamm et al., 2003).

Another critical determinant for the direction of repair is the extent of DNA end-processing (Cejka, 2015). While NHEJ requires either no or minimal DNA end processing, all HR sub pathways are initiated by the DNA resection step to expose 3' overhang single-stranded DNA (ssDNA) coated by RPA. This step of DNA end resection



**Figure 11: Overview of DSB repair pathways.** A schematic illustrating the major double-strand break (DSB) repair pathways: homologous recombination (HR), non-homologous end joining (NHEJ), alternative end-joining (alt-EJ), and single-strand annealing (SSA). The choice of DSB repair pathway primarily depends on whether DNA end resection occurs. When end resection takes place, HR, alt-EJ, and SSA can compete to repair the DSBs. In contrast, when end resection is inhibited, NHEJ is favored. Key repair factors involved in each pathway are indicated in the scheme. (Gelot et al., 2015)



is critical to steer DSB repair towards HR, likely to ensure that they have a donor template in the S and G2/M phases. (Chapman et al., 2012; Shibata, 2017; Symington & Gautier, 2011). This regulatory mechanism is also influenced by the cell cycle, as key resection factors are activated specifically during the S/G2 phases by CDKs and are repressed in G1 (Caspari et al., 2002; Huertas et al., 2008; Ira et al., 2004). In human cells, the choice of repair pathway is further regulated by the antagonistic interplay between 53BP1 and BRCA1, which respectively repress and promote resection (Bouwman et al., 2010; Bunting et al., 2010; L. Feng et al., 2015; Nacson et al., 2018).

The complexity of DSB damage influences repair pathway choice, with HR preferred for complex lesions induced by heavy ion radiation in the G2 phase (Shibata et al., 2011). HR is vital for addressing replication-associated DNA lesions, such as one-ended DSBs and protein blocks, due to NHEJ's limitations, underscoring HR's significance in replicative scenarios.

## B: Roles of HR pathway in dealing with replication-associated DNA damages

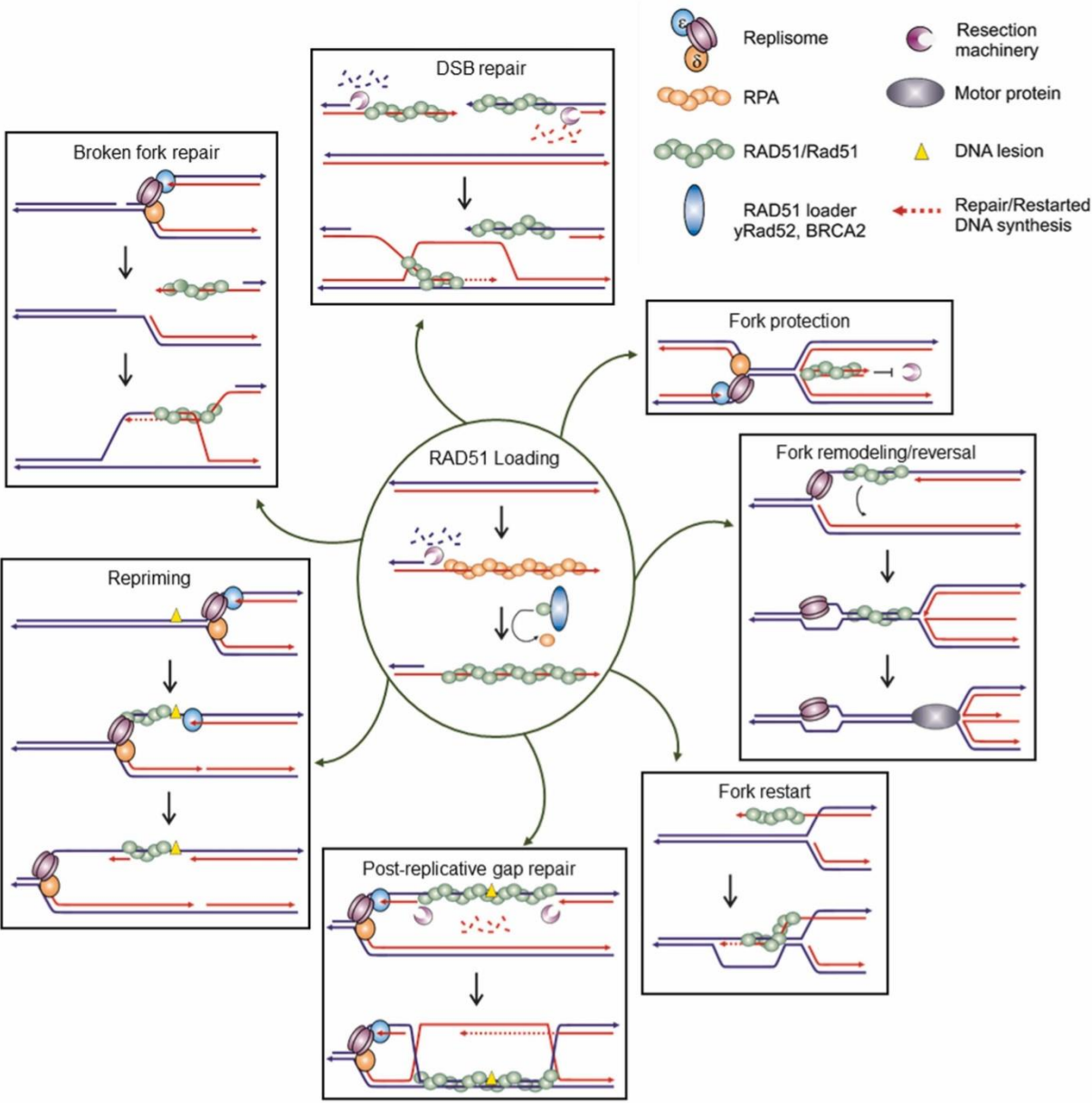
The inability to fully replicate the genome when cells enter mitosis can result in mitotic failure. To prevent this and ensure full chromosome replication, cells use various repair pathways to: i) restore replication competence at dysfunctional or broken forks, ii) maintain stalled fork integrity, and iii) fill in ssDNA gaps behind the forks ([Figure 12](#)). Below, I summarize how the homologous recombination (HR) machinery regulates these mechanisms to ensure continuous DNA synthesis and complete genome duplication.

### 1. Fork remodelling

Upon replication stress, replication forks may reverse locally, temporarily halting DNA synthesis. However, even undamaged forks can be reversed globally through signal transduction initiated from forks challenged by interstrand cross-links (Mutreja et al., 2018). This fork reversal transforms the fork into a four-branched DNA structure, similar to a Holliday junction (called "chicken foot"), with newly synthesized strands forming a regressed arm and parental strands re-annealing into a duplex.

This phenomenon was first thoroughly studied in bacteria. In *E. coli*, fork reversal follows replication perturbation, leading to enzymatic cleavage that produces a replicative double-strand break (DSB), which is toxic in the absence of homologous recombination (HR) (Michel et al., 2007; Seigneur et al., 1998; Septenville et al., 2012).

**Figure 12: The multifaceted functions of homologous recombination in DSB and replication-associated DNA damage.** See text for details. (Chakraborty et al., 2023)



In budding yeast, early electron microscopy studies showed that checkpoint-deficient cells treated with hydroxyurea (HU) accumulated four-branched structures, indicating reversed forks (M. Lopes et al., 2001; Sogo et al., 2002). It was thus considered that fork reversal is a pathological DNA structure. In fission yeast, fork reversal is prevented by the checkpoint kinase Cds1 via the phosphorylation of the endonuclease Dna2 that ensures the processing of the 3' or 5' flap at stalled forks. (Hu et al., 2012). Thus, the DNA replication checkpoint in yeast prevents fork reversal. More recent studies found that yeast cells treated with Camptothecin (CPT), an inhibitor of the topoisomerase I, which induces positive supercoils ahead of replication forks, also showed fork reversal (Menin et al., 2018; Ray Chaudhuri et al., 2012). Electron microscopy analyses of replication forks purified from mammalian cells treated with agents that induce nucleotide depletion, oxidative base damage, UV photoproducts, topoisomerase cleavage complexes, or DNA crosslinks revealed that around 25% of analyzed forks were reversed (Zellweger et al., 2015).

Several motor proteins in mammals, including members of the SNF2 helicase family such as SMARCAL1, ZRANB3, and HLTF, facilitate fork reversal and can catalyze replication fork remodelling both *in vitro* and *in vivo* (Achar et al., 2011; Bétous et al., 2012; Blastyák et al., 2010; Poole & Cortez, 2017). The RAD51 recombinase also participates in this process, with its depletion reducing reversed replication structures (Mijic et al., 2017; Zellweger et al., 2015). Notably, fork reversal relies on RAD51's strand exchange activity, not on the formation of a stable RAD51 nucleofilament (W. Liu et al., 2023; Mijic et al., 2017). Of note, it has been published that fork reversal does not require the strand exchange activity of Rad51 (Mason et al., 2019a, p. 201). In this study, it has been shown that fork reversal rather depends on a non-enzymatic function of Rad51 binding. Hence, it is important to mention that this point is rather conflictual in the literature obtained so far.

A recent report suggests that RAD51-mediated fork reversal allows bypassing the CMG helicase by annealing the parental strands behind the stalled fork, while SMARCAL1 extends this duplex, causing the nascent strands to anneal together (W. Liu et al., 2023). This mechanism enables fork reversal without unloading the replication machinery, keeping the helicase ready to resume DNA synthesis when conditions improve. Alternatively, RAD52 fortifies stalled replication forks during replication stress, shielding them from excessive reversal by SMARCAL1 by direct competition (Honda et al., 2023; Malacaria et al., 2019). Loss of RAD52 function results in defective replication restart, under-replicated regions, chromosome instability, and reliance on RAD51 for

replication completion, indicating a gatekeeper role in limiting excessive fork remodelling (Oehler et al., 2023).

Nevertheless, fork reversal is crucial for genome stability, providing time for DNA repair machinery to resolve issues and prevent DSB formation (Berti et al., 2013; Mutreja et al., 2018; Neelsen & Lopes, 2015; Ray Chaudhuri et al., 2012; Vujanovic et al., 2017). It also enables template switching for error-free DNA synthesis (Zellweger et al., 2015). Fork reversal acts as a reversible "holding state," quickly restoring normal replication once conditions improve. In human cells, two pathways restore reversed forks to a three-way structure: RECQ1-dependent branch migration, activated after the stress is removed (Berti et al., 2013; Zellweger et al., 2015). The other depends on the degradation of the reversed arm by WRN helicase and DNA2 nuclease, independently of EXO1, MRN, and CtIP resection (Thangavel et al., 2015).

## 2. Fork protection

In the reversed fork structure, the nascent strands form a reversed arm, creating a one-ended DNA end that mimics a DSB and is processed by nucleases like MRE11, EXO1, DNA2, and MUS81 (Berti et al., 2020; Lemaçon et al., 2017; Mijic et al., 2017; Thangavel et al., 2015). Stalled replication forks often have exposed ssDNA, making them vulnerable to nucleolytic degradation. HR factors, especially BRCA2 and RAD51, regulate fork degradation to preserve their integrity and stability. Without these factors, the reversed arm at fork reversal is degraded by the nuclease, as shown by DNA fiber assays and EM in human and *Xenopus* cells (Hashimoto et al., 2010; Ray Chaudhuri et al., 2016; Schlacher et al., 2011; Ying et al., 2012). This phenomenon is referred to as unprotected forks.

In fission yeast, Rad51's role in protecting arrested forks from nuclease attacks is vital for maintaining a DNA structure proficient for merging with an opposing fork (Ait Saada et al., 2017a). The DNA-binding mutant Rad51-T131P, forming an unstable filament, fails to protect replication forks from nuclease attack (Kolinjivadi et al., 2017; Mijic et al., 2017). Conversely, human cells expressing RAD51-II3A, retaining DNA binding but lacking strand exchange activity, effectively protects replication forks (Mason et al., 2019b, p. 2). This suggests that the role of RAD51 in ensuring fork protection does not depend on its strand exchange activity but on its ability to form a stable nucleofilament onto DNA. Evidence suggests that stable Rad51 nucleofilaments can directly protect forks from Mre11-dependent DNA degradation *in vitro*, whereas RPA cannot (Kolinjivadi et al., 2017). In humans, the protective function of RAD51 against nuclease attack largely involves its capacity to bind dsDNA instead of ssDNA.

This binding to dsDNA is regulated by the C-terminal domain of BRCA2 (Halder et al., 2022). BRCA2 C-terminus is also crucial for stabilizing the RAD51 filament on the regressed arm of a reversed fork (Kwon et al., 2023). Cells lacking BRCA2 suffers from the accumulation of unprotected forks, and this can be alleviated by RAD51 downregulation (Kolinjivadi et al., 2017; Lemaçon et al., 2017; Mijic et al., 2017). Thus, RAD51 not only promotes fork reversal independently of BRCA2 but also works with BRCA2 to shield the reversed fork from excessive degradation.

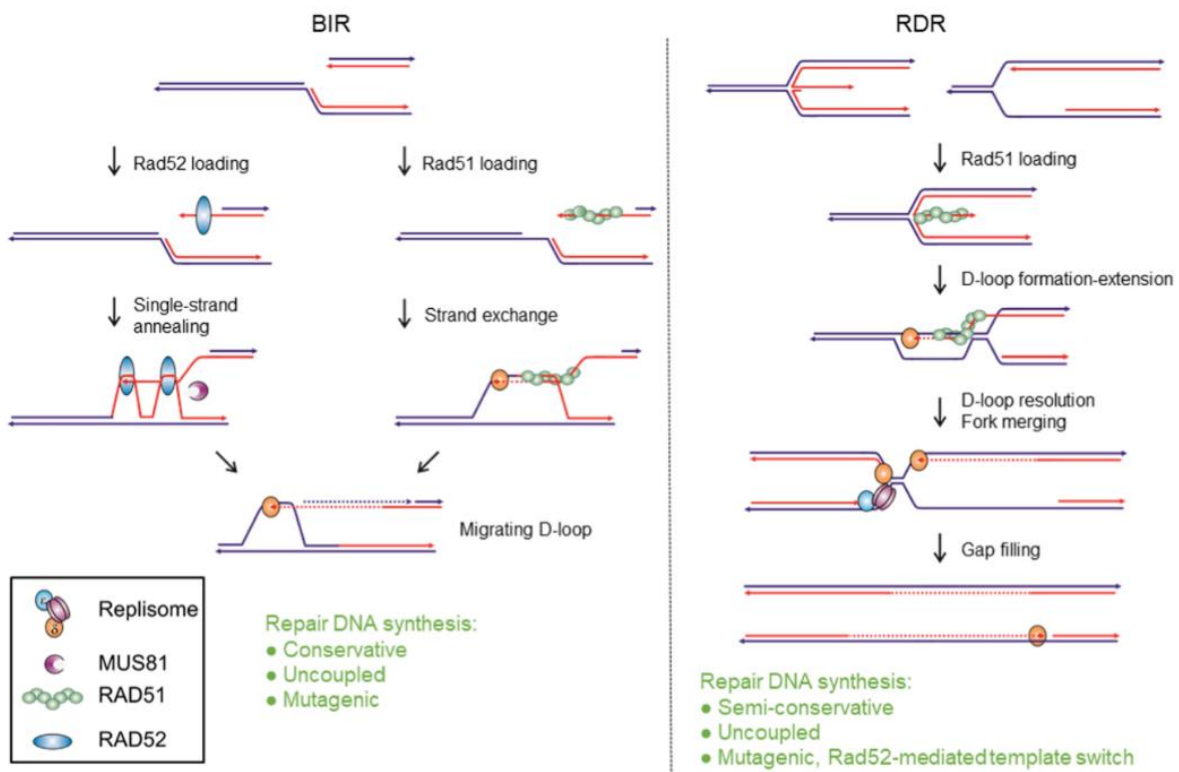
### 3. Fork repair and restart

When a replication fork stalls, collapses or becomes dysfunctional, it may be rescued by a converging fork from a newly activated origin nearby. As previously noted, HR factors play a crucial role in maintaining the integrity of halted replication forks. However, dysfunctional forks can also be restarted via HR and these restarted forks are rescued upon arrival of a converging fork. This is essential to prevent unfinished DNA replication and the risks associated with under-replication. Research in yeast, employing site-specific fork arrest assays, has shown that HR aids in completing DNA replication by restoring the DNA synthesis capability of dysfunctional forks through two key mechanisms: Break-Induced Replication (BIR) and Recombination-Dependent Replication (RDR) (A. Carr & Lambert, 2021). Notably, the DNA synthesis repair facilitated by either BIR or RDR presents characteristics distinct from those of canonical DNA replication.

#### 3.1 BIR

BIR has been extensively studied in the G2/M phase of yeast cells (Bosco & Haber, 1998; Malkova et al., 1996; Morrow et al., 1997). However, a later study showed that when a replication fork encounters a nick or a single-strand DNA break, it can become a broken fork, resembling a one-ended DSB. Without a converging fork, the dysfunctional broken fork may be repaired through Break-Induced Replication (BIR) to resume DNA synthesis ([Figure 13](#)) (Ait Saada et al., 2018; Mayle et al., 2015). BIR is initiated with an extensive 5' to 3' resection at the break site. The resulting 3' ssDNA overhang is coated by the Rad51 filament, which invades a homologous template. DNA synthesis occurs through a migrating D-loop and can extend over hundreds of kilobases, extruding the leading strand continuously from the D-loop (Donnianni & Symington, 2013; Saini et al., 2013; Wilson et al., 2013). This nascent ssDNA serves as a template for lagging strand synthesis. BIR is mechanistically distinct from canonical replication, being conservative rather than semi-conservative, and synthesis of leading

**Figure 13: Models of Break-induced Replication (BIR) and Recombination-dependent-replication (RDR).** On the left: Break-induced replication (BIR) can initiate from a broken replication fork during the S-phase or G2/M phase. Both Rad52-dependent and Rad51-independent BIR mechanisms are illustrated. On the right: RDR (Replication-Dependent Repair) begins from a single-stranded DNA (ssDNA) gap that is generated by the controlled degradation of the nascent strand, which can occur at a reversed fork or in the absence of fork reversal. (Chakraborty et al., 2023)



and lagging strands are asynchronous, facilitated by Pol  $\delta$  with the assistance of the non-essential subunit Pol32 (Donnianni et al., 2019; L. Liu et al., 2021; Lydeard et al., 2007). Instead of the CMG replicative helicase, BIR employs the Pif1 helicase, unwinding the DNA duplex and extruding newly synthesized DNA from the D-loop at a slower rate compared to canonical replication (Wilson et al., 2013). Pif1 allows the progression of BIR-mediated DNA synthesis, however, at a slower rate than the canonical replication: 0.5Kb/min vs 2Kb/min (L. Liu et al., 2021). Since BIR uses Pol32 dependent synthesis it is error-prone in nature. The mutagenic synthesis by BIR is limited to within a few kilobases from the break by Mus81 endonuclease and a converging fork. Lack of a timely converging fork or Mus81 may propel genome instability and cancer (Mayle et al., 2015). In vertebrate systems, such as in *Xenopus* egg extracts, the MRE11 nuclease and RAD51 are essential for restoring replisome integrity following fork collapse and breakage, highlighting the necessity of a homologous recombination-mediated mechanism to repair and restart broken replication forks (Hashimoto et al., 2010).

In Rad51-deficient yeast cells, a less efficient BIR pathway is observed. Unlike Rad51-dependent BIR, this Rad51-independent pathway requires significantly less homology for repair initiation and relies on Rad52's single-strand annealing activity to anneal resected broken DNA ends with a homologous region (Davis & Symington, 2001; Malkova et al., 2001). Although a minor pathway in budding yeast, recent evidence suggests that a RAD51-independent form of BIR plays a more prominent role in human cells during mitotic DNA synthesis (MiDAS). MiDAS is the process of DNA synthesis occurring during mitosis to repair under-replicated DNA. It serves as a critical mechanism to ensure complete replication of the genome before cell division, thereby maintaining genomic stability (Bhowmick et al., 2016; Minocherhomji et al., 2015). MiDAS is initiated at "difficult-to-replicate" sites like common fragile sites by MUS81- and EME1-dependent cleavage of unresolved and late replication intermediates, serves as a "last chance" mechanism to resume DNA synthesis and complete genome duplication before chromosome segregation (Bhowmick et al., 2016; Bhowmick & Hickson, 2017; Minocherhomji et al., 2015). It was initially reported as a RAD51 and BRCA2-independent but RAD52 and POLD3 dependent form of BIR (Bhowmick & Hickson, 2017; Sotiriou et al., 2016). However, a recent report identified an unexpected role of RAD51 in promoting MiDAS, which relies on protecting the under-replicated DNA in mitotic cells (Wassing et al., 2021).

However, completed DNA replication by BIR to ensure proper chromosome segregation come at the risk of increased mutations. Such mutations include complex



genome rearrangements, frameshift mutations from frequent template switches, accumulation of mutations and DNA damage at exposed ssDNA, translocations from ectopic invasion, half-crossovers from intermediate resolution, and loss of heterozygosity (Costantino et al., 2014; Deem et al., 2011; Elango et al., 2019; Osia et al., 2022; Sakofsky et al., 2014).

### 3.2 RDR

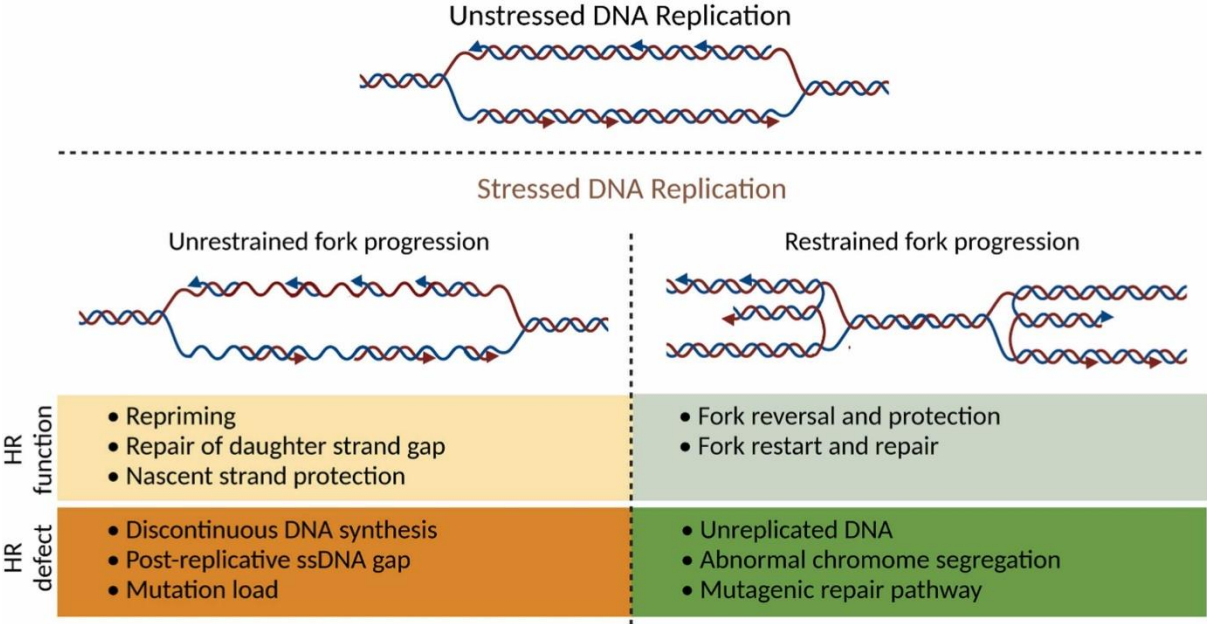
In the last decade, research has revealed that HR-mediated replication fork restart can occur independently of double-strand breaks (DSBs) ([Figure 13](#)) (Lambert et al., 2010; Mizuno et al., 2009a; Petermann et al., 2010; Zellweger et al., 2015). This process, termed recombination-dependent replication (RDR), has been predominantly investigated in fission yeast using a site-specific replication fork barrier (RFB) that induces fork collapse without causing a DSB (Lambert et al., 2010; Mizuno et al., 2009a).

In the initial phase of recombination-dependent replication (RDR), dysfunctional forks undergo reversal, creating a DNA end suitable for Ku protein binding. MRN-Ctp1 mediates initial resection, displacing Ku from the reversed arm and facilitating further long-range resection (Lambert et al., 2010; Miyabe et al., 2015; Teixeira-Silva et al., 2017). Rad51-dependent strand invasion follows, setting the stage for DNA synthesis. Unlike break-induced replication (BIR), DNA synthesis in RDR remains semi-conservative (Miyabe et al., 2015; Naiman et al., 2021a). In fission yeast experiments, forks restarted by RDR can resume activity within 15-20 minutes during the S-phase and may extend up to 20 Kb before merging with a converging canonical fork (Miyabe et al., 2015; Naiman et al., 2021a; Nguyen et al., 2015).

Further studies indicate that while Rad51 recombinase primarily initiates RDR, the template switch during fork progression relies on Rad52's single-strand annealing activity (Kishkevich et al., 2022). This non-canonical DNA synthesis is associated with various genetic instabilities. Ectopic recombination events during fork restart can lead to genomic rearrangements like translocations (Lambert et al., 2005). Conversely, correctly restarted forks are more error-prone, exhibiting increased replication slippage (RS), recombination between direct repeats, and the formation of dicentric and acentric isochromosomes at inverted repeats (Iraqi et al., 2012a; Jalan et al., 2019; Mizuno et al., 2013).

Recent research indicates that RDR might aid in completing DNA replication in human cells. Cells experiencing mild replication stress utilize an HR pathway involving RAD51 and RAD52 to sustain DNA synthesis until the G2-M transition, lessening genome

Figure 14: Replicative HR functions ensure complete genome duplication. (Chakraborty et al., 2023)



under-replication and replication stress-induced mitotic abnormalities. Unlike the MiDAS model, this resilient DNA synthesis doesn't rely on MUS81, suggesting that fork breakage isn't essential for maintaining DNA replication in G2 cells. (Mocanu et al., 2022).

While often used interchangeably, it's crucial to note that BIR and RDR are two distinct pathways. BIR, a specialized form of RDR, begins with a DSB, whereas RDR commences from an ssDNA gap. However, both entail non-canonical DNA synthesis and pose a higher mutation risk than conventional DNA synthesis. This underscores the trade-off between completing chromosome duplication and the heightened risk of genome instability from mutagenic repair mechanisms.

#### 4. Repriming and gap filling

When the replisome encounters a DNA lesion that inhibits normal replicative polymerases, specialized tolerance pathways come into play to maintain continuous fork progression. These pathways involve translesion synthesis (TLS) polymerases navigating past the lesion or re-initiating DNA synthesis downstream through repriming. In human cells, PRIMPOL facilitates repriming, while in budding yeast, it's the Primase-Pol alpha-Ctf4 complex (Bianchi et al., 2013; Fumasoni et al., 2015; García-Gómez et al., 2013; Mourón et al., 2013). Recent findings hint at homologous recombination (HR) factors influencing repriming through non-recombinogenic roles (Benureau et al., 2022). For example, in human cells lacking the TLS polymerase Eta, an increase in RAD51 accumulation behind replication forks following UV irradiation was noted, without a rise in recombination-like DNA intermediates (Benureau et al., 2022). Additionally, experiments with B02, an inhibitor disrupting RAD51 filament formation, suggest that RAD51 supports efficient repriming independently of strand invasion, possibly through interactions with Pol alpha (Di Biagi et al., 2023; Kolinjivadi et al., 2017).

In human cells, HR-mediated repair of post-replicative gaps is observed. After mild exposure to benzo(a)pyrene diol epoxide (BPDE), RAD51 forms foci in response to bulky DNA adducts. This RAD51 recruitment at post-replicative gaps occurs independently of replication fork stalling or collapse, facilitated by PRIMPOL repriming and MRE11- and EXO1-dependent resection (Piberger et al., 2020).

Overall, successful genome duplication depends on HR's replicative functions in multiple ways ([Figure 14](#)). First, by preserving halted replication fork integrity, HR ensures accurate termination, promotes fork repair and restart, and prevents genome

under-replication during mitotic transition. Second, by reducing the buildup of post-replicative gaps, HR supports uninterrupted DNA synthesis, thus preserving genomic stability.

### **III. SUMOylation is an important regulator of the DNA damage response and DNA repair**

#### **A. Post-translational modifications (PTMs)**

Before delving into the specifics of SUMOylation, it is important to note that PTMs are covalent modifications that change the properties of a protein to regulate its function, stability, localisation and interactions (Walsh et al., 2005). Common PTMs include phosphorylation, acetylation, glycosylation and ubiquitination. Other important modifications include SUMOylation, which is similar to ubiquitination, but is usually involved in stress responses, and will be discussed in details in the sub-sections III.B, III.C and III.D. Overall, PTMs allow cells to rapidly and precisely adjust protein function in response to various signals, making them essential for processes like cell signaling, DNA repair, and immune responses (Deribe et al., 2010; Mann & Jensen, 2003).

Out of the well known PTMs, one of the widely studied PTMs, called ubiquitination was first identified by Goldknopf in 1977, which led to the beginning of a new kind of post-translational signalling era (Goldknopf et al., 1977). Just as any other PTMs, ubiquitination is referred to as the process of covalent attachment of ubiquitin to substrate proteins. Ubiquitination is a complex PTM and has been shown to be regulated by more than 1000 proteins in human cells (Clague et al., 2015). In this process, ubiquitin is attached to substrate proteins through a three-step enzymatic cascade involving E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and various E3 ubiquitin ligases (detailed in section C.2.1) (Deshaies & Joazeiro, 2009; Pickart, 2001; Schulman & Harper, 2009; Ye & Rape, 2009). These E3 ligases facilitate the transfer of ubiquitin to target proteins. Ubiquitinated proteins are then recognized by receptors containing ubiquitin-binding domains (UBDs), while deubiquitinases (DUBs), a specialized family of proteases, remove ubiquitin modifications (Husnjak & Dikic, 2012; Komander et al., 2009). Large-scale proteomic studies have identified tens of thousands of ubiquitination sites across thousands of proteins, suggesting that most proteins undergo ubiquitination at some point in their cellular lifespan (Wagner et al., 2011). Over the years, researchers have identified that ubiquitination can be attached as single moieties or form polymeric chains which can trigger different outcomes respectively (Komander & Rape, 2012). Ubiquitination can form various branched and mixed linkage chains that needs to reach an 'ubiquitination threshold', in order to be

targeted for proteasomal degradation. A second layer in ubiquitination signalling is the presence of alternative ubiquitin modification. One of which is called SUMOylation and was not discovered until the late 1990s. The process was first identified through studies on the protein RanGAP1 (Ran GTPase-activating protein 1), which was found to be modified by a small ubiquitin-like protein. This small protein was initially termed SUMO (Small Ubiquitin-like Modifier) due to its structural similarity to ubiquitin, though it serves different functions (Mahajan et al., 1997). Key papers from 1996 to 1997 described SUMO modification, particularly the work by Melchior et al., who demonstrated the modification of RanGAP1 by SUMO1 (Lapenta et al., 1997; Matunis et al., 1996). This discovery marked the beginning of a new understanding of post-translational modifications distinct from ubiquitination, but still crucial in regulating a wide array of cellular processes, such as nuclear transport, gene expression, and protein stability.

## B: What is SUMOylation?

SUMOylation is another highly dynamic post-translational modification (PTM), which is involved in diverse cellular functions ranging from regulation of protein activity, stability, and localization (Geiss-Friedlander & Melchior, 2007). SUMOylation is also important for the regulation of DNA lesion repair within the cell (Cremona et al., 2012). The following chapter is dedicated to a detailed discussion on the key players of the SUMO cycle with a special focus on their importance in response to replication stress.

### 1. Mechanism and players

SUMOylation is one such PTM, which includes a covalent addition of a small ubiquitin-like modifier (SUMO) to target proteins. Similar to ubiquitination, SUMOylation is highly conserved amongst all eukaryotes. Yeast harbours a single SUMO polypeptide (ScSmt3 and SpPmt3 in budding and fission yeast respectively), whereas the mammalian counterpart harbours several SUMO paralogs (SUMO 1-5) (A. Chen et al., 1998; D. Guo et al., 2004; Lapenta et al., 1997; Y.-C. Liang et al., 2016; Mahajan et al., 1997; Matunis et al., 1996; Tanaka et al., 1999). Components involved in the SUMO pathway in humans and yeast are listed in Table 2.

The SUMO particle is first synthesized as an immature precursor. Immature SUMO precursors are successively cleaved by specific proteases to expose a C-terminal diglycine motif which is necessary for the following step of conjugation (Pichler et al., 2017). After processing, mature SUMO particles enter the SUMOylation cycle through a cascade of enzymatic steps ([Figure 15](#)). The first step involves SUMO adenylation, followed by the formation of a thioester bond with the E1 activating enzyme. The

**Table 2: Players of the SUMO pathway in humans, *S. cerevisiae* and *S. pombe*.** (Schirmeisen et al., 2021a)

SUMO Pathway Component	Humans	<i>S. cerevisiae</i>	<i>S. pombe</i>
Small ubiquitin-like modifier (SUMO)	SUMO-1, SUMO-2, SUMO-3, SUMO-4, SUMO-5	Smt3	Pmt3
Activating enzyme (E1)	SAE1 SAE2	Aos1 Uba2	Rad31 Fub2
Conjugating enzyme (E2)	Ubc9	Ubc9	Hus5
SUMO ligase (E3)	SP-RING type	PIAS1, PIAS2, PIAS3, PIAS4 Mms21	Siz1, Siz2 Mms21 Zip3
	other	RanBP2 * [69] HDAC4 [70], KPA1 [71], Pc2 [72], Topors [73]	
SUMO-targeted ubiquitin ligase (STUbl)	RNF4 RNF11	Slx5-Slx8 Uls1	Rfp1/Rfp2-Slx8 Rrp2 (predicted)
Sentrin/SUMO-specific protease (SENP)	SENP1 °,*, SENP2 °,*, SENP3, SENP5 ° SENP6, SENP7	Ulp1 °,* Ulp2	Ulp1 °,* Ulp2

\* Localized at the nuclear pore complex. ° Involved in SUMO maturation.

heterodimeric E1 enzyme consists of the SAE1-SAE2 subunits in humans, Aos1-Uba2 in budding yeast and Rad31-Fub2 in fission yeast ([Table 2](#)). SUMO is successively transferred to the E2 conjugating enzyme, forming another thioester bond. E2 conjugating enzymes are UBC9 in humans, ScUbc9 in budding yeast, and SpHus5 in fission yeast (Desterro et al., 1999; Gareau & Lima, 2010; Lois & Lima, 2005; Olsen et al., 2010).

Although the E2 enzyme can recognize and interact with some substrates, the subsequent E3 ligase is responsible for enhancing efficient SUMO transfer to the substrate through two mechanisms. Typically, E3 ligases bridge the SUMO-loaded E2 and the substrate, bringing them into close proximity (Hay, 2005; Pichler et al., 2004; Tatham et al., 2003). Alternatively, when the E2 enzyme can directly interact with the substrate, the E3 ligase binds the E2-SUMO complex and stimulates its ability to transfer SUMO to the substrate (Reverter & Lima, 2005). Consequently, E3 ligases promote the formation of an isopeptide bond between the C-terminal glycine of SUMO and the acceptor lysine of the target, ensuring substrate specificity (Gareau & Lima, 2010; Tozluoğlu et al., 2010).

These E3 SUMO ligases are divided into distinct families based on their structure and mechanism of action.

The Siz/PIAS-type proteins are the majorly studied family of SUMO E3 ligases. They contain a conserved SP-RING (Siz/PIAS-RING) domain required for their SUMO ligase activity (Hochstrasser, 2001; E. S. Johnson & Gupta, 2001; Y. Takahashi et al., 2001). The conserved RING (Really Interesting New Gene) finger domain is a specialised zinc-binding domain rich in cysteine or histidine residues found in many proteins, particularly those involved in protein ubiquitination (Freemont, 2000). It plays a crucial role in the function of E3 ubiquitin ligases, which catalyze the transfer of ubiquitin molecules to target proteins by an allosteric activation mechanism (C. A. Joazeiro et al., 1999). The RING domain in E3 ubiquitin ligases is responsible for facilitating the transfer of ubiquitin from an E2 ubiquitin-conjugating enzyme to a substrate protein by acting as a scaffold to bring E2 and the substrate together. The RING domain aligns the ubiquitin's C-terminal glycine on the E2 enzyme with the lysine residue on the substrate, enabling the transfer of ubiquitin. (*Hydrolases*, 2022; C. A. P. Joazeiro & Weissman, 2000).

Human cells have several members (PIAS 1-4), while budding yeast has two (ScSiz1 and ScSiz2), and fission yeast has one (SpPli1) (C. D. Chung et al., 1997; E. S. Johnson & Gupta, 2001; Rytinki et al., 2009; Watts et al., 2007; Xhemalce et al., 2004b). Moreover, another type of SUMO E3 ligase exhibiting E3 SUMO activity is MMS21 in humans,

ScMms21 in budding yeast, and SpNse2 in fission yeast. Despite harbouring the SP-RING enzymatic domain, they are unrelated to the Siz/PIAS family ([Figure 16](#)). Originally, both SpNse2 and ScMms21 were parts of the large, essential Smc5/6 complex involved in DNA repair (Andrews et al., 2005a; Potts & Yu, 2005; X. Zhao & Blobel, 2005). By far, only the SP-RING family of SUMO E3 ligases is evolutionarily conserved from yeast to humans.

The second type of SUMO E3 ligases is the well-characterized, mammal-specific nucleoporin RANBP2. They lack the SP-RING-type E3 ligase structure, but contain two internal repeats named IR1 and IR2 which can bind to UBC9 (Pichler et al., 2004). RANBP2 promotes SUMOylation by positioning the SUMO-loaded E2 in an optimal orientation for efficient transfer without making direct interactions with the substrates (Reverter & Lima, 2005; Werner et al., 2012).

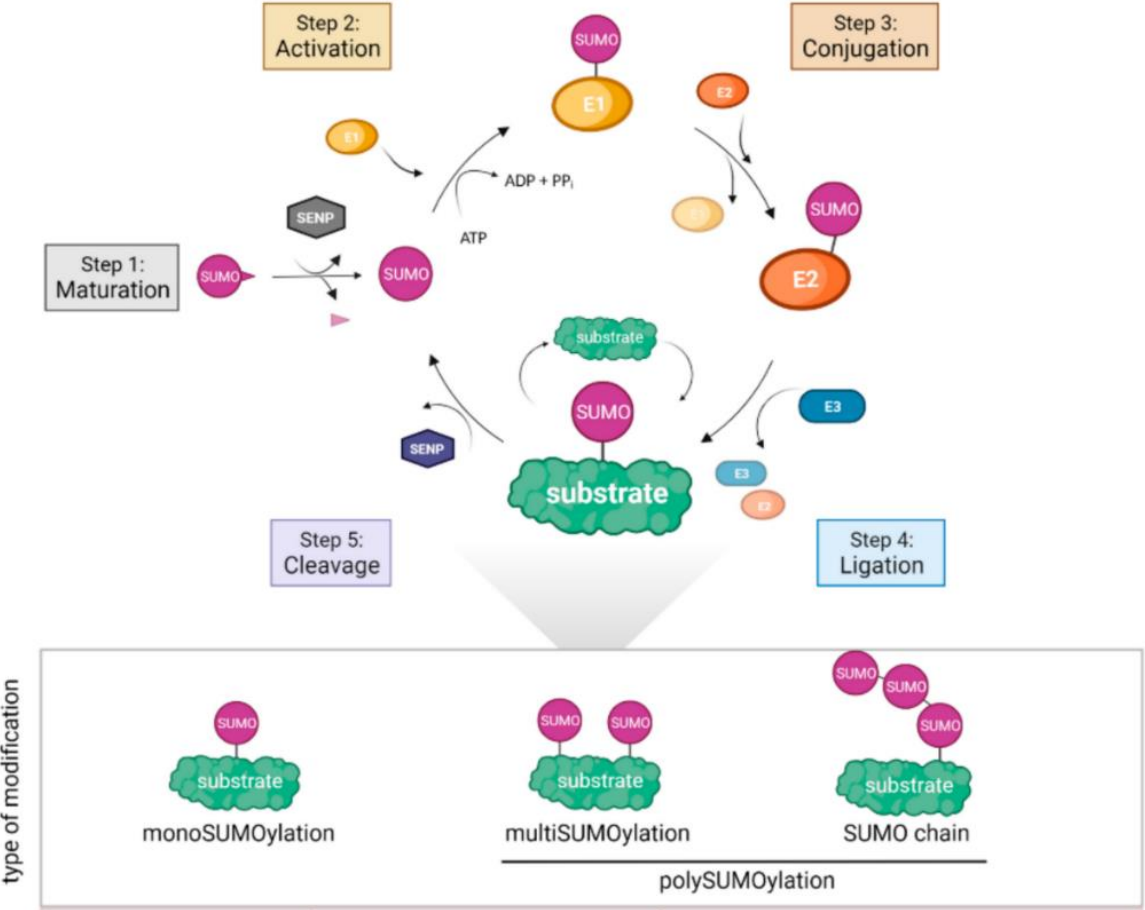
The SUMO machinery primarily targets lysines within a consensus motif of  $\Psi$ KxD/E, where  $\Psi$  is a hydrophobic residue such as I, V, or L, and X is any amino acid (Rodriguez et al., 2001; Sampson et al., 2001). Successive mass spectrometric data revealed additional consensus motifs, including inverted or extended core motifs that enhance E2 affinity and increase modification in vitro (See [figure 17](#)) (Hendriks & Vertegaal, 2016). Additionally, stressed conditions like heat shock or proteasomal inhibition might affect stringency in SUMOylation of lysines at non-consensus motifs (Hendriks et al., 2014). Nevertheless, multiple non-SUMOylated proteins also contain the SUMOylation consensus motifs, signifying that the presence of these motifs alone cannot designate it to be a SUMO substrate.

SUMOylation can be of various types. SUMO particles can attach as a monomer on a single acceptor lysine in the substrate, generating monoSUMOylation or they can attach on multiple lysines in the same substrate, referred to as multiSUMOylation. Additionally, SUMO can also form polymeric chains (polySUMOylation), where successive SUMO particles are conjugated to an internal lysine of the previous SUMO particle in the chain (See [Figure 15](#)) (Jansen & Vertegaal, 2021; Tatham et al., 2001).

As with other PTMs, SUMOylation is a highly dynamic and reversible process ([Figure 18](#)). Enzymes called SUMO-specific proteases aid in maintaining the reversibility of SUMOylation. SUMO proteases carry out deconjugation of SUMO from targets, which cleaves the isopeptide bond between the terminal glycine of SUMO and the substrate lysine (L. Shen et al., 2006). Additionally, certain SUMO proteases perform another essential function via their hydrolase activity by processing precursor SUMO to its mature form (Hay, 2007). All SUMO proteases discovered to-date belong to the Ulp/SENPs family of cysteine proteases.



**Figure 15: The SUMO pathway.** Top: An overview of the enzymes involved in the covalent attachment of SUMO to its substrates. First, SUMO is processed by SUMO-specific proteases (Step 1), followed by its transfer to the E1 activating enzyme (Step 2) and then to the E2 conjugating enzyme (Step 3). Finally, an E3 SUMO ligase catalyzes the conjugation of SUMO to the substrate (Step 4). SUMOylation can be reversed by SUMO-specific proteases (Step 5). Bottom: Illustration of the different types of SUMO modifications. (Schirmeisen et al., 2021a)



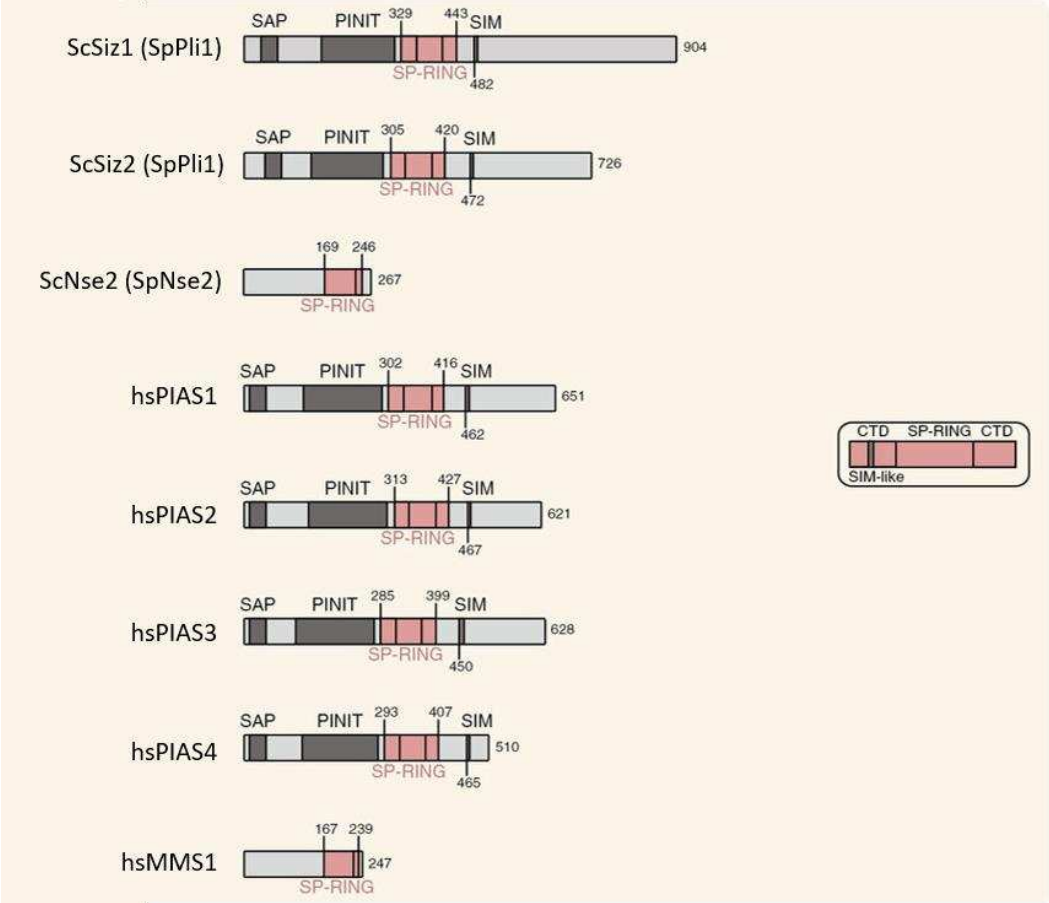
They share a conserved catalytic domain typically located at the C-terminal of the protein (Hickey et al., 2012; Kunz et al., 2018, p. 20; Mukhopadhyay & Dasso, 2007).

The first discovered SUMO protease was the budding yeast ScUlp1, followed by ScUlp2 (S.-J. Li & Hochstrasser, 1999, 2000). Fission yeast also contains two deSUMOylases: SpUlp1 and SpUlp2. Genetic studies in budding yeast revealed distinct SUMOylated substrates accumulated in ScUlp1- or ScUlp2-deficient strains, displaying different phenotypes (Bylebyl et al., 2003; S.-J. Li & Hochstrasser, 2000, 2003). This revealed that the two yeast SUMO proteases might harbour distinct substrate choices (Schwienhorst et al., 2000). Apart from the deSUMOylating activity, Ulp1 is also necessary for generating mature SUMO particles, while ScUlp2 preferentially cleaves SUMO chains, regulating substrate polySUMOylation (Eckhoff & Dohmen, 2015; Hickey et al., 2012). Spatial localization of Ulp1 and Ulp2 also largely influences substrate specificity (see below) similar to their counterparts in budding yeast. In fission yeast, Ulp1 is associated more with the processing of the precursor SUMO particles rather than the deconjugation of SUMO particles from substrates (D. L. Taylor et al., 2002). The main deconjugation activity is attributed to the SUMO protease, Ulp2. It is demonstrated to be required for DNA repair in *S.pombe* by reducing the accumulation of SUMO conjugates in mutants that accumulate SUMOylated proteins (Kosoy et al., 2007b).

In human cells, six SENP (sentrin-specific protease) family members have been identified (Geiss-Friedlander & Melchior, 2007; Mukhopadhyay & Dasso, 2007). SENP1, SENP2, SENP3, and SENP5 are related to yeast Ulp1, while SENP6 and SENP7 are closer to Ulp2 (Hickey et al., 2012; Kunz et al., 2018). Mammalian SENPs perform both SUMO maturation and deconjugation in a paralog-specific manner. SENP1 is most active on SUMO-1, SENP2 prefers SUMO-2, and SENP5 processes SUMO-3 precursors. For deSUMOylation, SENP1 and SENP2 release all SUMO isoforms, while SENP3 and SENP5 favours SUMO2/3 removal. Finally, SENP6 and SENP7 preferentially cleave SUMO2/3 chains (Kunz et al., 2018).

Therefore, SUMO proteases balance free and conjugated SUMO particles, with their interaction with SUMO E3 ligases determining cellular SUMOylation levels.

**Figure 16: Schematic representation of SP-RING family E3 SUMO ligases in yeast and humans.** The common motif essential for E3 activity is the SP-RING. Other shared domains include SAP, PINIT, and the SIM motif. Yeast Nse2 and mammalian MMS21 both possess the SP-RING motif, but otherwise, they differ significantly. Adapted from Pichler et al., 2017.



## 2. Functions

SUMOylation has distinct and non-compensatory functions with various molecular consequences. (Figure 19) (Geiss-Friedlander & Melchior, 2007; K. A. Wilkinson & Henley, 2010; J. Zhao, 2007, p. 200).

Attachment of SUMO can mask the binding surface of target proteins thus blocking the interactions with different cofactors. This may alter the enzymatic activity, prevent transcription factors from binding to chromatin or prevent another post-translational modification. For instance, SUMOylation of the E2 ubiquitin-conjugating enzyme inhibits its interaction with the E1 ubiquitin enzyme, reducing the ubiquitination of substrate proteins (Hardeland et al., 2002; Pichler et al., 2005).

Conversely, SUMOylation can create new binding sites for partners with specific SUMO interaction motifs (SIMs). While the interaction between SUMO and SIM is weak, it gets strengthened by multiple SIMs binding to SUMO chains (Hecker et al., 2006; Husnjak et al., 2016). These interactions have various outcomes, such as recruiting the budding yeast Srs2 helicase to the replication forks via SUMOylated PCNA to prevent recombination (Pfander et al., 2005). Additionally, SUMO-mediated interactions with transport complexes can also promote nuclear import and export of certain cargo proteins (Santiago et al., 2013).

On one hand, such interactions can influence substrate stability where SUMO chains may attract SIM-containing E3 ubiquitin ligases, leading to ubiquitination and degradation (refer to section B.2). On the other hand, SUMOylated proteins can be protected from deSUMOylation or degradation by other factors competing with SUMO proteases or ubiquitin ligases (Psakhye et al., 2019a; Y. Wei et al., 2017). These factors include SIM-containing ATPases or segregases that can extract SUMOylated proteins from chromatin via its translocase activity (e.g., budding yeast Uls1 and fission yeast Rrp1/2) (Lescasse et al., 2013; Y. Wei et al., 2017). SUMOylation may also induce conformational changes in the modified target. These changes reveal new binding sites or alter existing ones, which affect interactions with partner proteins or their DNA binding affinity (Geiss-Friedlander & Melchior, 2007).

**Figure 17: SUMO consensus motifs.** Left: A schematic representation of the main SUMOylation consensus motifs. Grey shading highlights hydrophobic residues; blue shading indicates lysine residues modified by SUMO; and red shading represents negatively charged residues. Right: Visualization of all known SUMOylation consensus motifs. Amino acids that are enriched are displayed above the x-axis, while those that are depleted are shown below the x-axis. The degree of enrichment or depletion of each amino acid is reflected by the height of the corresponding letter. (Hendriks & Vertegaal, 2016)

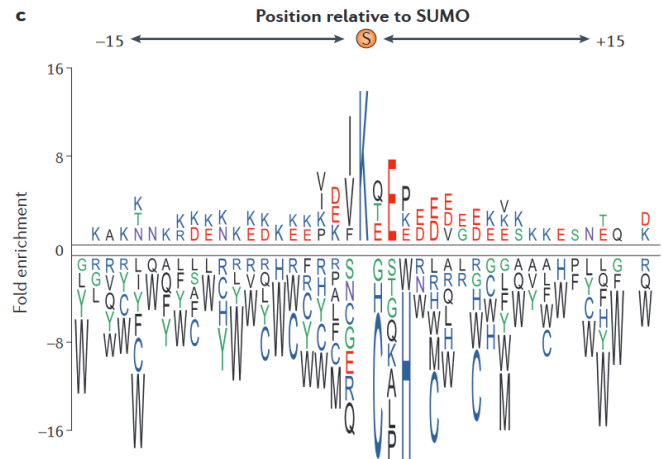
Core motif  
 [IV]-K-X-E

Full motif  
 [IVF]-K-[QTEP]-[ED]-[PKE]

Hydrophobic motif  
 [VIP]-X-[IV]-K-X-E

Negatively charged motif  
 K-X-E-X-[ED]\*5

Inverted motif  
 [ED]-X-K



SUMOylation is involved in a wide range of cellular processes including DNA replication, DNA damage repair, chromatin remodelling, nuclear trafficking, protein degradation, cell cycle progression, gene expression, and signal transduction (Hay, 2005; E. S. Johnson, 2004; Seeler & Dejean, 2017). This underscores the crucial role of SUMOylation in cellular fitness and survival. Indeed, a proteomic study reveals SUMOylation of over 6700 proteins in human cells, which comprise nearly 25% of the human proteome (Hendriks et al., 2017, p. 201). However, only a small fraction of the total cellular pool of these substrates is SUMOylated (<1%) at any given time, especially in unstressed cells (Hay, 2005; E. S. Johnson, 2004).

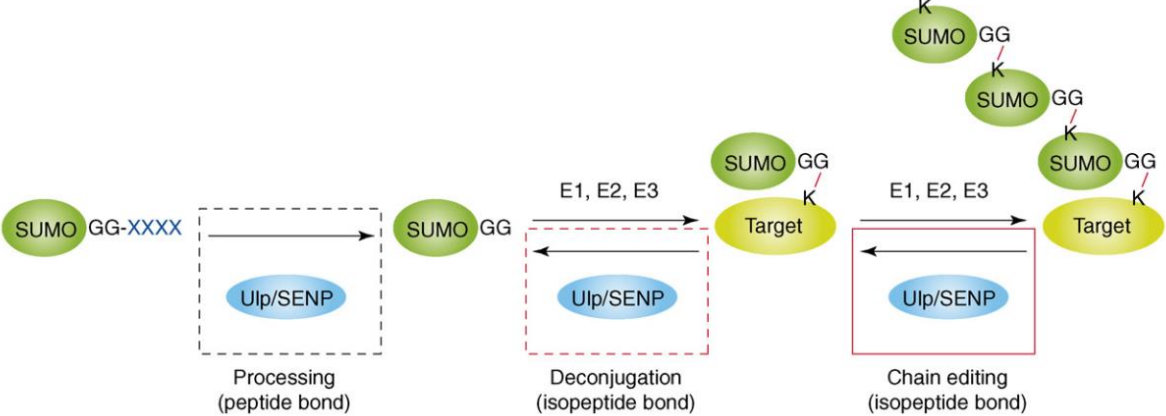
Hence, the cardinal question is: how can a small pool of SUMOylated proteins lead to significant effects? SUMOylation as a process is reversible and occurs in cycles. Although a rapid deconjugation by SENPs can shift the equilibrium towards the unmodified form, the previous transient SUMO modification can still achieve maximal downstream response. One model proposes that an initial wave of SUMOylation recruits other interactors, which remain on the substrate after SUMO cleavage. While, another model suggests that SUMOylation drives the substrate to functional complexes or subcellular compartments, persisting after deSUMOylation (Sahin et al., 2022). Some processes require synchronous action by multiple components (i.e. DNA damage repair, chromatin remodelling, and transcription), where the effect of each SUMOylation event adds up cumulatively giving a synergistic effect. Additionally, certain DNA metabolism factors may be SUMOylated only when engaged with chromatin, explaining low in vivo SUMOylation levels (Sarangi & Zhao, 2015).

## C: SUMOylation and ubiquitination: the maintenance of protein homeostasis

### 1. A comprehensive look at the SUMO-Targeted Ubiquitin Ligase (STUbL)

As mentioned above, SUMOylation has diverse cellular functions. They serve as targeting signals recognized by members of a novel class of E3-ubiquitin ligases termed SUMO-targeted ubiquitin ligases (STUbL) as mentioned in section III.A.2 (Perry et al., 2008; Uzunova et al., 2007).

**Figure 18: Activities of SUMO specific proteases.** The Ulp/SENPs play a crucial role in the SUMO maturation process by catalyzing the processing of SUMO precursors to reveal the C-terminal di-glycine motif (dashed black box). Additionally, Ulp/SENPs are essential for deconjugating various SUMO forms from substrates (dashed red box) and for chain editing (solid red box). (Nayak & Müller, 2014)



## 1.1 Structure

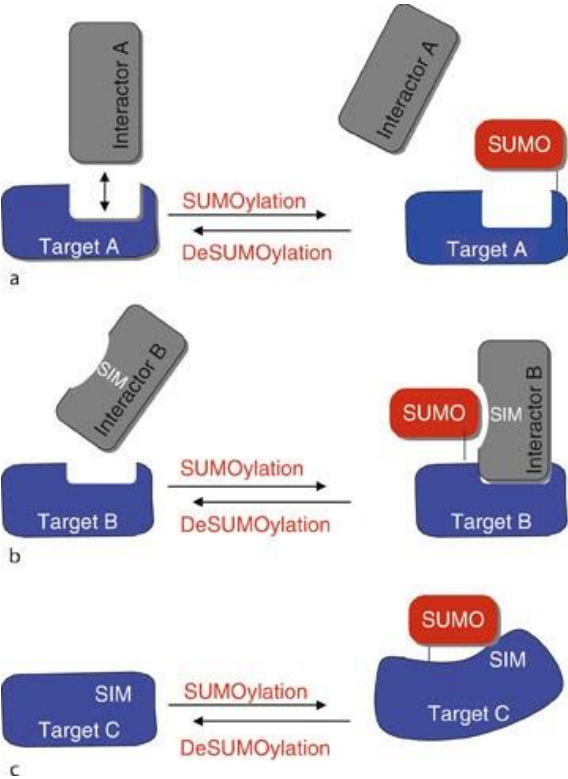
The initial discovery of STUbLs was driven by their interaction with SUMO or the presence of the SIM motif within them (li et al., 2007; Prudden et al., 2007a; H. Sun et al., 2007). SIMs are short peptide sequences that have been classified as SIMa, SIMb, or SIMr according to their exact amino acid sequence ([Figure 20](#)) (Miteva et al., 2010). The SIM domain has a hydrophobic core typically consisting of four amino acid residues and often conforms to either an 'hXhh' or an 'hhXh' consensus. The 'h' represents an amino acid with a large non-polar, aliphatic side chain (I, V, or L) and 'X' can be any amino acid (Beauclair et al., 2015; Song et al., 2005; Q. Zhao et al., 2014). Negatively charged residues, such as aspartates, glutamates, and/or phosphoserines, often flank the hydrophobic core. Effective and empirically validated SIM prediction algorithms are based on the same consensus as mentioned above (Q. Zhao et al., 2014). SIMs are generally found in an unstructured context and are believed to form a short  $\beta$  strand structure that binds to a groove created by a  $\beta$  strand and an  $\alpha$  helix of SUMO (Miteva et al., 2010; Reverter & Lima, 2005). Functionally dissected STUbLs so far, share two main features: SIMs that mark them as SUMO interactors and a RING domain that interacts with an E2 ubiquitin-conjugating enzyme ([Figure 21](#)). Most studied STUbLs possess multiple SIMs, enabling cooperative binding to multiple SUMO units and show a preference for substrates with SUMO chains (Tatham et al., 2008; Uzunova et al., 2007). Once the STUbL recognises SUMOylated proteins via its SIM motif, it can promote their ubiquitination and subsequent deSUMOylation or degradation (Prudden et al., 2007a). This activity of transferring ubiquitin from the E2 enzyme to the lysine residue of the substrate is a function of the enzymatic RING domain present in STUbL. The RING domain is composed of zinc-fingers that coordinate two zinc atoms in a cross-brace active structure within seven conserved cysteine and one histidine side chain of the RING structure ([Figure 22](#)) (Borden et al., 1995; Saurin et al., 1996). All STUbLs studied so far, possess multiple SIMs but contain only a single RING domain. ([Figure 21](#)).

## 1.2 Characterization of STUbL function across species

STUbLs are a unique class of E3 ubiquitin ligases as their action allows a specified crosstalk between the SUMO and the ubiquitin system (Perry et al., 2008; Prudden et al., 2007a; Uzunova et al., 2007). They have been characterised across various organisms as summarised below.



**Figure 19: Molecular consequences of SUMOylation.** SUMOylation influences protein-protein interactions by three non-mutually exclusive mechanisms: a) Masks a site of interaction, b) New binding site formation, and c) Induction of structural changes in the substrate protein. (Geiss-Friedlander & Melchior, 2007)



## *S. cerevisiae*, Uls1

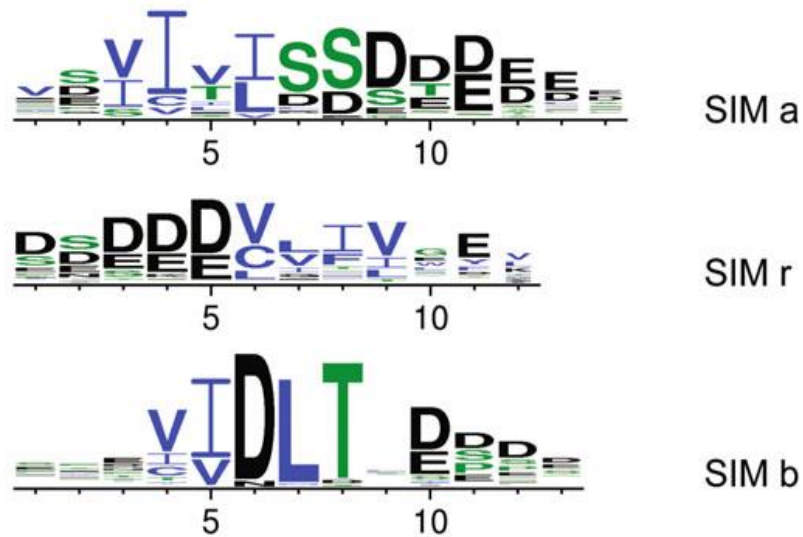
Uls1 is one of the two existing RING finger ubiquitin ligases that was identified to ubiquitylate SUMO conjugates by yeast two-hybrid (Y2H) interaction (Uzunova et al., 2007). Uls1 is a large protein, consisting of four predicted SIMs in the N-terminal part, a Swi2/Snf2-like translocase domain and a RING finger domain in its C-terminal part ([Figure 21](#)) (Dresser et al., 1997; Hannich et al., 2005; Uzunova et al., 2007; Z. Zhang & Buchman, 1997). Uls1 deletion leads to the accumulation of high molecular weight (HMW) SUMO conjugates, which is aggravated upon deletion of the other ubiquitin ligase, Uls2 (described below) in budding yeast (Uzunova et al., 2007). This highlights the idea of overlapping functions between Uls1 and Uls2, offering a proteolytic control over cellular SUMOylated conjugates. Additionally, mutations in Uls1, Uls2 or factors affecting the proteasome are synthetically lethal when combined with mutants of the SUMO pathway due to the inability to remove SUMO conjugates in these conditions (Makhnevych et al., 2009).

Other activities involve, the Swi2/Snf2 domain of Uls1, which overlaps functionally with Rdh54 and Rad54 and aids in DNA damage repair and recombination (Chi et al., 2011; Shah et al., 2010). Uls1 helps dismantle Rad51-dsDNA complexes during double-strand break repair, minimizing toxic accumulations, especially in the absence of Rad54 and Rdh54 (Chi et al., 2011). Intriguingly, Uls1 inactivation rescues the synthetic lethality observed when the Sgs1 helicase and the Mus81 resolvase are inactivated, a situation in which the disentanglement of HR intermediates is compromised, thus emphasising the role of Uls1 in HR (Kramarz et al., 2017).

Another study revealed that Uls1 inhibits NHEJ at telomeres by removing non-functional, poly-SUMOylated Rap1, thereby preventing chromosomal end fusion (Lescasse et al., 2013; Pardo & Marcand, 2005). Mutations in Rap1 SUMOylation sites bypass Uls1's requirement for recognition and dissociation of poly-SUMOylated Rap1 from DNA for degradation. Both translocase and ubiquitin ligase activities of Uls1 are essential for this function in telomere maintenance (Lescasse et al., 2013). Besides, Uls1 translocase activity aids in downregulating aberrant NHEJ at DSBs within telomeric repeats by relocating them to the nuclear envelope (Marcomini et al., 2018).

In terms of cellular localisation, Uls1 is found both in the nucleolus and in the nucleoplasm. In the nucleolus, Uls1 interacts with the nucleolar protein Ebp2 in a SUMO-dependent manner. The N-terminal SIM domain of Uls1 is required for the interaction with Ebp2, which is essential for rRNA maturation and ribosomal subunit assembly (SHIRAI & MIZUTA, 2008).

**Figure 20: Residue conservation in the three types of SIMs shown in a sequence logo representation.** Residue conservation for the three SIM types is depicted using a sequence logo representation. The overall height of each position in the logo reflects its information content, while the height of individual residues indicates their frequency at that position. Residues are color-coded as follows: black for charged residues, green for polar residues, and blue for hydrophobic residues. (Miteva et al., 2010)



## *S. cerevisiae*, Slx5-Slx8

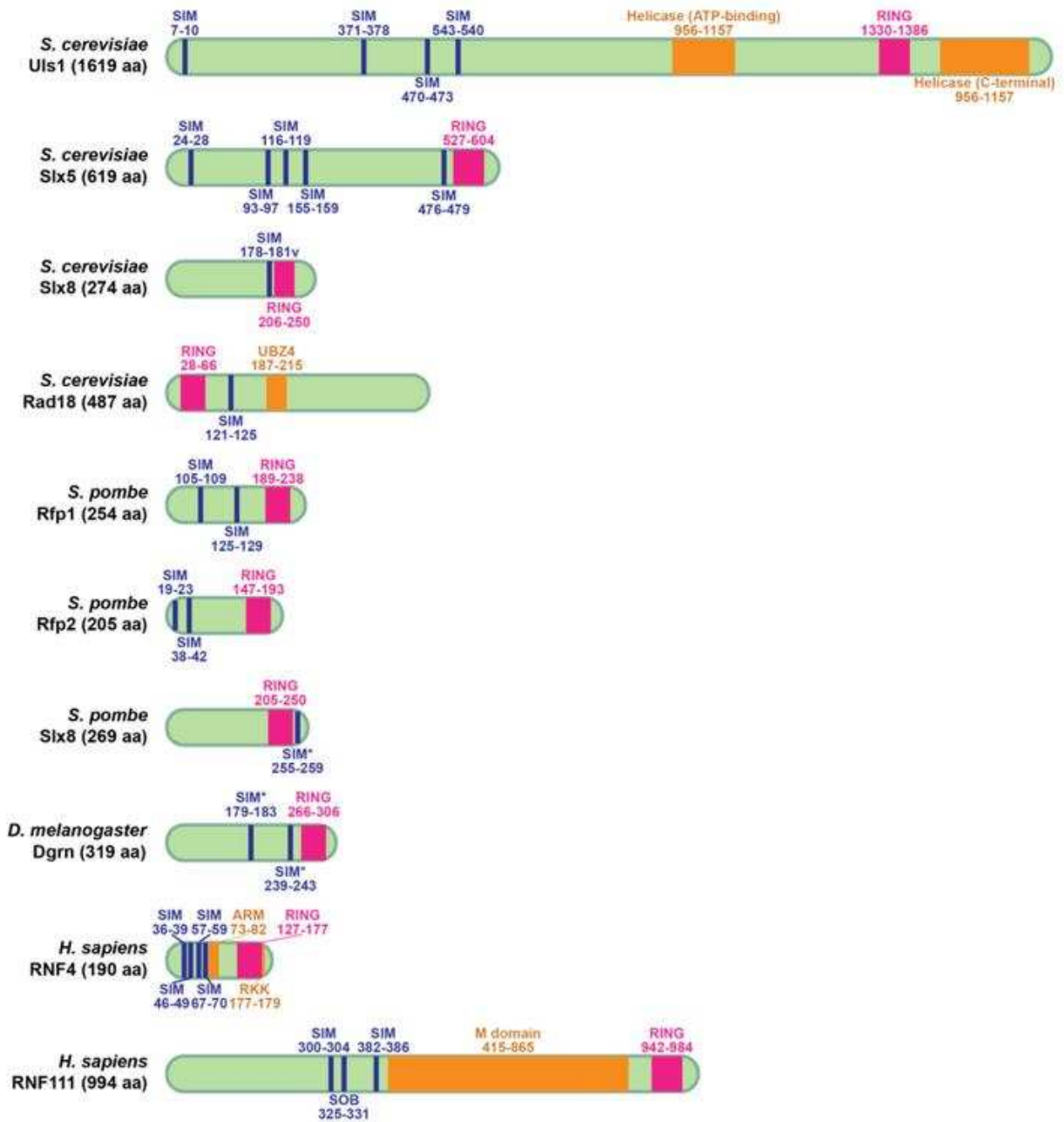
The other STUBL complex in budding yeast is the Slx5-Slx8 heterodimer, which was also identified via its interaction with SUMO using a Y2H assay (Hannich et al., 2005; Uzunova et al., 2007; Xie et al., 2007). Each monomer contains a RING finger domain, which facilitates the transfer of ubiquitin from an E2 ubiquitin-conjugating enzyme to a substrate protein. In Slx5 and Slx8, this domain is essential for their function as E3 ubiquitin ligases. They are named synthetic lethality of unknown [X] function 5 and 8 (Slx5 and Slx8) due to their co-lethality with the RecQ helicase Sgs1, (Mullen et al., 2001). Slx5 STUBL consists of multiple SIM domains of the 'a' and 'b' type. However, Slx8 bears a single SIM 'a' motif (refer to [Figure 21](#)). These SIM domains aid the binding of Slx5 and Slx8 to HMW-SUMO conjugates which form SUMO chains (Mullen & Brill, 2008). The Slx5-Slx8 heterodimer, in cooperation with the E2 ubiquitin-conjugating enzyme Ubc4 or Ubc5, acts upon poly-SUMOylated proteins to target them towards proteasomal degradation (Uzunova et al., 2007). Additionally, evidence suggests that Slx5-Slx8 preferentially attaches ubiquitin to the terminal lysine of SUMO chains, but is also capable of ubiquitylation of monoSUMOylated moieties (Mullen & Brill, 2008). Hence, mutations in Slx5 and Slx8 also lead to the accumulation of SUMOylated proteins as seen with Uls1, highlighting their role in the regulation of the SUMO pathway by processing SUMOylated adducts (Z. Wang et al., 2006).

In the context of DNA repair, the Slx5-Slx8 STUBL is crucial for the cell survival of mutants lacking the Sgs1 helicase. Accumulation of SUMO conjugates is a characteristic phenotype of the *sgs1* mutant. The co-lethality between *slx5* and *slx8* with *sgs1* is in fact due to the accumulation of SUMOylated proteins to toxic levels. This co-lethality is observed to be suppressed by the inactivation of the Ulp2 SUMO protease, which indicates that Slx5-Slx8 and Ulp2 act in an antagonistic manner. Triple mutants of *slx5*, *sgs1* and *ulp2* displayed an even higher accumulation of SUMOylated proteins than the corresponding double mutant. Authors suggest that loss of Ulp2 protease suppresses the toxicity of the SUMOylated proteins that accumulate in the *slx5Δ-slx8Δ* cells by allowing poly-SUMO chains extension on target proteins. This additional modification might channel target proteins into alternative pathways for proteolytic degradation that require the formation of even longer SUMO chains ([Figure 23](#)) (Mullen et al., 2001, 2011; Mullen & Brill, 2008).

Slx5 and Slx8 are also involved in multiple other processes. First, they suppress gross chromosomal rearrangements (GCRs). Deletion of *SLX5* or *SLX8* increases GCRs by 296 and 152-fold, respectively (Putnam et al., 2009; C. Zhang et al., 2006). Furthermore, Slx5-Slx8 has implications in nuclear protein quality control. For instance, the mutated

**Figure 21: Domain structures of SUMO-targeted Ubiquitin Ligases from yeast, flies and humans.**

See text for details. (Adapted from Chang et al., 2021)



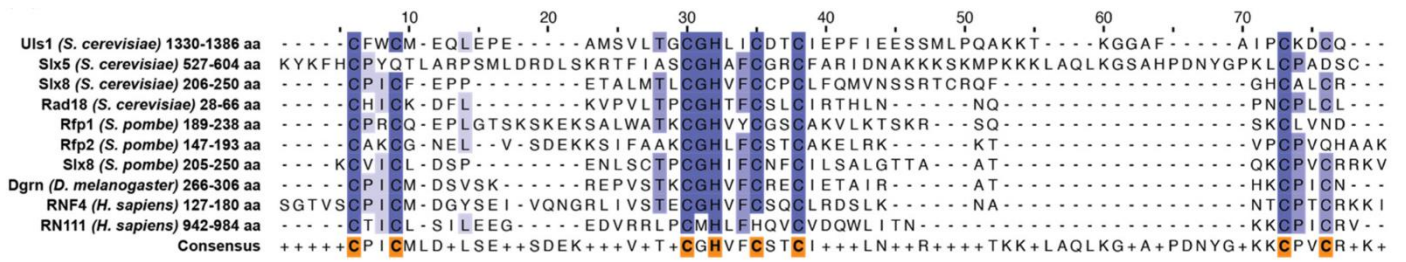
form of the transcriptional regulator Mot1, is a SUMO-dependent substrate of Slx5-Slx8 for degradation (Z. Wang & Prelich, 2009). This prevents aberrant transcriptional regulation by the Mot1 mutant.

Slx5-Slx8 functions are critical factors during normal DNA replication too. The STUbL Slx5-Slx8 regulates the turnover of the cell cycle regulatory Dbf4-dependent kinase (DDK) (Psakhye et al., 2019a). SUMOylated DDK binds to chromatin for normal origin firing and this phenomenon is unrelated to replication stress. The DDK SUMOylation is controlled by the SUMO protease Ulp2 at the replication origins to prevent premature Slx5/Slx8-mediated degradation. SUMOylated DDK degradation is necessary to prevent re-replication and facilitate replication termination. Another example of Slx5-Slx8 function during replication is in the turnover of replisome subunits, like the CMG helicase. (Psakhye et al., 2019b).

#### *S. cerevisiae*, Rad18

Another budding yeast STUbL is Rad18 ([Figure 21](#)), which is not conserved in humans. Interestingly, the identification of Rad18 as an STUbL came from the evidence of physical interaction between the SUMO E2 enzyme Ubc9 and Rad18 itself (Hoegge et al., 2002). Primarily Rad18 recognizes and ubiquitinates SUMOylated PCNA to further initiate DNA damage tolerance (DDT) pathways (Parker & Ulrich, 2012, p. 20). The interaction between the SIM domain of Rad18 and SUMOylated PCNA enhances the Rad18 ubiquitin ligase activity. The deletion of the SIM domain of Rad18 results in a phenotype similar to that of the DDT-deficient mutants. These phenotypes include sensitivity towards alkylating agents like MMS and UV irradiation. Deletion of the SIM domain in Rad18 leads to the loss of PCNA ubiquitination and thereby impacts the ability to activate DDT mechanisms such as translesion synthesis (TLS) (Kannouche & Lehmann, 2004). Therefore, the interaction of SUMOylated PCNA with Rad18 via the SIM domain, indicates that the SIM domain optimizes Rad18 activity. However, this SIM-dependent enhancement of Rad18 is not observed in humans, as mammalian Rad18 lacks a SIM domain (Parker & Ulrich, 2012). However, in mammalian cells, Rad18 forms a complex with Pol $\eta$ , and this association is essential for normal DNA damage tolerance (Barkley et al., 2012; Day et al., 2010; Watanabe et al., 2004). The assembly of the Rad18–Pol $\eta$  complex is tightly regulated by the checkpoint kinases, which integrate translesion synthesis (TLS) with S-phase progression. Additionally, it has been shown that the interaction between Pol $\eta$  and Rad18 is crucial for targeting Rad18 to proliferating cell nuclear antigen (PCNA) and promoting efficient PCNA monoubiquitination. (Durando et al., 2013).

Figure 22: Sequence alignment of the RING domains of STUBs. (Adapted from Chang et al., 2021)



## *S. pombe*, Rfp1- and Rfp2-Slx8

In fission yeast, the STUbL complex consisting of Slx8 and either of the redundant RING finger proteins Rfp1 or Rfp2, is crucial for genome stability and DNA damage repair. Rfp1 and Rfp2 are the functional homologs of Slx5 in budding yeast, although they lack the E3 ligase activity (Figure 21). Recruitment of Slx8 (proficient in E3 ubiquitin ligase function) to Rfp1 and Rfp2 through the RING-RING domain interaction creates the functional enzyme. *rfp1Δ rfp2Δ* double mutants display severe growth defects and are extremely sensitive to hydroxyurea (HU) (Prudden et al., 2007a; H. Sun et al., 2007). Consistently, fission yeast *slx8Δ* mutants grow slowly and are sensitive to UV light, HU, MMS, and CPT (Prudden et al., 2007a; Steinacher et al., 2013a). This indicates that mutations impairing this complex result in the accumulation of toxic SUMO conjugates and importantly, the observed growth defects are suppressed by the deletion of the E3 SUMO ligase Pli1.

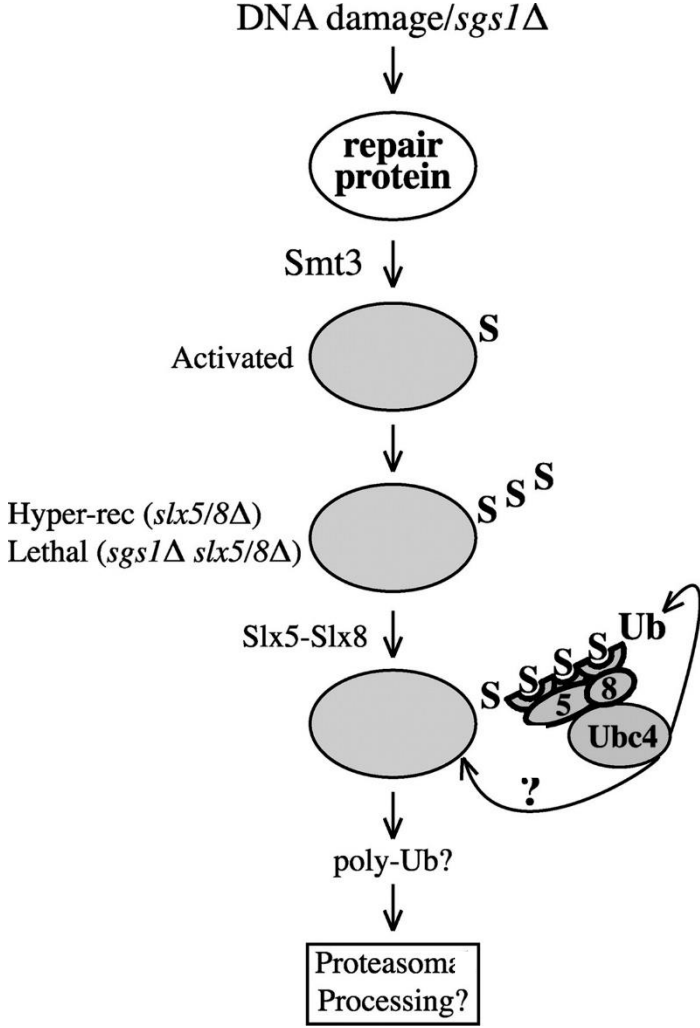
Topoisomerase I (Top1) was identified as a relevant substrate of Slx8 in fission yeast. TOP1-SUMO conjugates accumulate in Slx8 mutants in a Pli1-dependent manner, suggesting that Rfp1/2–Slx8 down-regulates SUMO conjugates (Steinacher et al., 2013a).

Rfp1/2–Slx8 binds SUMO conjugates via multiple SIMs and can ubiquitylate SUMOylated proteins like Rad60, a conserved protein involved in the DNA damage response. Absence of Rfp1/2–Slx8 shows increased Rad52 foci formation, indicating an important role in preventing DNA damage accumulation. Additionally, this STUbL complex is required to remove SUMOylated proteins from DNA repair sites in response to DNA damage or replication fork arrests (Heideker et al., 2009).

Other examples of STUbL-dependent regulation of DNA damage and genome stability include Slx8-mediated repair of covalent Top1-DNA adducts (Top1-cc). The repair of Top1-cc is facilitated by the Rad16-Swi10 endonuclease and does not depend on Pli1. However, it requires the Nse2-based SUMO ligase. (Heideker et al., 2011). In terms of chromosome segregation, *rfp1Δ rfp2Δ* double mutants display fragmented chromosomes, elongated nuclei, and asymmetric positioning of nuclei, whereas Slx8 mutants exhibit mis-segregated chromosomes and multinucleated cells. These phenotypes are also suppressed by deleting the E3 SUMO ligase Pli1, indicating that SUMO conjugates are the underlying sources of genome instability in the absence of the Slx8 STUbL (Steinacher et al., 2013a; H. Sun et al., 2007).



Figure 23: Slx5–Slx8 Ub ligase modifies the SUMO chain to limit its length or to direct it to the proteasome following Ub chain elongation via an unknown mechanism. (Adapted from Mullen & Brill, 2008)



### *S. pombe*, Rhp18

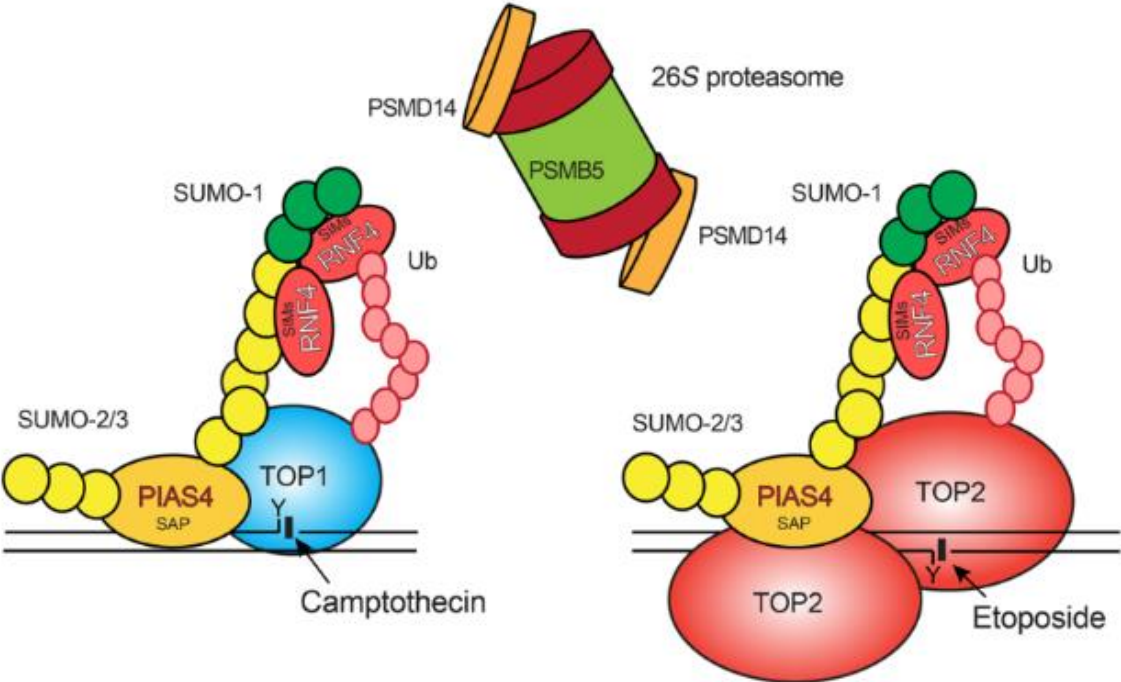
The homologue of the budding yeast Uls1 protein, Rad18 is known as Rhp18 in fission yeast. Rhp18 is an E3 ubiquitin ligase that plays a key role in the DNA damage tolerance pathway, particularly in post-replication repair (PRR) (Verkade et al., 2001). In fission yeast, Rhp18 binds to the multi-BRCT domain protein Brc1, which is involved in maintaining genome integrity and cell viability and brings about tolerance to replication stress by PRR (Reubens et al., 2017). Importantly, as observed in budding yeast for Rad18, Rhp18 in fission yeast plays a crucial role in the ubiquitination of PCNA (Frampton et al., 2006). Rhp18 is required specifically for PCNA mono-ubiquitination, which is a critical step in facilitating TLS and allows replication to proceed past DNA lesions. Hence, *rhp18* $\Delta$  cells are sensitive to UV irradiation due to their inability to ubiquitinating PCNA, which is vital for cell survival (Frampton et al., 2006; Ramasubramanian et al., 2010). Additionally, Rhp18 works in concert with the DNA repair protein, Rad60 to repair DSBs formed in response to MMS treatment and maintains checkpoint arrest until the damage is repaired (Morishita et al., 2002). This highlights the role of Rhp18 in modulating the timely completion of DNA replication in fission yeast in response to DNA damage.

### *Homo sapiens*, RNF4

The human RNF4 protein, also known as small nuclear RING finger protein (SNURF), is an E3 ubiquitin ligase that has been studied very extensively. (Lallemand-Breitenbach et al., 2008; Prudden et al., 2007a; Tatham et al., 2008; Weisshaar et al., 2008). RNF4 contains four SIM domains which can bind SUMO along with two structural features including the arginine-rich motif (ARM) and arginine-lysine-lysine (RKK) motif (Figure 21) (Grocock et al., 2014; Kuo et al., 2014). Out of the four SIMs in RNF4, SIM2 and SIM3 have the highest affinity towards SUMO chains, which are responsible for downstream substrate ubiquitination. Mutations in SIM2/3 leads to decreased SUMO affinity and elimination of ubiquitination activity (Keusekotten et al., 2014; Xu et al., 2014). RNF4 has a preference for binding to tetra-SUMO over di- or mono-SUMOylated substrates. (Aguilar-Martinez et al., 2016; Kung et al., 2014). Upon binding to poly-SUMO chains, RNF4 dimerizes, which induces the activation of its E3 ubiquitin ligase activity (Rojas-Fernandez et al., 2014). This dimerization step is essential as RNF4 cannot function as monomers (Liew et al., 2010).

In term of activity, RNF4 is crucial for NHEJ repair in mammals (Galanty et al., 2012; Kuo et al., 2016; K. Luo et al., 2012, 2015; R. Vyas et al., 2013, p. 201). Reports suggest that RNF4 depletion can affect recruitment of the NHEJ-factor XRCC4 onto DNA

Figure 24: Model for the SUMO-Ub-proteasome pathway in the repair of TOP-DPCs. (Dhingra & Zhao, 2019)



(Pfeiffer et al., 2017). Moreover, RNF4 is also required for the recruitment and persistence of proteins like 53BP1 at DSB sites, required for the DDR mediated DSB repair (Grocock et al., 2014). In HR, RNF4 targets and removes SUMOylated factors from DSB sites to ensure the disassembly of repair factors once their purpose is served (Pfeiffer et al., 2017; Yin et al., 2012). RNF4 is also required for the turnover of SUMOylated CtIP to restrict excessive resection facilitating HR (Han et al., 2021). Additionally, RNF4 contributes to the removal of RPA1 from single-stranded DNA (ssDNA), allowing Rad51 to bind and proceed with HR (Galanty et al., 2012; Yin et al., 2012). This highlights the robust role of RNF4 in HR and NHEJ.

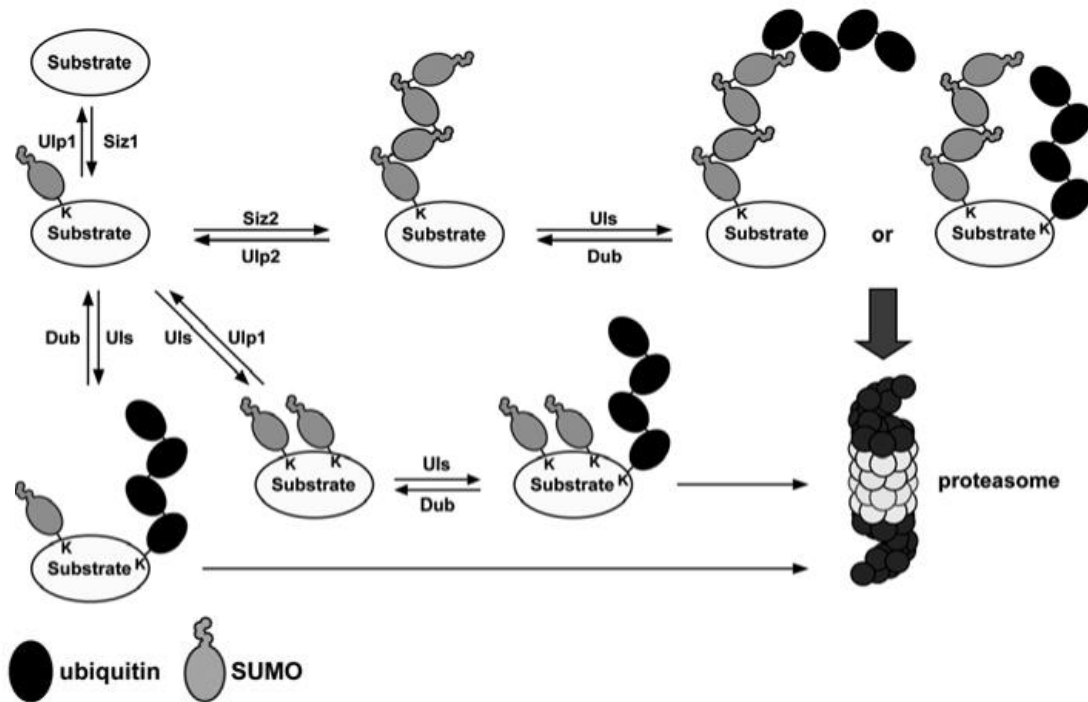
Other functions of RNF4 include its major role in the removal of topoisomerase DNA-protein crosslinks known as TOP-DPCs. SUMOylation of TOP1-DPC and TOP2-DPC by PIAS4, signals RNF4 recruitment and subsequent degradation of such structures ([Figure 24](#)) (Y. Sun et al., 2020). RNF4 knockdown increases aberrant partition of chromosomes leading to chromosomal bridges and segregation errors in mitosis. (Cuijpers et al., 2017). This emphasizes upon the multifaceted role of RNF4-mediated ubiquitination in maintaining genome stability.

#### *Homo sapiens, Arkadia/RNF111*

Another RING finger protein called Arkadia (RNF111) was first detected as a novel STUbL from the bioinformatics tool called String (H. Sun & Hunter, 2012). Apart from having two SIMs and one RING domain, it also contains a unique middle region as shown in [Figure 21](#). The middle region is comparatively large and contributes to its bigger size as compared to RNF4. The fact that RNF4 and RNF111 have unique sequences also hint towards non-redundant roles between them. This hypothesis is supported by the fact that RNF111 knockdown has no effect upon the ubiquitination of TOP1-DPC, a role specific for RNF4 (Y. Sun et al., 2020). The RNF111 STUbL has a higher preference towards binding SUMO1-capped SUMO2/3 chains over pure SUMO2/3 chains, which is again not the case for RNF4 (Sriramachandran et al., 2019). In contrast to RNF4, RNF111 is suggested to exist as a monomer from solution structure and size exclusion data (Chasapis et al., 2012; J. D. Wright et al., 2016).

STUbL activity of RNF111 is involved in various different cellular processes. RNF111 aids in nucleotide excision repair (NER) by operating with the E2-Ubiquitin conjugating enzyme UBC13-MMS2. RNF111 alongside UBC13-MMS2 SUMOylates xeroderma pigmentosum complementation group C (XPC) after UV damage. This facilitates the recruitment of XPC to UV-induced DNA damage, enhancing NER efficiency (Poulsen et al., 2013).

**Figure 25: Ubiquitin-dependent proteolytic control of SUMO conjugates in budding yeast.** The schematic illustrates a model for regulating the SUMOylated state of substrate proteins in budding yeast. On one side, SUMOylation is controlled by the activity of SUMO ligases, such as Siz1 and Siz2. On the other side, deSUMOylation is managed by Ulp1 and Ulp2, which exhibit different activities toward SUMO chains. Additionally, SUMOylation can trigger ubiquitylation through SUMO-recognizing ubiquitin ligases, leading to subsequent degradation by the proteasome. Ubiquitin (Ub) may be attached either to lysine residues of the substrate or to the SUMO chain itself. (Miteva et al., 2010)



RNF111 also plays a role in translesion synthesis (TLS) following UV-induced DNA damage. DNA polymerase eta (pol  $\eta$ ), which interacts with mono-ubiquitinated PCNA, undergoes SUMOylation of damaged DNA by PIAS1, facilitating RNF111-mediated turnover post-replication. Knockdown of RNF111 increases pol ' $\eta$ ' foci formation, expression and SUMOylation. RNF111 directly targets pol  $\eta$  via its SIM and RING domains, independent of RNF4's STUbL activity (Guérillon et al., 2020). Altogether, RNF111 helps limit mutagenesis during TLS to maintain genomic stability.

RNF111 contains at least three functional SIMs, similar to RNF4, which bind strongly to SUMO1 or SUMO2 chains, suggesting that it recognizes poly-SUMO signals. Additionally, it targets promyelocytic leukemia (PML) protein, with its depletion causing accumulation of poly-SUMOylated PML upon arsenic trioxide (ATO) treatment (Erker et al., 2013). This effect is synergistic with RNF4 depletion, indicating that both proteins contribute to ATO-induced, SUMO-dependent degradation of PML. This highlights that RNF4 and RNF111 can have some overlapping roles alongside their respective unique functions.

## 2. When SUMOylation meets ubiquitination: the protein turnover pathway

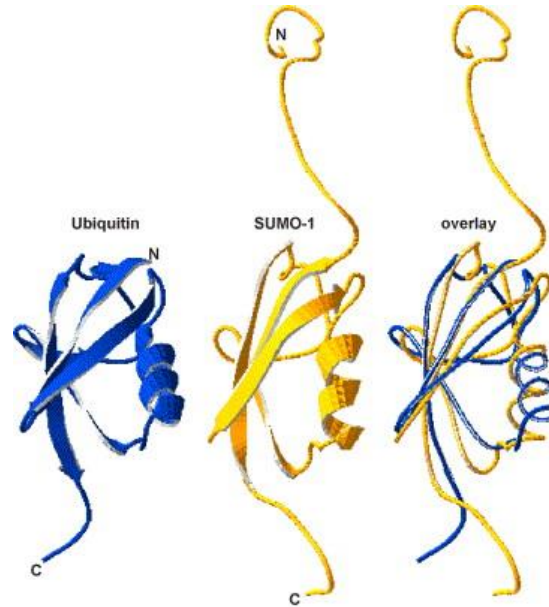
It is no longer surprising that PTMs such as SUMOylation are intricately associated with genome maintenance and damage response (S. Su et al., 2020). This is ideal due to the ability to rapidly and reversibly change the properties of proteins to materialize certain functions and fine-tuning pathways without the requirement for de novo protein synthesis. Ubiquitination as mentioned earlier, is another such PTM, which contributes to different signalling pathways. In the section below, I emphasize upon the crosstalk between SUMOylation and ubiquitination in maintaining protein homeostasis within cells.

### 2.1 SUMOylation precedes ubiquitination

As mentioned earlier, STUbL is responsible for the ubiquitination of SUMO-modified substrates, subsequently bridging SUMO and ubiquitin signalling pathways (Figure 25). It participates in the catalysis of various different ubiquitin chains, which results in distinct proteolytic and non-proteolytic outcomes for the target proteins (Han et al., 2023).

The process of ubiquitination occurs through a cascade of sequential steps similar to that of SUMOylation. It involves a series of steps with three main types of enzymes: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin ligase (Maspero & Polo, 2016). Initially, E1 activates ubiquitin in an ATP-dependent process and transfers it to E2. This leads to the formation of a thioester bond between E2's

**Figure 26: Comparison of the structure of ubiquitin and SUMO based on the example of human SUMO-1.** Both proteins possess a characteristic tightly packed  $\beta\alpha\beta\beta\alpha\beta$  ubiquitin-like fold. Notably, SUMO is differentiated by a long and flexible N-terminal tail, which is absent in ubiquitin. (Jürgen Dohmen, 2004).



active cysteine and ubiquitin's C-terminal carboxyl group. The E3 ubiquitin-ligase then catalyzes the final transfer step by bringing together the ubiquitin-loaded E2 and a specific substrate to facilitate the formation of a covalent bond between the substrate's lysine  $\epsilon$ -amino group and ubiquitin's C-terminal glycine residue (Dove et al., 2017; Maspero & Polo, 2016; Pao et al., 2018; Streich Jr & Lima, 2016). Further ubiquitination can occur at additional substrate sites with a situation of multiple monoubiquitination or extension of ubiquitin residues can take place upon an existing ubiquitin moiety by forming polyubiquitin chains. Ubiquitin contains seven lysine residues (K6, K11, K27, K29, K33, K48, K63) and an N-terminal methionine (M1), which can link to another ubiquitin, creating different signals (Branigan et al., 2015; Bremm et al., 2010; Saeki, 2017). These varieties of ubiquitin codes typically regulate protein stability or modulate protein interactions and activities. For example, K48-linked chains usually target proteins for degradation, while monoubiquitination or K63-linked chains signal various cellular processes like DNA repair (Castañeda et al., 2016; Jung et al., 2013). Humans have two E1 enzymes, UBA1 and UBA6, while budding yeast and fission yeast have only one, named ScUba1 and SpUba1 respectively. Similarly, humans have around 40 E2s while around 11 of them are present in yeast. Conversely, E3 ubiquitin ligases are a large family, with over 600 in humans and around 60 to 100 in yeast (Finley et al., 2012; Kim et al., 2007; D. C. Scott et al., 2016; M. Wang & Pickart, 2005). There are different types of E3 ligases, including the HECT (Homologous to the E6AP Carboxyl Terminus), RING (Really Interesting New Gene), and RBR (RING-between-RING) family members (Finley et al., 2012; Metzger et al., 2012). All STUbLs characterised till date, fall under the RING-type E3 ubiquitin ligase category.

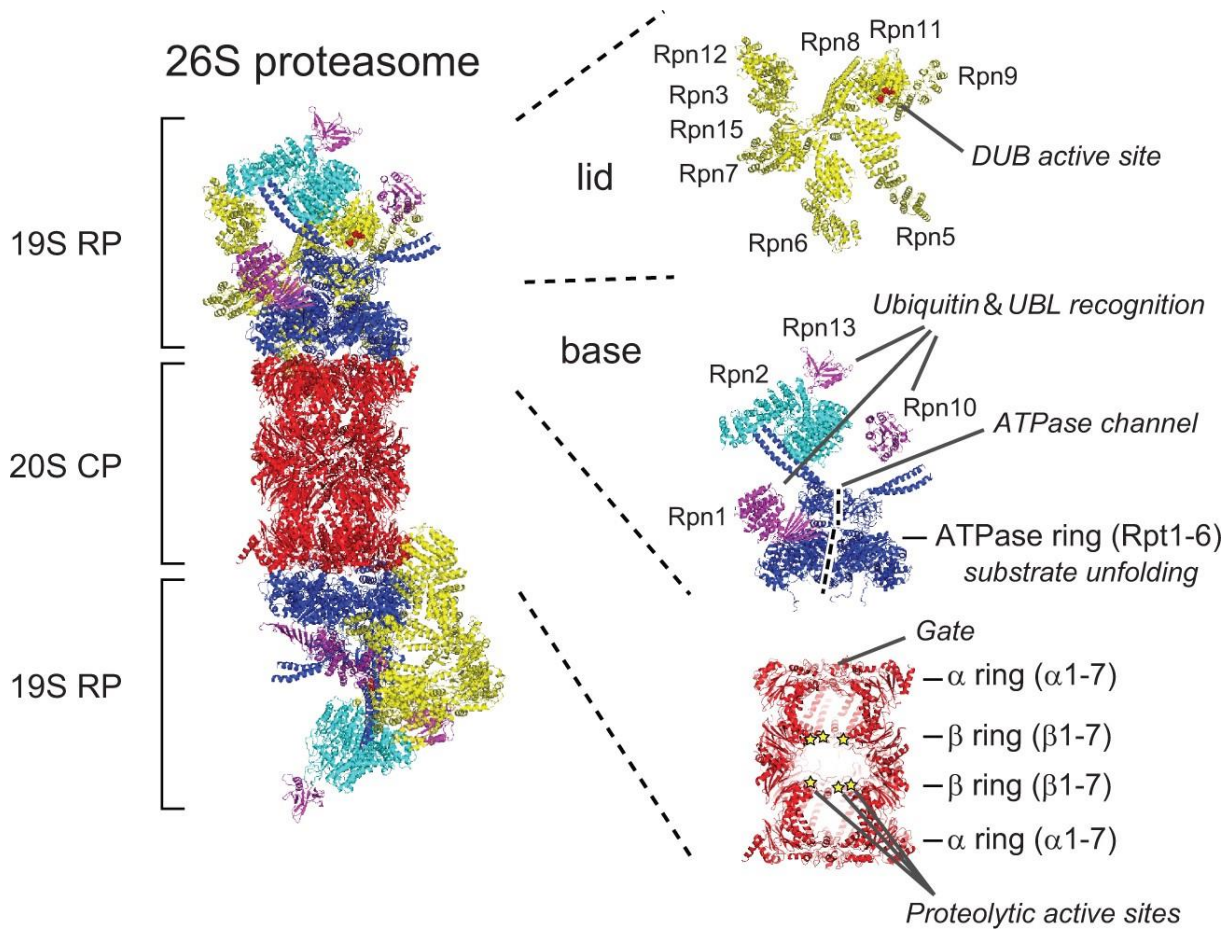
Finally, deubiquitinases (DUBs) are ubiquitin-specific proteases, which facilitate the removal of ubiquitin for maintaining the reversible nature of ubiquitination as also observed in SUMOylation (I. Chen, 2016). It involves at least 19 proteins in yeast and many more in higher eukaryotes that hydrolyze the peptide bond at G76 of the ubiquitin domain (E. S. Johnson et al., 1995). There are two classes of deubiquitinating enzymes (DUBs): ubiquitin C-terminal hydrolases (UCH), which process ubiquitin-fusion proteins and small peptides, and ubiquitin-specific processing proteases (UBP), which remove ubiquitin from larger proteins and disassemble polyubiquitin chains (Larsen et al., 1996; C. C. Liu et al., 1989; Papa & Hochstrasser, 1993; Tobias & Varshavsky, 1991; K. D. Wilkinson et al., 2002; N. Zhang et al., 1993).

## 2.2 SUMOylation vs ubiquitination: the subtle difference

SUMOylation and ubiquitination have a complementary yet distinct role in regulating protein function. Firstly, the structure of ubiquitin is slightly different from SUMO. Both ubiquitin and SUMO contain a characteristic tightly packed  $\beta\beta\alpha\beta\beta\alpha\beta$  ubiquitin-like



**Figure 27: Structure and key subunits of the 26S proteasome.** The 26S proteasome is a 2.5-MDa protease complex composed of a 20S core particle (CP) and one or two 19S regulatory particles (RP). The RP includes three ubiquitin/UBL receptors, a deubiquitylation (DUB) enzyme, and hexameric ATPase subunits that are involved in processing substrate proteins. These components are organized into two sub-assemblies known as the lid and the base. The proteolytic active sites are located within the chamber of the CP. Substrate proteins are first unfolded and then translocated into these active sites through the channels of the ATPase and CP. (Saeki, 2017)

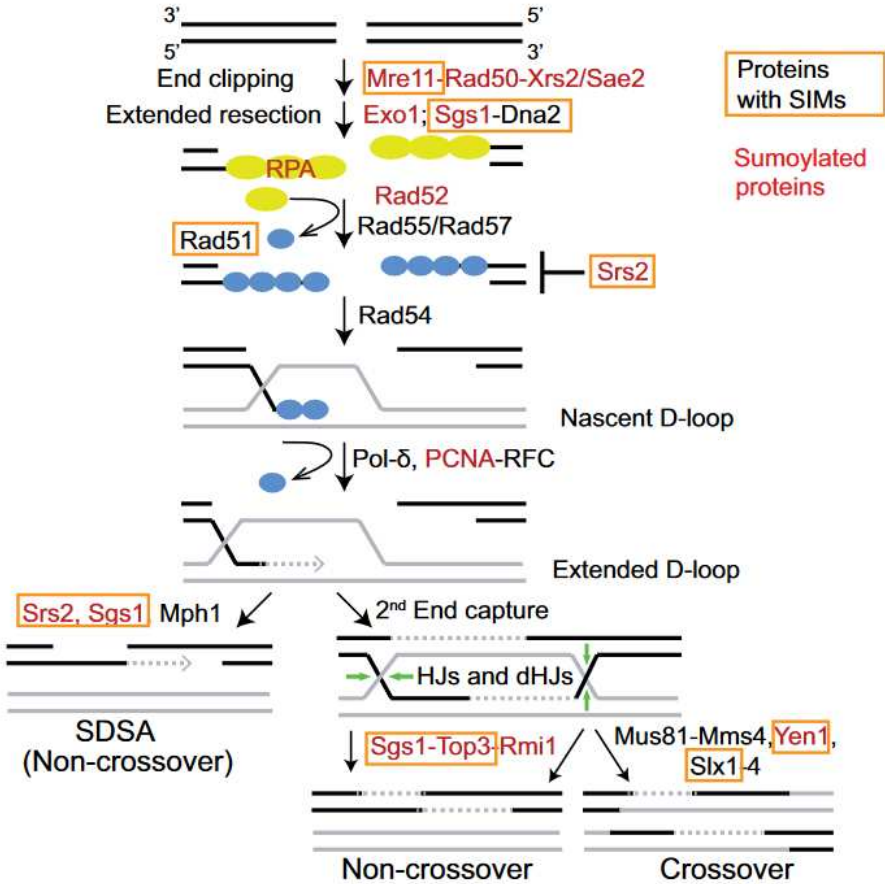


fold. However, SUMO is distinguished by a long and flexible N-terminal tail, not found in ubiquitin ([Figure 26](#)) (Bayer et al., 1998). In terms of enzyme diversity, SUMOylation has a single E2 enzyme (Ubc9) and a limited number of E3 ligases (discussed in detail in sections III-A.1 and III-B.1) as compared to ubiquitination, which harbours a plethora of E2 and E3 enzymes (as mentioned above). Additionally, the substrate specificity of ubiquitin linkages is largely determined by the E2-E3 pairs, with the E3 ligases being particularly diverse to accommodate various substrates (Kim et al., 2007). On the other hand, SUMOylation heavily relies on the specificity of E3 ligases to determine substrate targeting, as Ubc9 can only transfer SUMO to certain motifs without E3 assistance (Reindle et al., 2006; Tozluoğlu et al., 2010). Overall, the enzymatic mechanism of SUMOylation is simpler, with fewer enzymes involved, and is often more dependent on specific interaction motifs. Ubiquitination, however, involves a more complex array of enzymes and interactions, allowing for a wider variety of ubiquitin chains and functions (Al-Hakim et al., 2010).

### 2.3 Degradation of ubiquitinated substrates

The final step in the maintenance of protein homeostasis is brought about by the large, multi-subunit protease complex, called the 26S proteasome. The proteasome functions by degrading polyubiquitinated substrates in order to maintain the turnover of misfolded, damaged or fated proteins marked for removal from the cell. Parallel to the degradation of ubiquitinated substrates, the proteasome can also recognize and degrade certain non-ubiquitinated proteins (Y. Murakami et al., 1992; Ravid & Hochstrasser, 2008). PolyUb chains linked through K48–G76 isopeptide bonds are the principal signal for proteasomal proteolysis (Chau et al., 1989, p. 198; Finley et al., 1994, p. 1). The multicatalytic 26S proteasome consists of a 20S core particle (CP) with catalytic activity and a 19S regulatory subunit (RP) (Baumeister et al., 1998). The 20S CP, a barrel-shaped structure of four rings (two outer  $\alpha$ -rings and two inner  $\beta$ -rings), contains catalytic sites on the  $\beta$ -subunits (Groll et al., 1997). Each end of the 20S CP can be capped by a 19S RP, which recognizes ubiquitinated proteins. Recognition is followed by opening of the  $\alpha$ -ring orifice, unfolding of the substrates, and insertion into the 20S CP for degradation using ATP ([Figure 27](#)) (F. Beck et al., 2012). Degraded proteins releases short peptides and reusable ubiquitin back into the cell (Finley, 2009; Smith et al., 2006). Dysregulation of the ubiquitin proteasome system (UPS) can play a significant role in the onset of neoplastic transformation. Additionally, UPS has emerged as a promising source for new therapeutic interventions targeting genomic instability with potential applications in cancer treatment and management (Morgan & Crawford, 2021).

**Figure 28: SUMO-based control of the homologous recombination machinery.** This scheme provides an overview of double-strand break (DSB) repair by homologous recombination, focusing solely on budding yeast proteins. For detailed information, please refer to the text. (Dhingra & Zhao, 2019)



## D: Replication Stress and SUMOylation

### 1. SUMO-based regulation of homologous recombination

The unique feature of SUMOylation to rapidly and reversibly alter the stability, activity, and localization of target proteins, makes it a crucial fine-tuning regulator in genome maintenance pathways. SUMO is essential for viability in most eukaryotes, except in fission yeast where *Sppmt3*-deleted cells have severely compromised growth while still being viable. Disruptions in SUMO metabolism can lead to severe pleiotropic phenotypes, including lethality (Geiss-Friedlander & Melchior, 2007). This section focuses on the SUMO-based regulation of the homologous recombination (HR) machinery involved in processing double-strand breaks (DSBs) and stalled replication forks.

Early studies in yeast revealed that defects in the SUMO pathway caused hypersensitivity to DNA-damaging agents and replication inhibitors. Mutations in E2 conjugating enzymes (ScUbc9 and SpHus5) and SUMO E3 ligases (ScSiz1-2, ScMms21, and SpNse2) resulted in such hypersensitivities (Cremona et al., 2012; Jentsch & Psakhye, 2013; Maeda et al., 2004; Sacher et al., 2005; Watts et al., 2007; X. Zhao & Blobel, 2005). In budding yeast, Ubc9- and Mms21-mediated SUMOylation prevents pathological Rad51-dependent cruciform structures at damaged forks during resumption of replication (Branzei et al., 2006).

In human cells, many SUMO pathway players like UBC9, PIAS, and MMS21, along with SUMO1 and SUMO2/3, are quickly recruited to sites of DSBs and stalled forks (Galanty et al., 2009; Morris et al., 2009; R. Vyas et al., 2013). Mutations in these genes increase sensitivity to agents causing DSBs or replication stress. For instance, mutated UBC9 inhibits DNA damage-induced RAD51 nuclear foci formation (Shima et al., 2013). Moreover, depletion of PIAS1 or PIAS4 impairs DSB repair by HR, while MMS21 protects cells from DNA damage-induced apoptosis (Galanty et al., 2009; Morris et al., 2009; Potts & Yu, 2005). This indicates the necessity of SUMO conjugates at DNA damage sites.

However, SUMOylation can be a double-edged sword. Mutations causing SUMO conjugate accumulation also sensitize cells to DNA damage and replication stress (Branzei et al., 2006; Galanty et al., 2009; Maeda et al., 2004; Morris et al., 2009; Schwienhorst et al., 2000; Srikumar et al., 2013; X. Zhao & Blobel, 2005). As mentioned above, fission yeast lacking STUbL subunits SpRfp1 and SpRfp2 show slow growth and sensitivity to HU or MMS (Kosoy et al., 2007b; Prudden et al., 2007a; H. Sun et al., 2007). Deleting SpSlx8 leads to high sensitivity to genotoxic agents, but deleting SpPli1

rescues these phenotypes, suggesting toxic SUMO conjugate accumulation (Prudden et al., 2007a; Steinacher et al., 2013a). In humans, RNF4 depletion, which inactivates STUbL, results in defective DSB repair by HR (Galanty et al., 2012). This is due to RNF4's role in the removal of SUMOylated chromatin-bound factors upon prolonged fork collapse which is subsequently converted to DSBs (Ragland et al., 2013). RNF4 also removes SUMOylated BLM from collapsed forks to resume DNA synthesis (N. Ellis et al., 2021). Dysregulation in SUMO homeostasis can thus affect DNA repair capabilities and cell survival.

Studies in yeast and mammals have identified numerous SUMO targets among replisome components and DNA repair proteins, including HR factors. SUMOylation levels in these factors rise in response to replication stress or DNA damage (Cremona et al., 2012; Jentsch & Psakhye, 2013; Psakhye & Jentsch, 2012; Watts et al., 2007; X. Zhao & Blobel, 2005).

The following examples of key SUMOylated factors illustrate how SUMOylation fine-tunes various steps of recombination-mediated repair by affecting the fate of modified targets ([Figure 28](#)). These insights are crucial for understanding the molecular mechanisms involved in relocating replication stress sites to the nuclear periphery (see sections 2.1 and 2.2).

### *Resection*

In response to DSB or fork stalling, DNA end processing takes place in two stages: the MRN and CtIP (MRX and ScSae2/SpCtIP in yeast) initially perform preliminary trimming. This is followed by extensive resection by either EXO1 or the BLM-DNA2 complex (Sc/SpExo1, ScSgs1/SpRqh1, Sc/SpDna2 in yeast) (Mimitou & Symington, 2011). In budding yeast, the MRX complex is essential for the SUMOylation of numerous HR factors acting downstream of the resection, likely by generating ssDNA which allows for the recruitment of SUMO ligases (I. Chung & Zhao, 2015; Cremona et al., 2012; Psakhye & Jentsch, 2012). Additionally, other components of the resection machinery are also SUMOylated.

In budding yeast, ScSae2 SUMOylation enhances its solubility and promotes DNA end resection, in response to DNA damage (Sarangi et al., 2015). The nuclease-helicase ScDna2 undergoes SUMOylation at multiple sites, which is suggested to facilitate its targeting to damage sites and promote DNA end resection. Conversely, SUMOylation specifically attenuates the nuclease activity of ScDna2, while its helicase activity remains unaffected. Furthermore, SUMOylation of ScDna2 appears to promote its degradation, creating a feedback loop to prevent excessive resection (Ranjha et al., 2019).

In humans it was initially reported that SUMOylation of CtIP would promote its recruitment to DSB sites, thus facilitating DNA end resection (Soria-Bretones et al., 2017). Further studies revealed that CtIP SUMOylation also protects the integrity of stalled replication forks (Locke et al., 2021). This protection mechanism involves the degradation of SUMOylated CtIP after being ubiquitinated by STUbL to prevent excessive DNA resection during replication fork stalling (Han et al., 2021). Moreover, SUMOylation regulates the stability of human EXO1 under both normal and stressed conditions. EXO1 is SUMOylated in vivo by the E3-SUMO ligases PIAS1/PIAS4, which is a prerequisite for its ubiquitin-mediated degradation. Conversely, EXO1 is a substrate of the SENP6 SUMO protease, depletion of which promotes EXO1 degradation. Thus, the coordinated action of SUMO-conjugating and deSUMOylating enzymes provides a balanced regulation of EXO1 activity (Bologna et al., 2015).

#### *Rad51 filament formation*

SUMOylation of RPA takes place upon binding of the RPA complex to ssDNA overhangs in yeast and humans. In the budding yeast system, RPA SUMOylation occurs upon exposure to DNA-damaging agents by the SUMO ligase ScSiz2, (I. Chung & Zhao, 2015; Cremona et al., 2012; Dhingra et al., 2019; Psakhye & Jentsch, 2012). In humans, the RPA complex is modified by SUMO-2 and SUMO-3, which further aids in RAD51 foci formation. Specifically, the SUMO protease, SENP6 interacts with RPA during unperturbed S-phase, maintaining it at a hypoSUMOylated level. Replication stress leads to the dissociation of the complex and subsequent accumulation of SUMOylated RPA. This modification enhances RAD51 recruitment and accelerates RPA replacement by RAD51 (Dou et al., 2010). A later study reported that in cells expressing non-SUMOylable RPA or in cells depleted of RNF4, RAD51 failed to replace RPA (Galanty et al., 2012). Thus, STUbL-mediated turnover of RPA at the DNA damage site is crucial for efficient HR initiation.

Rad52 SUMOylation is a conserved process observed in yeast, as well as in human cells (J. C. Y. Ho et al., 2001; Sacher et al., 2006, p. 200). In budding yeast, SUMO modification of ScRad52 is enhanced by the MRX complex, the SUMO-conjugating enzyme ScUbc9, and the SUMO ligase ScSiz2 (E. S. Johnson, 2004; Sacher et al., 2006; X. Zhao & Blobel, 2005). It was later published that RPA-bound ssDNA promoted ScRad52 SUMOylation, but this was not the case for Rad51-coated ssDNA. Thereby, suggesting that ScRad52 SUMOylation occurs before Rad51 nucleofilament formation (Altmannova et al., 2010). Although SUMOylation did not alter ScRad52 oligomerization or its interactions with RPA and Rad51, it significantly lowered ScRad52's affinity for ssDNA and dsDNA by reducing its DNA annealing activity. This modification was described to prompt

ScRad52's dissociation from DNA to favor appropriate repair pathways or provide a mechanism for a dynamic exchange of ScRad52 on DNA (Altmannova et al., 2010). Later, it was found that SUMOylated ScRad52 recruits the Cdc48 segregase to promote its displacement from DNA by reducing its interaction with Rad51 (Bergink et al., 2013)

Another noteworthy interaction is the strong interplay between SUMOylation and Rad51 functions. Early studies showed that mammalian RAD51 interacts with the UBC2 SUMO conjugating enzyme and the SUMO-1 protein (H. Saitoh et al., 2002; Z. Shen et al., 1996). The interaction with SUMO-1 is suggested to be non-covalent and occur via the SIM of RAD51 (Song et al., 2004). It was later observed that this SIM plays a crucial role in DNA repair by attracting RAD51 to DNA damage sites. Moreover, RAD51 accumulation at DNA damage sites also requires the E2 SUMO ligase UBC9 and the E3 SUMO ligases PIAS1/PIAS4 (Shima et al., 2013). It has also been recently confirmed that RAD51 is directly SUMOylated (Hariharasudhan et al., 2022). This study demonstrated that the SUMO E3 ligase activity of TOPORS promotes RAD51 SUMOylation both in vitro and in vivo. Knockdown of TOPORS led to decreased RAD51 recruitment to DNA lesions and reduced HR-mediated repair efficiency. Interestingly, SUMOylation-deficient RAD51 was less capable of associating with BRCA2, likely explaining the HR repair deficiency in cells expressing this mutant. Altogether, RAD51 SUMOylation is critical for its recruitment to DNA lesions and promotes HR-mediated repair.

On the other hand, Srs2 helicase, an antagonist of HR mediator proteins, can both bind to SUMO and be SUMOylated at the same time. Budding yeast ScSrs2 contains a SIM domain, known for binding to SUMOylated PCNA, a feature not yet confirmed in SpSrs2. This interaction initially recruits ScSrs2 to stalled replication forks where it removes ScRad51 from DNA (Papouli et al., 2005; Pfander et al., 2005). Later, ScSlx5/8 and ScUls1 were shown to associate with SUMOylated Srs2 and reduce its levels at stalled replication forks by targeting it for degradation (Kramarz et al., 2017; Urulangodi et al., 2015). This mechanism limits Srs2-mediated inhibition of Rad51 when HR is required to rescue replication defects. In humans, Srs2 is recruited at SUMOylated PCNA, which protects forks and controls spontaneous genome rearrangements (Burkovics et al., 2013; Gali et al., 2012). Other factors that are SUMOylated include Sgs1 and BLM (Branzei et al., 2006; Eladad et al., 2005). While the SUMOylation of yeast Sgs1 promotes recombination specifically at telomeres, aiding in telomere maintenance, the SUMOylation of human BLM enhances its interaction with RAD51, promoting homologous recombination at stalled replication forks (C.-Y. Lu et al., 2010; Ouyang et al., 2009). Both mechanisms underscore the critical roles of SUMOylation in the regulation of DNA repair pathways and the preservation of genomic stability in different contexts.

### *Clearance of HR intermediates*

The 3' ssDNA generated by end resection is utilized by the recombinase Rad51 for homology search. Rad51-mediated homology search leads to the formation of joint DNA structures such as D-loops and Holliday junctions (HJs). Processing these DNA intermediates into linear products involves several conserved enzymes to complete HR repair. In budding yeast, the STR complex (Sgs1-Top3-Rmi1) dissolves HJs throughout the cell cycle, while Mus81-Mms4 and Yen1 act during mitosis and anaphase, respectively (West et al., 2015). SUMOylation affects all three HJ processing enzymes. Firstly, STR SUMOylation by Smc5/6 SUMO E3 enzyme facilitates its accumulation at DNA repair sites, with Esc2 (SUMO-like domain-containing protein) enhancing this SUMOylation (Bermúdez-López et al., 2016; Bonner et al., 2016; S. Li et al., 2021; Suhandynata et al., 2021). Secondly, the Mms4 nuclease is a mitotic substrate of the Slx5/8 STUbL. This is important as the inability to remove Mms4 leads to the accumulation of its active form on chromatin in the next G1 phase. Active Mms4 causes abnormal processing of replication-associated recombination intermediates and delays the activation of the DNA damage checkpoint (Waizenegger et al., 2020). Finally, the Slx5/8 STUbL also regulates the degradation of the Yen1 nuclease. SUMOylation of active Yen1 helps in limiting crossover formation and hence, suppresses chromosome segregation defects in various mutants (Bauer et al., 2019; Talhaoui et al., 2018).

### **2. SUMOylation mediates chromatin mobility and relocation of DNA damage sites**

Difficult-to-repair DSBs and collapsed replication forks are relocated to the nuclear periphery, where they interact with the nuclear pore complexes (NPCs). This relocation facilitates recombination-dependent repair or replication restart in order to maintain genome integrity (Kramarz et al., 2020a; Lamm et al., 2020, 2021a, p. 202; Nagai et al., 2008a; Pinzaru, Lamm, et al., 2020; Whalen & Freudenreich, 2020a). During replication stress or DNA damage, factors involved in homologous recombination are also subjected to SUMOylation, which is essential for regulating the functional activity (Cremona et al., 2012; Psakhye & Jentsch, 2012). Notably, NPCs serve as centers for SUMO activity, being enriched with multiple proteins associated with the SUMO pathway. Such proteins include SIM-containing STUbLs and SUMO proteases (Panse et al., 2003; X. Zhao et al., 2004). This highlights SUMOylation as a crucial link to enhance recombination-mediated repair in a spatially protected manner at the NPCs. Multiple studies have shown that the mechanisms of relocation of DSBs or replication stress sites depend on SUMOylation.



## 2.1. DSBs

In *Drosophila* cells, the SUMO ligase Nse2 and the STUbL enzyme Dgrn are required for the relocation of heterochromatic DSBs to the nuclear periphery. Additionally, SUMOylation impedes HR progression in heterochromatin domains by blocking the recruitment of Rad51 and thus prevents potential aberrant recombination between repeated sequences. At the nuclear periphery, STUbL likely mediates the ubiquitination and subsequent degradation of so far unidentified SUMOylated proteins, which may in turn promote Rad51 loading and repair (Caridi et al., 2018; Chiolo et al., 2011; Ryu et al., 2015a, 2016a). STUbl, Dgrn also aids in the relocation of SUMOylated heterochromatic DSBs to Mps3 located in the inner nuclear membrane (Koi and Spag4 in flies) (Ryu et al., 2015a).

In budding yeast, DSBs in the ribosomal DNA relocate outside the nucleolus for HR repair, facilitated by SUMOylation of HR proteins (Lisby & Rothstein, 2015; Torres-Rosell et al., 2007). This process is dependent on ScMms21 and ScSiz2 SUMO ligases, with ScRad52 as a key SUMOylated target. Preventing ScRad52 SUMOylation leads to nucleolar ScRad52 foci and hyper-recombination, indicating that ScRad52 SUMOylation prevents harmful recombination in the rDNA loci by promoting nucleolar exclusion (Torres-Rosell et al., 2007). Another work from yeast showed that persistent DSBs are recruited to the nuclear periphery in a SUMO-dependent manner. In the S-phase, monoSUMOylation by ScMms21 promotes DSBs relocation towards the nuclear envelope protein ScMps3. On the other hand, polySUMOylation in G1 recruits the SUMO-targeted ubiquitin ligase ScSlx5/Slx8 which promotes the relocation of persistent DSBs to the NPC. In this context, the formation of SUMO chains depends on both ScMms1 and ScSiz2 and deletion of either SUMO ligase inhibits the relocation of DSBs to the NPCs (Horigome et al., 2016a; Nagai et al., 2008a).

Similar to persistent heterochromatin DSBs, eroded telomeres are also moved to the nuclear periphery for repair (Seeber & Gasser, 2017). Eroded telomeres in budding yeast accumulate SUMO-conjugates on RPA and telomeric components. SUMOylation further targets the eroded telomeres to the NPC by the ScSlx5/Slx8 STUbL. At the NPC, ScUlp1-mediated deSUMOylation takes place which promotes Rad51-independent repair and generation of type II survivors (Churikov et al., 2016b; Géli & Lisby, 2015; Khadaroo et al., 2009). Moreover, POT1 mutation at the telomere that fosters tumorigenesis displays synthetic lethality with pathways involved in SUMOylation of replication/repair factors and the disassembly of the NPC. This hints towards a dependence on SUMO-mediated repair at the NPC (Pinzaru, Kareh, et al., 2020a). Finally, SUMOylated Rad52 also promotes the repair of eroded telomeres via a Rad51-

dependent pathway, while SUMOylated Rad59 aids repair through a Rad59-dependent pathway (Charifi et al., 2021).

These studies indicate that SUMOylation coordinates the nuclear positioning of DSBs, ensuring HR-mediated repair occurs in a safer environment.

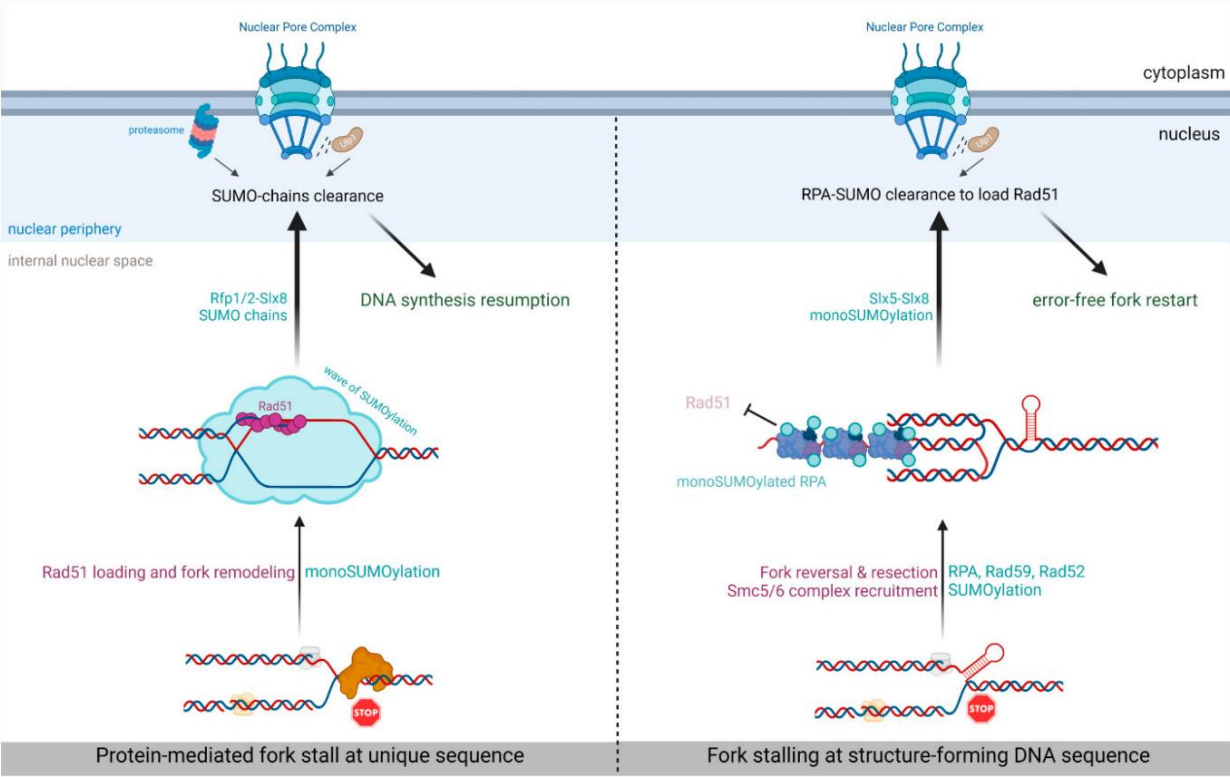
## 2.2. Replication stress sites

### *Forks stalled by tri-nucleotides repeats*

In budding yeast, replication forks collapsed at CAG repeats relocate and anchor to the NPC in a process dependent on SUMOylation ([Figure 29](#)). This mechanism involves the Smc5/6-associated ScMms21 and the ScSlx5 STUbL proteins (X. A. Su et al., 2015a; Whalen & Freudenreich, 2020a). Interestingly, expressing a mutated SUMO particle that eliminates polySUMOylation did not reduce relocation rates, suggesting that monoSUMOylation alone is sufficient for promoting relocation. In addition, SUMOylated HR proteins help in the relocation of these collapsed forks to the nuclear periphery (Whalen et al., 2020a). Some specific targets of SUMOylation upon fork collapse include ScRad52, ScRad59, and the RPA subunit. Although individual SUMOylation-deficient mutants in these proteins only partially impair relocation, SUMOylation of all three proteins is necessary for efficient relocation and double mutants displayed additive effects (Whalen et al., 2020a). This implies that SUMOylation of these proteins may facilitate their interaction with the nucleoporin, Nup84-bound ScSlx5, which tethers the collapsed fork to the NPC. While ScRad59 and RPA were shown to be SUMOylated by Mms21, no differences were observed in the SUMOylation pattern of Rad52 in Mms21 mutants lacking its ligase domain. This suggests that the SUMOylation observed may result from different stimuli, although a minor role for the other SUMO ligase ScSiz2 cannot be excluded.

Besides promoting relocation, SUMOylation at the collapsed fork has another role. SUMOylated RPA, loaded onto previously processed ssDNA overhang, prevents ScRad51 loading before NPC anchorage. Possibly, SUMOylation of RPA subunits may inhibit ScRad51 filament formation by changing the kinetics of the RPA filament disruption, or alternatively, by modifying ScRad51's interaction with its loader ScRad52 (Whalen et al., 2020a). The exclusion of ScRad51 from stalled forks in the interior of the nucleus is thought to prevent recombination events that might be detrimental at the early stages of fork stalling. At the NPC, ScSlx5/Slx8 STUbL enzyme can ubiquitinate SUMOylated proteins at the fork, leading to their degradation. This in turn could alleviate the inhibition of Rad51 binding and facilitate its access to ssDNA to stimulate HR-mediated fork restart (Whalen et al., 2020a).

**Figure 29: SUMO-driven routing of replication stress sites towards the nuclear periphery.** Left panel: DNA-bound, protein-mediated fork arrest in the fission yeast system. Right panel: Structure-mediated fork stalling in budding yeast. (Schirmeisen et al., 2021a)



In summary, SUMOylation drives the relocation of collapsed forks induced by repeats towards NPCs, enabling a Rad51-dependent pathway of fork restart.

#### *Forks stalled at DNA-bound protein complex*

In fission yeast, when replication forks stall at a specific site known as a replication fork barrier (RFB), they move to the nuclear periphery during S-phase and attach to the NPC (Kramarz et al., 2020a). Similar to the situation in budding yeast, SUMOylation plays a crucial role in determining the fate of these stalled forks, with both beneficial and detrimental effects ([Figure 29](#)).

SUMOylation, facilitated by the SpPli1 SUMO E3 ligase, is essential for protecting dysfunctional forks and guiding them towards the nuclear periphery. Unlike in the case of collapsed forks at CAG tracts, monoSUMOylation alone is not adequate to trigger relocation, highlighting the necessity of SUMO chain formation. The absence of the subunits of STUbL, including SpSlx8 as well as SpRfp1 and SpRfp2 (similar to ScSlx5 in budding yeast), leads to arrested replication forks being no longer enriched at the nuclear periphery. Interestingly, in strains with mutated SpSlx8, the active RFB exhibited increased mobility (indicating a lack of anchorage), a phenomenon not observed in the absence of Pli1. Thus, SUMO chains accumulating at arrested forks promote relocation to the nuclear periphery and facilitate their anchorage to the NPC in a STUbL-dependent manner (Kramarz et al., 2020a). This is crucial for removing poly-SUMOylation by the SUMO protease, Ulp1 from the relocated forks to enable HR-dependent DNA synthesis. Nevertheless, specific SUMO targets in this process have not been identified yet.

Consequently, NPCs play a crucial role in enabling the resumption of DNA synthesis by clearing SUMO conjugates from arrested forks. This resumption of DNA synthesis is facilitated by two factors enriched at the nuclear periphery: the Ulp1 SUMO protease, which clears off the SUMO chains and ensures efficient restart of DNA synthesis whereas, the proteasome sustains the progression of restarted forks (Schirmeisen et al., 2023).

Overall, these findings underscore the vital role of NPCs in removing DNA repair/replication factors, which could hinder the resumption of DNA synthesis at stalled forks, when SUMOylated. The SUMO-driven relocation of perturbed replication forks serves as a critical protective mechanism to uphold genome stability. Although variations exist in the mechanisms involved in different types of stalled forks, the underlying principles and outcomes of these processes remain consistent across various systems.

## IV. The intricate crosstalk between the centromere and STUbL

### A: The centromere: A recap

The centromere is a specialized region of the eukaryotic chromosome that plays a crucial role in chromosome segregation during cell division (McKinley & Cheeseman, 2016; Yanagida, 2005). The centromere serves as the attachment site for the kinetochore, a protein complex that interacts with the spindle fibres emanating from the two spindle poles of the dividing cell during mitosis. The attachment of spindle fibres ensure accurate chromosome segregation so that each daughter cell receives an identical set of chromosomes. The structure and function of the centromere can be detailed as follows:

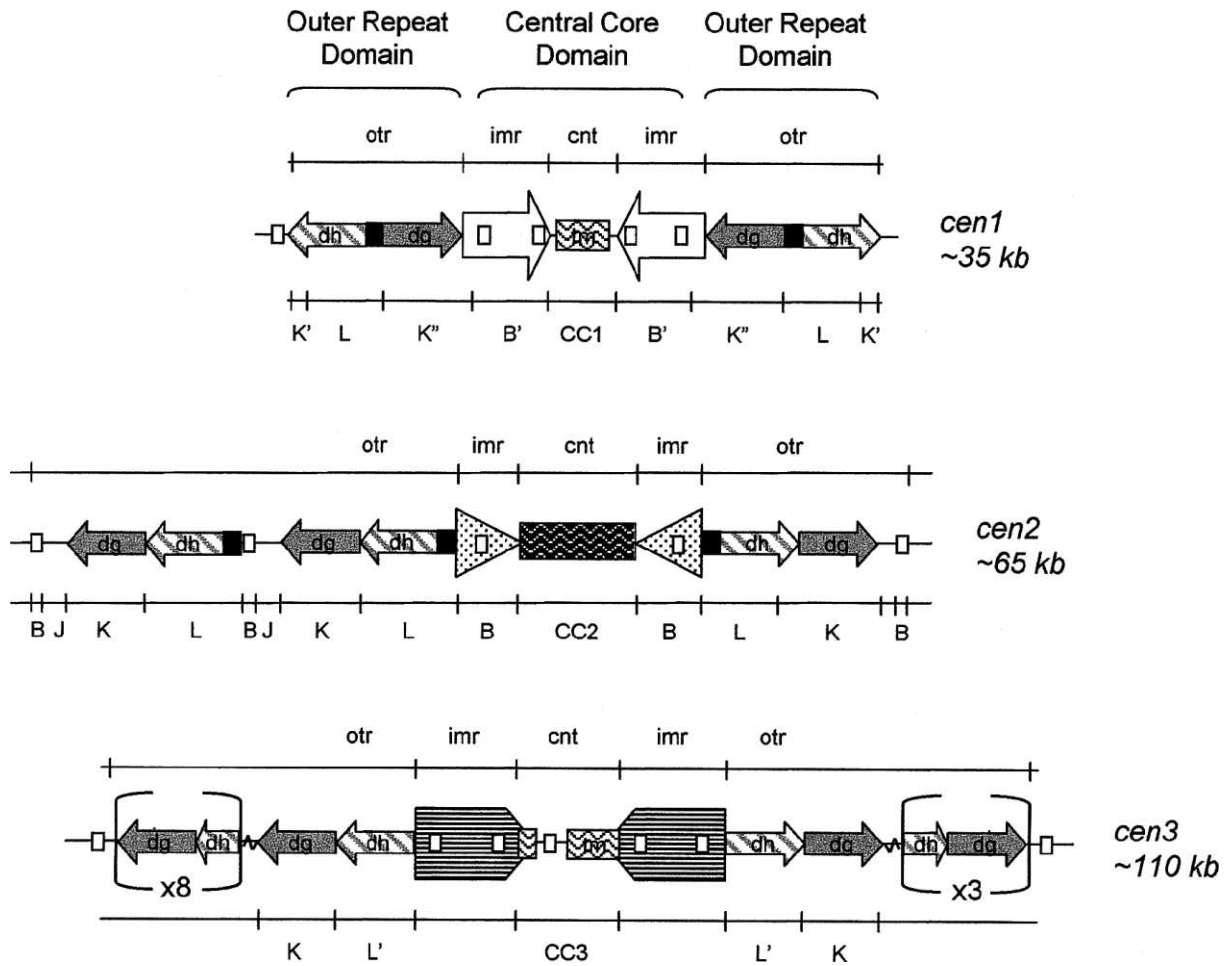
#### 1. Structure

During cell division, one of the most critical steps is ensuring the equal distribution of the duplicated genome to the daughter cells (Prosser & Pelletier, 2017; Winey & Bloom, 2012). The process of guiding the separation of sister chromatids is brought about by an array of microtubules, which nucleate from the microtubule-organising centers (MTOCs) (Rincón & Monje-Casas, 2020; J. Wu & Akhmanova, 2017). MTOCs are present at the two opposite poles of a dividing cell and are composed of nine sets of microtubule triplets arranged in a ring, called centrioles (Avidor-Reiss & Gopalakrishnan, 2013). In metazoans, these polar MTOCs typically consist of two perpendicular centrioles and pericentriolar material (PCM), forming the organelle known as centrosome. Centrosomes in metazoans are cytoplasmic structures (Bobinnec et al., 1998). In yeast, the orthologous structure to the centrosome is known as the spindle pole body (SPB) (Jaspersen & Winey, 2004). Unlike centrosomes, the SPB is embedded within the nuclear envelope, as yeast undergoes closed mitosis (Rüthnick & Schiebel, 2018).

Microtubules nucleated from the centrosomes/SPB attach to the chromosomes at specialised regions called centromeres, which were first identified in the 1930s (Darlington & Hall, 1997). For a long time, centromeres have been interchangeably used with kinetochores, until they were proven to perform separate functions (Brinkley & Stubblefield, 1966; Jokelainen, 1967; Luykx, 1965; Schrader, 1939). It is now widely accepted that centromeres are chromatin structures whereas, kinetochores are proteinaceous structures that bind to the centromere (Cooke et al., 1993).

Centromeric regions typically consist of repetitive DNA sequences (Pardue & Gall, 1970). In humans, this is primarily composed of alpha satellite DNA, which is a highly

**Figure 30: The DNA structure of fission yeast centromeres.** Centromeres have a central core (cnt) surrounded by innermost repeats (imr) and outer repeats (otr). Cen1, Cen2 and Cen3 are the three centromeric regions displaying significant variability. The annotation dg/dh/tm used by Yanagida and colleagues, and K, L, CC that is used by Clarke and colleagues are both given to indicate the relationship between the two systems of nomenclature for marking repeat sequences, unique sequences and sequence with similarity. (Pidoux & Allshire, 2004)



repetitive sequence with interspersed short and long non coding DNA elements (Alexandrov et al., 1993; Willard, 1985; Willard & Waye, 1987). In budding yeast, centromeric sequences are relatively short, about 125 base pairs in length (Clarke & Carbon, 1980; Hegemann & Fleig, 1993). They consist of three DNA elements named CDEI, CDEII, and CDEIII (Clarke & Carbon, 1985; Fitzgerald-Hayes et al., 1982). However, centromeres in fission yeast are larger and more complex compared to those in budding yeast, spanning across 40-100 kilobase pairs (Carbon & Clarke, 1990).

Fission yeast has three chromosomes and thereby contain three centromeres. All the three centromeric-regions have a conserved central core and flanking inverted repeats (Chikashige et al., 1989). However, the nucleotide sequences are not conserved and contain significant variability. Centromere 1 (*cen1*) spans across 35 kb, whereas, *cen2* and *cen3* occupy 65 kb and 110 kb respectively ([Figure 30](#)) (Steiner et al., 1993; K. Takahashi et al., 1992). Each of the 3 centromeres consist of a central core domain flanked by inner and outer repetitive elements. The central core is approximately 4-7 kb, containing unique sequences (Clarke et al., 1986). The inner repeats (*inr*) flank the central core, consisting of inverted repeats. Whereas, the outer repeats (*otr*) are composed of heterochromatic sequences, including the *dg* and *dh* repeats (Chikashige et al., 1989; Pidoux & Allshire, 2004; K. Takahashi et al., 1992). The *dh* and *dg* repeats are typically 1-5 kb pairs in length and contain highly repetitive sequences. These repeats are rich in AT base pairs and contribute to the formation of heterochromatin through the binding of specific proteins for a functional centromere (Kagansky et al., 2009; K. Takahashi et al., 1992). In fission yeast, centromeric heterochromatin structures were detected using high-pressure freezing electron microscopy techniques (Kniola et al., 2001).

The centromere-specific DNA could be either unique to the chromosome, as observed in the central core region (*cc2*) on chromosome II (Clarke et al., 1993). Alternatively, centromeric DNA could also have regions of homology as observed in the central core region of chromosome I and III (*cc1* and *cc3*) (S. Murakami et al., 1991; Steiner et al., 1993).

Fission yeast serves as an outstanding model to study centromere biology since it is suitable for genetic and biochemical analysis. Research suggests that centromeres in fission yeast resemble those of higher eukaryotes, in contrast to the centromeres in budding yeast (Clarke, 1990). This is because centromeres in fission yeast contain distinguished centromere-specific, repetitive DNA sequence, which are analogous to higher eukaryotes. Similarly, kinetochore attachment to centromeres in metazoans are dependent on multiple spindle attachment as in fission yeast (Ding et al., 1993; Joglekar

et al., 2008). They are called the regional centromeres (Pluta et al., 1995). Conversely, budding yeast has a point centromere with a single microtubule attachment making it more distant from the higher eukaryotes (Peterson & Ris, 1976).

Additionally, the surrounding structure of the centromeric chromatin is the region of pericentric heterochromatin domain which is critical for sister chromatid cohesion (Bernard et al., 2001; Nonaka et al., 2002). In fission yeast, the boundary between centromeric and pericentric chromatin is demarcated by two types of elements: a single tRNA gene and inverted repeats that flank the pericentric chromatin (Ayoub et al., 2000; Noma et al., 2006; K. C. Scott et al., 2006). These elements function as barriers to prevent the spreading of heterochromatin into euchromatic regions.

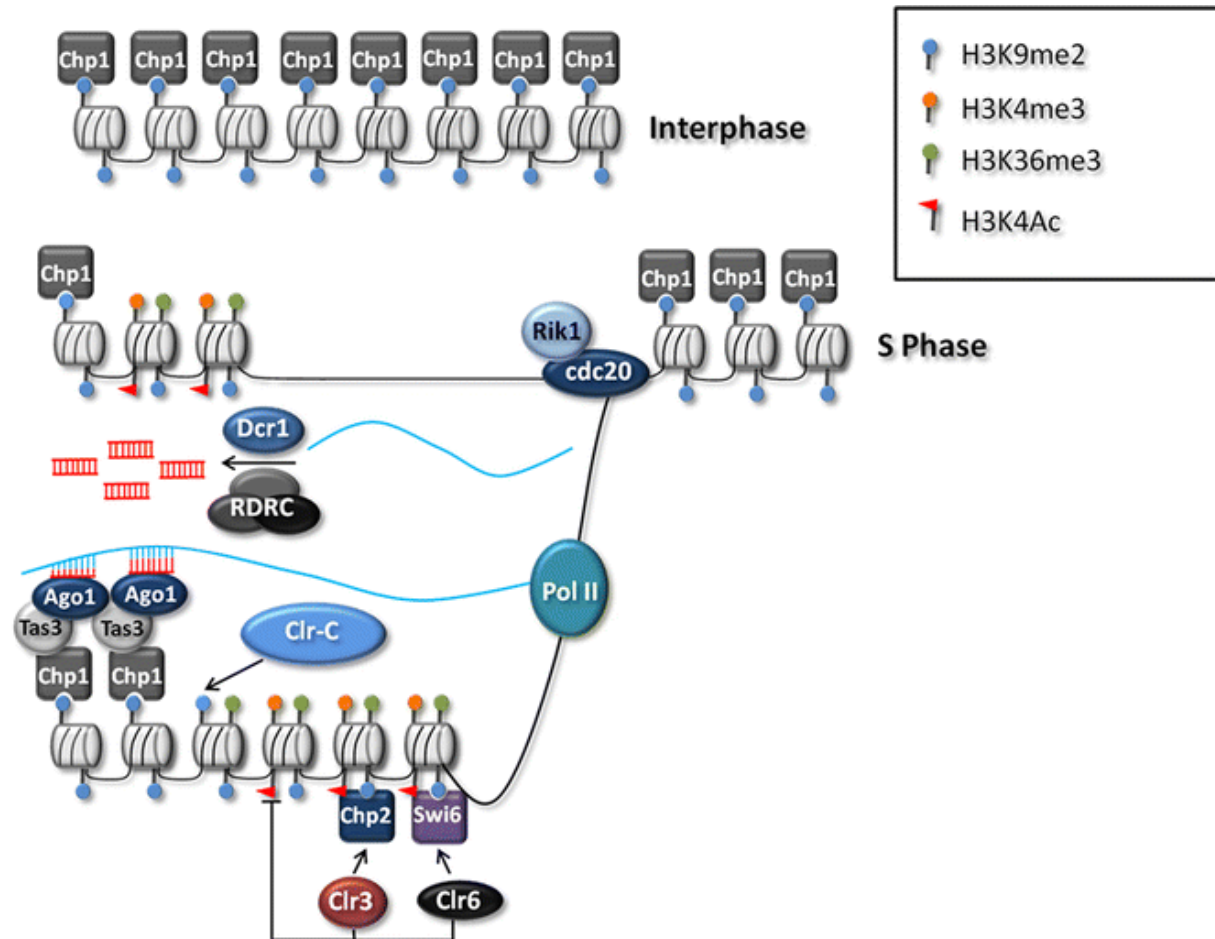
## 2. Functions

Centromeres are important structures within the chromosomes that ensure genomic stability and maintain cellular functions (Cleveland et al., 2003). They form the assembly site for kinetochores, which are the sites for mitotic and meiotic spindle attachment (Bernard et al., 1998; A. Hayashi et al., 2006; Pidoux & Allshire, 2004). Spindle attachment to kinetochores function in separation of sister chromatids during cell division. Functional spindle attachment facilitates chromosome movement and ensures equal distribution of chromosomes to the following generation (Dobie et al., 1999; Pidoux & Allshire, 2000; Rieder & Salmon, 1998; S. Saitoh et al., 1997). Therefore, centromeres are crucial for genetic stability. Defects in segregation of chromosomes cause various human disease (Potapova & Gorbysky, 2017). Chromosome segregation defects in meiosis can result in aneuploid embryos with lesser or more chromosomes than normal (Lamb & Hassold, 2004). On the other hand, errors in mitotic chromosome segregation can lead to chromosome lagging and asymmetric nuclear division, contributing to tumor formation (Ba et al., 2007; Ishii et al., 2008; Lengauer et al., 1997).

In fission yeast, assembly of a functional kinetochore upon the centromere depends on the histone protein Cnp1, which is a centromeric histone variant of the conventional histone protein H3 (Allshire & Karpen, 2008; K. Takahashi et al., 2000). Centromeric H3-containing nucleosomes are specifically associated with centromere formation and is essential for the assembly of the kinetochores (Castillo et al., 2007). Surprisingly, the pericentromere replicates early in the S phase and loss of heterochromatin causes this domain to become very sensitive to replication fork pausing, stalling or collapse, leading to gross chromosome rearrangements (Nakagawa & Okita, 2019; Okita et al., 2019). During S phase, canonical histone H3 is incorporated into nucleosomes across the genome, including centromeres. Consequently, Cnp1 is incorporated at centromere-specific nucleosomes after DNA replication, in the G2 phase of the cell



**Figure 31: Propagation of centromeric heterochromatin during DNA replication.** Heterochromatin is marked by H3K9me, which recruits RNAi components and Clr-C. During S-phase, replication dilutes H3K9me, allowing transcription and active marks like H3K4Ac to appear, reducing Chp1 binding and RITS association. Swi6 and Chp2 bind to H3K9me, recruiting deacetylases to remove H3K4Ac, while Clr4 is recruited to re-establish H3K9me and RNAi converts centromeric transcripts to siRNAs, propagating heterochromatin. (Alper et al., 2012).



cycle (Shukla et al., 2018). The centromeric nucleosomes are reassembled with the help of specific chaperones and loading factors like Scm3, Mis6, Mis16 and Mis18 (Folco et al., 2024; T. Hayashi et al., 2004; Williams et al., 2009). These factors help to localise Cnp1 at the centromere. Further, Scm3 and Mis18 are involved in enriching Cnp1 (CENP-A in humans) at the centromere and removal of the canonical H2B and H3 histones by Cnp1 in order to form functional centromeres (Ogiyama et al., 2013; Williams et al., 2009).

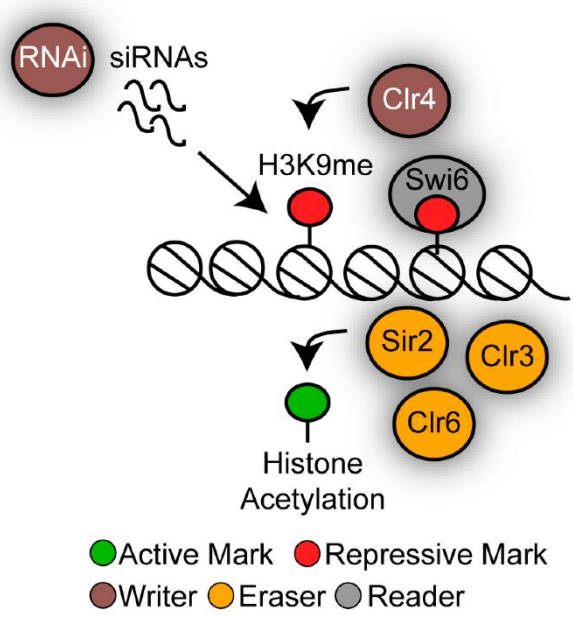
No specific sequence is established to be important for centromere function in fission yeast. It is rather the entire central core and the outer repeat that is required for chromosome segregation, which creates a unique structure to make it functional (Baum et al., 1994; Folco et al., 2008; Marschall & Clarke, 1995).

### *Epigenetic control of centromeres*

Fission yeast centromeres are epigenetically regulated to maintain centromere identity and prevent impaired kinetochore assembly (Karpen & Allshire, 1997). Epigenetic regulation of centromere function involves a complex interplay of histone modifications, non-coding RNA and protein complexes that establish and maintain a heterochromatic state of a functional centromere. This includes methylation of histone H3 at lysine 9 (H3K9Me) by the single methyl-transferase, Clr4 in *S. pombe* (Cutter DiPiazza et al., 2021; Nakayama et al., 2001a; Yamada et al., 2005). H3K9 methylation is a hallmark for heterochromatin formation and serves as a binding site for proteins like Swi6 (Human HP1 homolog) and Chp2, which aid in chromatin compaction by bridging neighbouring nucleosomes (Canzio et al., 2011; Sadaie et al., 2004). This further prevents binding of the transcription machinery and silence gene expression (Bannister et al., 2001; Partridge et al., 2002; Sadaie et al., 2008; K. Zhang et al., 2008). Swi6 helps in spreading and maintaining the heterochromatic state by binding to newly methylated H3K9 residues (M. T. Hayashi et al., 2009; P.-C. Li et al., 2013). Moreover, Swi6 recruits cohesin to the outer centromeric repeats to maintain sister chromatid cohesion and it has been observed that physical tethering of chromatids to the centromere can bypass Swi6 requirement (Yamagishi et al., 2008).

Initially, centromeres were thought to be transcriptionally inactive. However, it was later demonstrated that centromeric transcription does occur for a brief period during the S-phase of the cell cycle, when histone marks are removed, allowing RNA Pol II access ([Figure 31](#)) (E. S. Chen et al., 2008; Kloc et al., 2008). Transcription of centromeric repeats produces non-coding RNAs that are processed by the RNAi machinery (Verdel & Moazed, 2005). These non-coding RNAs are essential for targeting the RNAi component called the RNA-induced transcriptional silencing (RITS) complex to

Figure 32: Histone deacetylases Sir2, Clr3 and Clr6 maintain the hypoacetylated histone state in fission yeast. (Buscaino, 2019)



centromeric regions, guiding the deposition of H3K9me marks at specific loci (Verdel et al., 2004; White & Allshire, 2008). Fission yeast strains lacking functional RNAi exhibit increased centromeric transcript levels, transcriptional de-repression of transgenes, reduced centromeric siRNAs, and a marked decrease in H3K9 methylation levels at centromeres (Volpe et al., 2002). The combined effect of which leads to the loss of centromeric heterochromatin and disruption of centromere function (Grewal, 2010). RNAi machinery is also required for maintaining the regulation of chromosome segregation in mitosis and meiosis in *S. pombe* (Hall et al., 2003). This defect in chromosome segregation is due to disruption in centromere cohesion in the RNAi mutants.

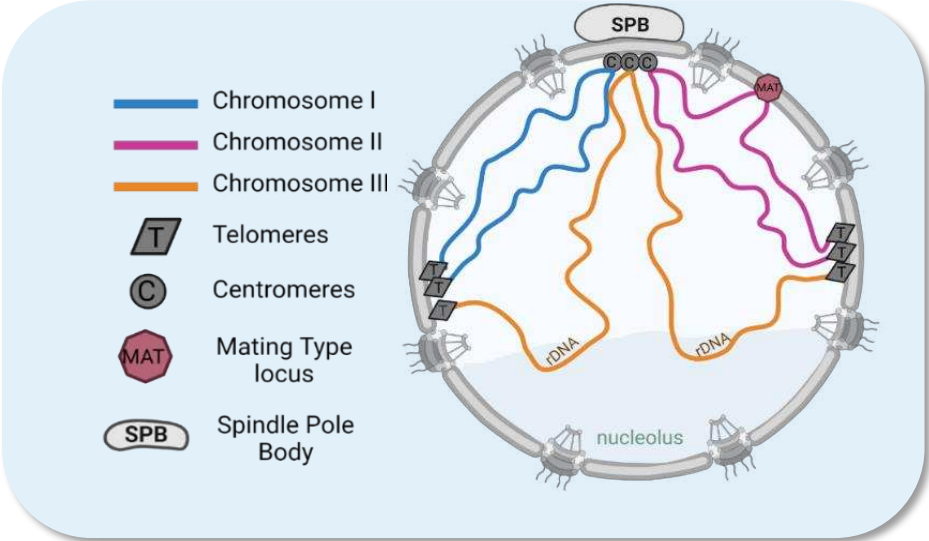
Furthermore, histone deacetylases (HDACs) that remove acetyl groups from histone tails also lead to a more compact and transcriptionally silent chromatin structure. Histone deacetylation by Clr3, Clr6 and Sir2 reinforces the heterochromatic state established by H3K9 methylation at the centromere (Figure 32) (Nakayama et al., 2001a). Inhibition of histone deacetylation results in de-repression of centromeric heterochromatin and chromosome loss (Ekwall et al., 1997). Other chromatin remodelling factors include the SHREC complex. This complex interacts with methylated histones and deacetylates them, aiding in the formation of silent chromatin (Sugiyama et al., 2007). It helps to maintain the repressed chromatin state at centromeres.

### *Centromere clustering*

In fission yeast, three heterochromatic regions including the centromeres, telomeres, and the mating type loci cluster at the nuclear periphery (Figure 33) (Alfredsson-Timmins et al., 2007; Funabiki et al., 1993; Mizuguchi et al., 2015a). Centromere clustering brings all the three chromosomes together and localizes them adjacent to the spindle pole body (SPB) at the nuclear periphery during interphase (Jin et al., 2000). The spatial organisation of clustering is an essential aspect for loading of centromeric proteins like Cnp1, thereby influencing centromere identity (W. Wu et al., 2022a).

Centromere clustering in *S. pombe* is dependent on the clustering protein Csi1, which forms the physical link between the kinetochore and the SPB at the nuclear envelope (Figure 34) (Hou et al., 2012a, p. 201, 2013, p. 2). Deletion of Csi1 leads to severe de-clustering of centromeres, causing them to disperse within the nucleus instead of remaining clustered near the SPB (Hou et al., 2012a, p. 201). These mutants exhibit a high frequency of chromosome loss during mitosis and experience delays in mitosis due to disruption in centromere clustering. Csi1 is the strongest known de-clustering mutant in fission yeast.

**Figure 33: Three heterochromatic regions including the centromeres, telomeres, and the mating type loci cluster at the nuclear periphery in fission yeast.** (prepared by previous PhD student, Kamila Schirmeisen)



Additionally, multiple other factors can affect centromere clustering. The Sad1 protein (LINC complex SUN domain protein), located at the nuclear envelope, interacts with Kms1 and Kms2 proteins associated with the SPB. This complex anchors kinetochores to the SPB and is essential for centromere clustering (Fernández-Álvarez et al., 2016; Hou et al., 2012a).

Similarly, Lem2 (LEM domain inner nuclear membrane protein) is an inner nuclear membrane proteins that interact with centromeric chromatin and contribute to proper centromere positioning and clustering (Tange et al., 2016).

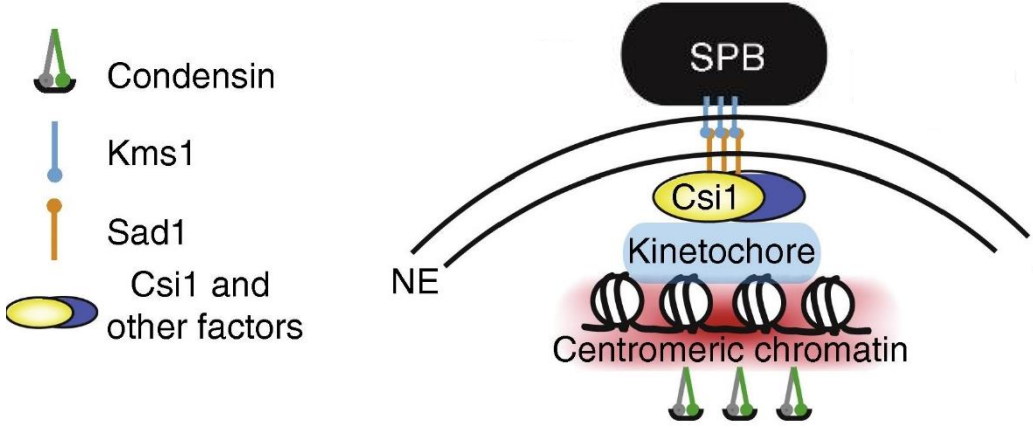
Overall, centromere clustering in fission yeast is a critical aspect of cellular function, influencing various processes from chromosome segregation and nuclear organization to genome stability and gene regulation.

## **B: SUMO control in the regulation and homeostasis of the centromere in *S. pombe***

In this section, I will discuss how centromere homeostasis is maintained, with special emphasis on SUMOylation. As explained in Chapter III.A, SUMO is a small protein, which can covalently bind and modify the function of target proteins. This is crucial in the regulation of various cellular functions.

In fission yeast, the SUMO pathway was shown to be required for the alleviation of chromosome segregation defects (Tanaka et al., 1999). SUMO deleted cells exhibited a plethora of problems including mitotic chromosome structure or segregation errors (Shayeghi et al., 1997). Since late 1990s, SUMOylation was suggested to participate in regulating centromeric heterochromatin. Initially, it was reported in humans that the PcG bodies, which are the regulators of homeotic gene expression could be centers for SUMOylation (Kagey et al., 2003). Research suggested that the PcG bodies which are located near centromeres, could be somehow linked to centromeric stability by promoting heterochromatin formation (Saurin et al., 1998). However, direct evidence was not presented until the mid-2000, where a study highlighted that deletion of *Pmt3*, which is the gene that encodes the SUMO protein in fission yeast, displayed defects in silencing of heterochromatin in the pericentric region of the centromere (Shin, Choi, et al., 2005). SUMO mutants also displayed altered histone modification patterns at heterochromatic regions (Shin, Choi, et al., 2005). The study revealed that deletion of the SUMO encoding gene, *pmt3*, leads to disruption in centromere silencing. In addition, they demonstrated that SUMO-conjugating enzyme, Hus5, is enriched at centromeric heterochromatin and the enrichment is dependent upon the methyl transferase, Clr4 and heterochromatin domain protein, Swi6. Both, Clr4 and Swi6 are

Figure 34: Csi1 forms the physical link between the kinetochore and the SPB at the nuclear envelope in fission yeast. (Adapted from Mizuguchi et al., 2015)



SUMOylated by Hus5 and defects in SUMOylation of Clr4 and Swi6 also causes a slight defect in centromere silencing. This study provided clear evidences for the significance of SUMO in centromere silencing and maintenance.

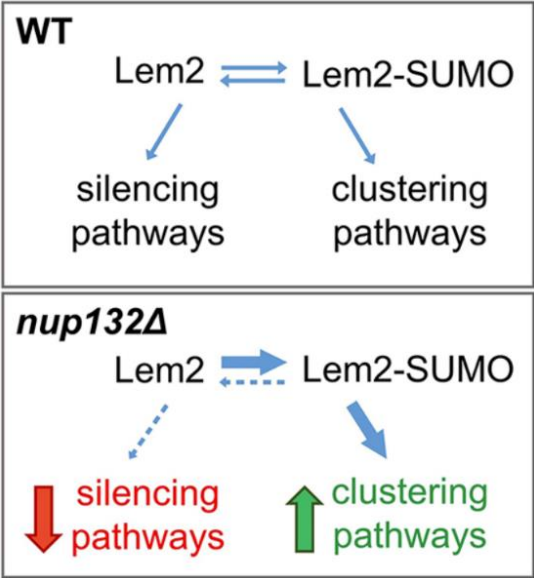
Moreover, inner nuclear membrane protein, Lem2, is also involved in centromere clustering along with Csi1 in parallel pathways (Barrales et al., 2016). Both Lem2 and Csi1 are subject to SUMOylation in fission yeast (Køhler et al., 2015a). Lem2 has been recently identified as a key factor, involved in centromeric defect in cells depleted for the nucleoporin, Nup132 (Strachan et al., 2023a). Nup132 deleted strains have lost the ability to tether Ulp1 (SUMO protease) to the nuclear periphery and thereby, leads to the degradation of Pli1 (E3-SUMO ligase) by auto-SUMOylation and successive degradation (Nie & Boddy, 2015, p. 201). This leads to a global reduction in SUMOylation, whereas an enrichment in poly-SUMOylation at the NPC due to the lack of Ulp1 at the periphery (Strachan et al., 2023a). Authors in a recent study point out that suppression of Lem2 SUMOylation by reduction of global SUMOylation can rescue centromeric silencing defects (Strachan et al., 2023a). Therefore, highlighting that increased SUMOylation at the nuclear periphery is a feature of aberrant silencing. However, despite the negative regulation on silencing, Lem2 hyper-SUMOylation unexpectedly enhances centromere clustering and restores clustering defects in Csi1-deficient cells (Strachan et al., 2023a). This displays how Lem2 SUMOylation can have paradoxical effects upon different centromeric functions and underscores how fine-tuning of SUMOylation is important for centromere regulation ([Figure 35](#)).

Additionally, Pli1 is specifically involved in silencing of the central domain of the centromere and thereby affects structure of the centromere (Xhemalce et al., 2004b). Deletion of Pli1 leads to sensitivity towards the microtubule-destabilising drug, thiabendazole (TBZ) and a concomitant loss of minichromosomes. This suggests a possible defect in the establishment or maintenance of a proper central core structure for the centromere or kinetochore. Furthermore, division of labor takes place between Pli1 and Swi6 for centromere maintenance. Pli1 is required for preserving the central core, whereas Swi6 is required for maintaining silencing of the outer repeats (Xhemalce et al., 2004b). This highlights the differential effects on maintaining the multifaceted activity of the centromere.

Another instance of SUMO-mediated centromere maintenance is brought about by the Rrp1 and Rrp2 DNA translocases, which harbour STUbL activity (Barg-Wojas et al., 2020). Following overexpression of Rrp1 and Rrp2, centromeric histone Cnp1 is mislocalized in *S. pombe*. Cnp1 is mis-incorporated into dg repeats of the centromere, which is not a natural locus for Cnp1 incorporation. This leads to increased silencing



Figure 35: Model for the impact of SUMOylation on Lem2 function in centromere clustering and silencing. (Strachan et al., 2023a)



and faulty centromere structure via modulation of histone dynamics. This study elucidated the critical role of the dynamic regulation of SUMOylation and emphasized upon the necessity of precise modulation at highly sensitive thresholds.

Altogether, multiple studies have shown that mutations or disruptions in the SUMOylation pathway can lead to defects in centromere maintenance and chromosome mis-segregation, underscoring the importance of SUMOylation in these processes.

## V. Experimental system

### A: Fission yeast as a powerful model for studying eukaryote biology

A model organism is a non-human species that is widely used to study particular biological processes. The goal of using model organisms is to gain insights into the functioning of other organisms, including humans, due to the shared biological pathways and genetic similarities. They are chosen for their simplicity, ease of manipulation, short generation times, and well-characterised genetics, making them valuable tools in research for studying development, disease, and basic cellular functions. Examples include unicellular organisms (bacteria, yeast), invertebrate animal models (roundworms, fruit flies), vertebrate animal models (frog, zebrafish, chicken) and mammalian model organisms (mice, rats) (Figure 36).

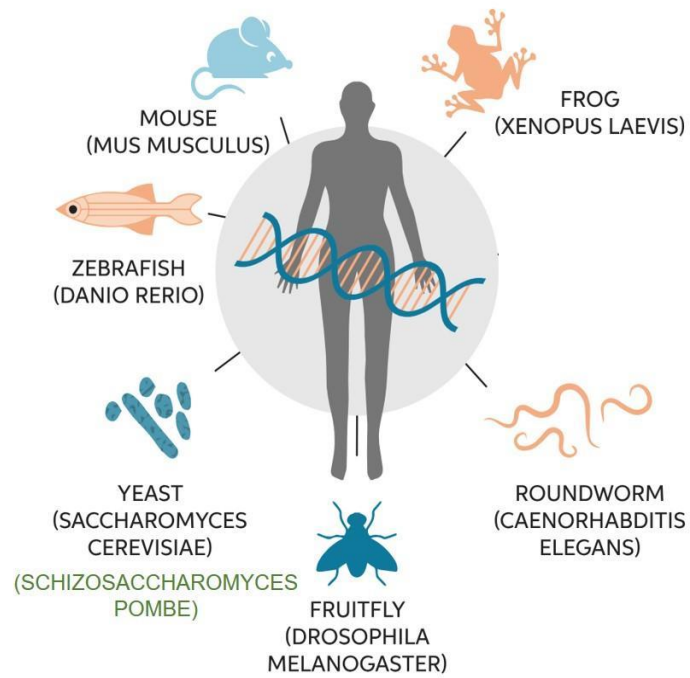
All these species share significant biological similarities with humans, leading to the belief that observations made in model organisms can reveal insights into the molecular mechanisms of human cells. Therefore, research using model organisms has the potential to enhance our understanding of the molecular causes of human diseases and expedite their diagnosis and treatment.

In research, two yeast species are frequently employed to investigate the molecular functions of eukaryotic cells: the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* (Figure 37). Although they share a common ancestor, these species have become evolutionarily distinct since they diverged around 400 million years ago (Heckman et al., 2001; Hedges, 2002). Notably, the evolutionary gap between budding and fission yeast is equivalent to that between fission yeast and mammals, placing both yeast species at a similar distance from humans (A. Vyas et al., 2021, p. 202). Moreover, the rapid evolution of budding yeast has resulted in the loss of certain genes and functions that fission yeast and metazoans have retained. For example, budding yeast has lost the RNA interference (RNAi) pathway, which enables it to harbor dsRNA killer viruses. (Drinneberg et al., 2011).

Therefore, fission yeast offers unique advantages for investigating the biological processes in complex eukaryotes including humans, making it a valuable "micromammal" model (Aravind et al., 2000; Forsburg & Rhind, 2006; Wood et al., 2002).

Fission yeast was first isolated from East African beer and described in 1893 by Paul Linder, who named it *Schizosaccharomyces pombe*. The term "shizo" means split/fission, reflecting the yeast's division method, while "pombe" means beer in Swahili (*Schizosaccharomyces Pombe Lindner, 1893, n.d.*). Research on fission yeast as a laboratory organism began in 1946 when Urs Leupold used *S. pombe* for genetic

**Figure 36: Common model organisms used in molecular biology.** (Adapted from [www.chegg.com/learn/topic/model-organisms](http://www.chegg.com/learn/topic/model-organisms)).



analysis of the mating-type system (Leupold, 1950). Around the same time in Edinburgh, Murdoch Mitchison used fission yeast to study growth and division (Mitchison, 1957, 1990). The fields of genetics and cell biology merged in the mid-1970s when Paul Nurse, after learning yeast genetics in Leupold's group, joined Mitchison's lab to explore cell cycle control. Together with colleagues, Nurse isolated yeast mutants with blocked cell cycle progression and altered size and division (P. Fantes, 1989). In 2001, Paul Nurse, Leland Hartwell, and Tim Hunt were awarded the Nobel Prize in Physiology or Medicine for their discovery of key cell cycle regulators.

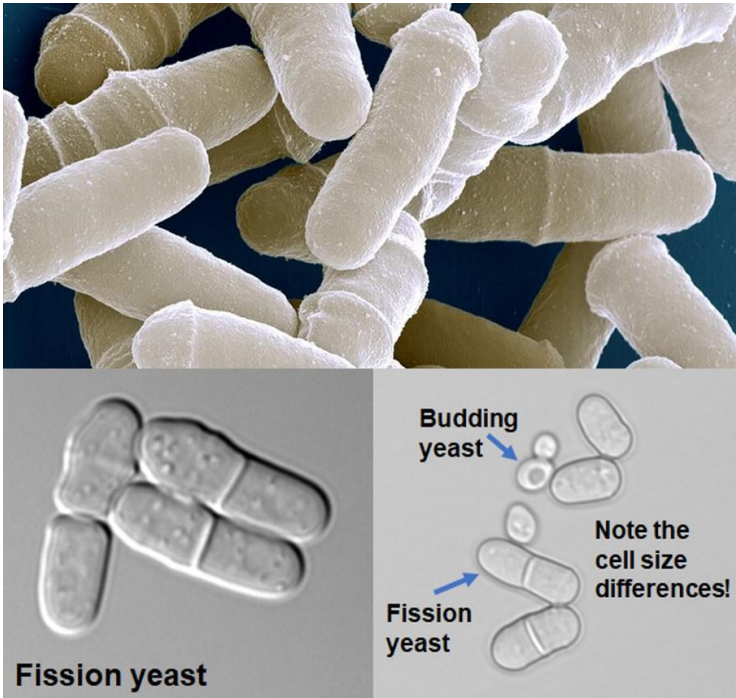
Over the subsequent decades, the fission yeast research community grew globally, contributing significant insights into the molecular mechanisms of various cellular processes like DNA replication, DNA repair, and the cell cycle (P. A. Fantes & Hoffman, 2016). A major milestone was achieved in 2002 when the entire genome sequence of *S. pombe* was published, making it the sixth eukaryotic species to have its complete genome sequenced (Wood et al., 2002).

Similar to budding yeast, the fission yeast genome is 13.8 Mb in size but is organized into three relatively large chromosomes of 5.7 Mb, 4.6 Mb, and 3.5 Mb, unlike the 16 smaller ones of the budding yeast. Of the 5,118 protein-encoding genes identified in fission yeast, 3,539 (69%) have human homologs, including 1,244 genes associated with human diseases (pombase.org).

Additionally, the chromatin structure in fission yeast exhibits several mammalian-like characteristics that are either absent or modified in budding yeast (Wood et al., 2002). These features include higher levels of chromosome condensation during mitosis, conserved heterochromatin proteins, larger origins of replication, and large centromeres with repetitive sequences, as well as highly organized telomere structures. Another similarity with mammals is in the gene structure. In human cells, most protein-coding genes contain exons and introns. Similarly, over 50% of fission yeast genes have one or more introns, compared to only 5% in budding yeast. Furthermore, gene splicing and epigenetic silencing mechanisms in fission yeast are very similar to those in mammalian cells, demonstrating a functional conservation in gene expression processes (A. Vyas et al., 2021).

The mitotic (vegetative) cell cycle of fission yeast consists of the G1, S, G2, and M phases ([Figure 38](#)) (Forsburg & Rhind, 2006; Mitchison & Nurse, 1985). Most cell growth occurs during the G2 phase, which makes up about 70% of the cycle. Upon reaching the necessary size, the cell enters the M-phase, where the nucleus divides into two smaller nuclei. Notably, the nuclear envelope remains intact during nuclear division, a process known as closed mitosis (Ding et al., 1997; McCully & Robinow, 1971). Following the

**Figure 37: Cellular morphology of fission yeast.** Transmission electron microscopy (top) and bright field microscopy (bottom) images of fission yeast cells. Comparison of the size and shape of budding and fission yeast cells (bottom right). ([www.mpg.de](http://www.mpg.de), [www.fast.kumamoto-u.ac.jp](http://www.fast.kumamoto-u.ac.jp))



M-phase, the cell enters G1, during which a medial septum forms to divide the cell at its midpoint (Hoffman et al., 2015). However, cytokinesis is not completed in the short G1 phase but occurs after DNA replication at the end of the subsequent S phase, resulting in two identical G2 daughter cells. Consequently, cells in G1 (with two nuclei, each containing a single genome: 2x1C) and G2 phase cells (with one nucleus containing a duplicated genome: 1x2C) have the same total DNA content, making it difficult to distinguish these phases by measuring cellular DNA content via flow cytometry.

Fission yeast primarily exists in the haploid form during the mitotic cycle. However, when faced with limited nutrients, cells can enter a stationary phase due to glucose starvation, or they can undergo the meiotic cycle upon nitrogen starvation (Hagan et al., 2016). The latter process involves the fusion of two haploid cells with opposite mating types (h+ and h-), resulting in the formation of a diploid zygote (Forsburg & Rhind, 2006). Subsequently, meiosis is initiated, leading to the production of four haploid spores, equivalent to human gametes. Upon restoration of appropriate nutrients, these spores germinate and re-enter the mitotic life cycle for vegetative growth. However, if nutrients are supplied to the conjugated cells before the initiation of the meiotic cycle (at the diploid zygote stage), they can continue vegetative growth as diploids (Egel & Egel-Mitani, 1974; Hayles & Nurse, 2018).

Apart from the mentioned similarities between fission yeast and mammalian cells, other advantages that make it an excellent model organism are as follows: Firstly, *S. pombe* is non-pathogenic, and its relatively short cell cycle allows for easy handling under standard laboratory conditions. Additionally, the small and easily manipulatable genome of fission yeast has facilitated the development of versatile experimental techniques for genomics and proteomics analyses. Consequently, over the past decades, fission yeast has emerged as a prominent model for unraveling the fundamental molecular mechanisms governing not only cell division and morphology but also DNA replication, repair, and recombination.

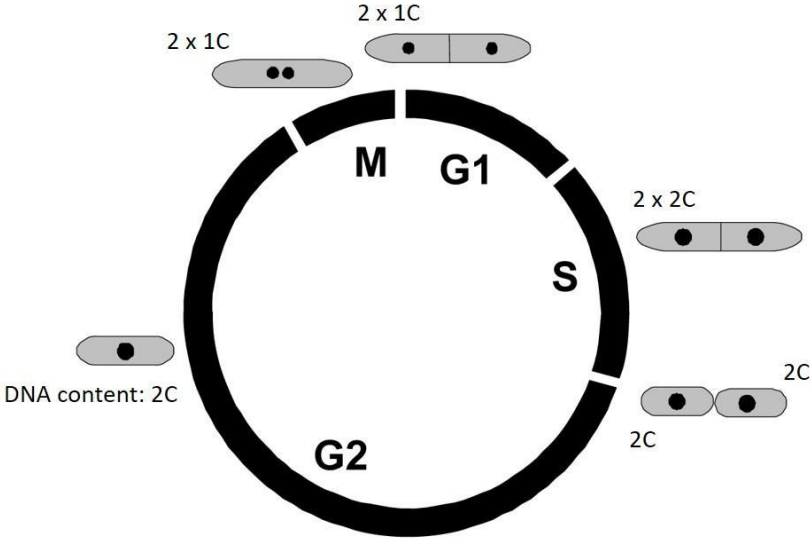
## B: Live cell microscopy in fission yeast

Fission yeast is an excellent model organism for studying cell biology with microscopy for several reasons:

### 1. *Simple, Rod-shaped Morphology:*

Fission yeast cells are cylindrical and rod-shaped, which makes them easy to visualize and analyze under a microscope. Their uniform shape and size (approximately 4 μm in

**Figure 38: Schematic representation of the fission yeast cell cycle.** The circle reflects the duration and relative positions of the different cell cycle phases: G1 (gap phase 1), S (DNA replication), G2 (gap phase 2) and M (mitosis). Sketches outside the circle indicate the morphology of cells at the corresponding phase. Black spots inside the cells represent the nuclei and the numbers indicate the total amount of DNA per cell (e. g. 1C = single complete genome). [Adapted from (Knutsen et al., 2011)]





width and 8-15  $\mu\text{m}$  in length) facilitate consistent and reproducible observations (Hagan et al., 2016).

## 2. *Well-defined Cell Cycle:*

The cell cycle stages in fission yeast are well-defined and synchronized, allowing for detailed study of cell cycle progression, mitosis, and cytokinesis. This is particularly useful for time-lapse microscopy and studying dynamic cellular processes (Mitchison & Nurse, 1985).

## 3. *Genetic Manipulability:*

Fission yeast is highly amenable to genetic manipulation. Researchers can easily introduce fluorescent tags to endogenous proteins under the control of the native promoter, enabling the visualization of specific proteins, organelles, and cellular structures in living cells using fluorescence microscopy (Bähler et al., 1998).

## 4. *Conservation of Eukaryotic Cell Processes:*

Many fundamental cellular processes, such as DNA replication, repair, recombination, and the regulation of the cell cycle, are conserved between fission yeast and higher eukaryotes, including humans. This makes findings in fission yeast highly relevant to understanding similar processes in more complex organisms (Forsburg & Rhind, 2006).

## 5. *Ease of Culturing:*

Fission yeast can be easily cultured in the laboratory under simple conditions. This allows for large-scale experiments and the ability to maintain and observe cells over extended periods (S. Moreno et al., 1991).

## 6. *Clear Visualization of Subcellular Structures:*

Fission yeast has distinct subcellular structures, such as the nucleus, spindle pole bodies (similar to centrosomes in higher eukaryotes), and a well-defined cell wall, which are easily observable with various microscopy techniques (Ding et al., 1993).

## 7. *Well-characterized Genome:*

The complete genome of fission yeast has been sequenced and is relatively small and simple. This allows for comprehensive genetic studies and the creation of mutant strains to study the effects of specific genes on cellular processes (Wood et al., 2002).

## 8. *Dynamic Studies:*

The relatively rapid cell cycle (about 2-4 hours) allows researchers to observe and record cellular events in real-time. This is particularly advantageous for studying

dynamic processes such as cell division, protein localization, and intracellular trafficking (Taddei et al., 2010).

### 9. *Compatibility with Advanced Microscopy Techniques:*

Fission yeast is suitable for advanced microscopy techniques such as super-resolution microscopy, live-cell imaging, and quantitative fluorescence microscopy. These techniques provide high-resolution images and quantitative data on cellular components and processes (Green et al., 2015).

Overall, the combination of its simplicity, genetic tractability, relevance to higher eukaryotes, and compatibility with advanced imaging techniques makes fission yeast an ideal model organism for studying cell biology using microscopy.

## C: Conditional replication fork barrier in fission yeast

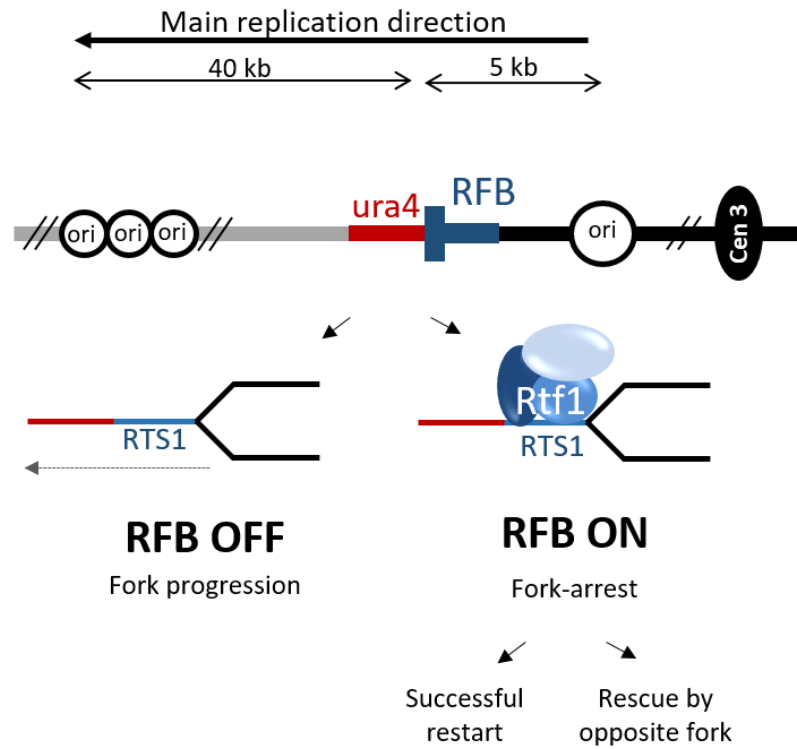
Replication forks can halt at either accidental or programmed replication fork barriers (RFBs) during the replication process. In bacteria, RFBs play a role in coordinating replication termination by causing replication forks to stall. For instance, the Tus protein binds to specific "Ter" sites and inhibits the activity of the replicative helicase (Hill & Marians, 1990; Khatri et al., 1989; E. H. Lee et al., 1989).

In eukaryotic cells, one of the well-studied RFBs is found in yeast. In budding yeast, programmed barriers within rDNA repeats prevent collisions between the replication and transcription machineries by facilitating predominantly unidirectional replication of the rDNA repeats (Brewer et al., 1992; Kobayashi & Horiuchi, 1996; Krings & Bastia, 2005).

Similarly, in fission yeast, a natural replication fork barrier exists at the mating type locus (*mat*) on chromosome II. This Replication Termination Sequence (RTS1) ensures unidirectional replication of the *mat* locus, which is crucial for optimal mating type switching (Codlin & Dalgaard, 2003; Dalgaard & Klar, 2001).

The arrest of the replication fork at RTS1 relies on the binding of a protein complex involving the key protein Rtf1 (Replication Termination Factor 1). In the absence of Rtf1, the RTS1 sequence undergoes normal replication without impeding the progress of replication forks (Dalgaard & Klar, 1999, 2001; Eydmann et al., 2008). RTS1 spans 859 base pairs and comprises two regions (Codlin & Dalgaard, 2003). The larger region, known as Region B, harbors four binding sites for Rtf1, making it crucial for barrier activation. Region A is thought to enhance the blocking function of Region B. Rtf1 binds to the RTS1 sequence and interacts with other proteins within the complex, such

**Figure 39: Schematic representation of the *RTS1*-RFB construct in fission yeast.** The *RTS1* sequence (in dark blue) has been integrated at the *ura4* locus (red) on chromosome III. Cen3 indicates the position of the centromere. Ori indicates the position of origins of replication. Numbers indicate the distance in kilobases between the replication origins and the RFB. When Rtf1 is expressed, it binds to the *RTS1* sequence and blocks the progression of replication forks migrating from Cen3 towards the telomere. Such arrested forks may be either rescued by the opposite forks or restarted by homologous recombination mechanisms (see text for details).



as Swi1 and Swi3, thereby acting as a physical barrier for the replication fork. An important characteristic of the RTS1 barrier is its ability to hinder the progress of the replicating fork in a polar manner: it selectively impedes forks traveling in one direction while leaving the opposite fork unaffected (Dalgaard & Klar, 2001). The distinction between the "restrictive" and "permissive" replication directions depends on the orientation of the RTS1 sequence.

The natural system of fission yeast RFB has been modified to function as a genetic tool for inducing specific fork stalling, allowing for the exploration of molecular mechanisms involved in arrested, collapsed, and restarted replication forks (Lambert et al., 2005). To achieve this, the RTS1 sequence has been integrated into chromosome III at the *ura4* locus (Figure 39). The majority of replication forks in this region originate from a strong replication origin about 5 kilobases upstream of the *ura4* locus, moving from the centromere-proximal side to the telomere. However, the closest origins on the telomere-proximal side are over 40 kilobases away and are considered weak origins.

For the system to be inducible, the native Rtf1 gene was positioned under the control of a thiamine-repressible nmt41 promoter. In the presence of thiamine, Rtf1 expression is suppressed, rendering the RTS1-RFB inactive (referred to as OFF condition). Conversely, upon thiamine removal, the nmt41 promoter is de-repressed, allowing Rtf1 expression and activation of the RTS1-RFB (referred to as ON condition). Rtf1 expression peaks 16 hours after thiamine removal. In this setup, the active RTS1-RFB blocks the progress of approximately 90% of forks originating from the strong origin on the centromeric-proximal side. These stalled forks can either be rescued by merging with converging forks or restarted via homologous recombination. The recombination-dependent restart of replication occurs within 20 minutes of fork arrest and subsequent recruitment of Rad52 and Rad51. Importantly, restarted forks undergo semi-conservative but non-canonical DNA synthesis, with both strands replicated by polymerase delta. This type of DNA synthesis is prone to replication errors, including replication slippage in micro homology regions, intra and inter-chromosomal template switches, and reversal of DNA replication orientation due to palindromic sequences (U-turn) (Naiman et al., 2021; Ait Saada et al., 2017; Iraqui et al., 2012; Miyabe et al., 2015; Mizuno et al., 2009, 2013; Nguyen et al., 2015; Teixeira-Silva et al., 2017; Tsang et al., 2014)).

In our laboratory and in collaboration with other teams, we have developed a range of genetic, cellular, and molecular assays to investigate the molecular processes and key factors involved in events at the replication fork blockage site, specifically the RTS1-RFB locus. These methods enable us to:

1. Assess the efficiency of recombination-mediated fork restart using the proxy-restart genetic assay. In this strategy we exploit the concept of HR-mediated fork restart being prone to replication slippage (RS) due to its association with a non-processive DNA synthesis. We can track RFB-induced RS induced by the active RFB by introducing a non-functional *Ura4* allele (named *ura4-sd20*) downstream of the *RTS1*-RFB. This allele contains a duplication of 20 nucleotides flanked by regions of micro-homology. Upon fork restart using the error-prone DNA synthesis, the replisome slips on the regions of microhomology leading to the deletion of the duplication and restoration of a functional *ura4+* gene to select for Ura<sup>+</sup> cells. The frequency of Ura<sup>+</sup> revertants reflects how often the *ura4-sd20* allele is replicated by a restarted replication fork in the cell population.
2. Examine replication intermediates formed at stalled forks using bi-dimensional gel electrophoresis (2DGE).
3. Monitor the recruitment of proteins to the stressed locus in individual cells through microscopy combined with a fluorescent reporter system, or in a cell population using chromatin immunoprecipitation (ChIP).
4. Track the fate and nuclear positioning of the locus in vivo through microscopy coupled with a fluorescent reporter system.
5. Additionally, to study the dynamics of HR-dependent DNA synthesis, we employ the polymerase usage sequencing (Pu-seq) approach in collaboration with Karel Naiman and Antony Carr. The Pu-seq approach allows the genome-wide mapping of the usage of Polymerases  $\delta$  and epsilon (Pol  $\epsilon$ ) during DNA replication (Daigaku et al., 2015). Pu-seq makes use of a pair of yeast strains mutated in either Pol  $\delta$  or Pol  $\epsilon$  that incorporate higher levels of ribonucleotides during DNA synthesis. The mapping of ribonucleotides in a strand-specific manner in strains mutated either for Pol  $\delta$  or Pol  $\epsilon$  allows the genome-wide tracking of polymerase usage. Combined with the *RTS1*-RFB, the Pu-seq method allows the monitoring of the usage frequency of each polymerase separately on both the Watson and Crick strands when the RFB is either inactive (RFB OFF, in an *rtf1* $\Delta$  genetic background) or constitutively active (RFB ON, Rtf1 being expressed under control of the *adh1* promoter to maximize fork arrest efficiency) (Naiman et al., 2021a).

These techniques have been instrumental in our studies and have been cited in our previous publications (Ait Saada et al., 2017a; Hardy et al., 2019; Kramarz et al., 2020a; Schirmeisen et al., 2023; Teixeira-Silva et al., 2017).

# OBJECTIVES

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SUMOylation is a reversible and dynamic process that affects the activity, localization and stability of a target protein. Recent publication from the team, in *S. pombe*, has showed that SUMO chains trigger the relocation of a single arrested replication fork to the nuclear periphery to anchor to the NPC (Kramarz et al., 2020a). This anchorage requires SUMO chains and the Slx8 STUbL pathway, but the exact SUMOylated targets continue to remain unknown. However, at the same time, SUMO chains limit the frequency of replication slippage during the progress of restarted forks. NPC anchorage promotes SUMO conjugates to be cleared off by the SUMO protease Ulp1 and the proteasome, whose activities are enriched at the nuclear periphery. The shift of replication forks to the nuclear periphery requires the strand exchange activity of the recombinase Rad51. This suggests that arrested replication forks need to be remodelled to be prone to anchorage. One possibility is that the formation of joint molecules by the recombinase Rad51 facilitates SUMO chains formation critical for relocation. Importantly, the absence of SUMO chains or the ability to perform mono-SUMOylation only, abrogates the relocation to the nuclear periphery, but allows a replication fork restart pathway to occur in the nucleoplasm without routing to the NPC. This suggests the existence of at least two spatially segregated RDR pathways, whose usage could be under SUMO control. One pathway, triggered by SUMO chains, would require an NPC anchorage; a second one, proficient when SUMO chains are not formed, would occur in the nucleoplasm. In this context, the objectives of my PhD were divided into two branches:

### **Development of *in vivo* approaches to track SUMO chains formation in the nuclear space.**

- Can we tag Slx8 STUbL with GFP?
- If yes, can we exploit the visualization of Slx8-GFP foci as a marker for tracking SUMO modifications in live cells?
- Is Slx8-GFP foci sufficient to track spontaneous and stress-induced SUMO chains formation in the nuclear space? If yes, how?
- If not, how to characterize the Slx8-GFP foci in terms of nuclear space and architecture? Does it mark any specific loci or nuclear region?
- What is the role of SUMO chains in the control of the aforementioned Slx8-GFP localization, if any?

### **Investigate the spatially segregated RDR pathway occurring in the nucleoplasm.**

- Does the modulation of joint molecules formation and stability affect the relocation to the nuclear periphery?
- Are there specific factors that are targets of SUMOylation?

- Can we identify a recombination-dependent replication pathway occurring in the nucleoplasm?
- If yes, what is the genetic dependency of this pathway

Altogether, during my PhD I focus on two major sub-topics: firstly, the characterization of the Slx8 STUbL within the nucleus with special emphasis upon the status of SUMOylation and secondly, towards a better understanding of how RDR is regulated within the nucleoplasm with respect to various HR factors and SUMOylation. Despite being relatively diverse, both the objectives help to put into perspective some ideas relating to SUMOylation and recombinogenic role of HR factors in fork restart.



# RESULTS

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The results obtained during my PhD are presented in the following sections:

### **Section I: Publication #1**

I had the opportunity to write a review from the team entitled "*The multifaceted functions of homologous recombination in dealing with replication-associated DNA damages*". I signed as a co-first author for this review with my colleague, Kamila Schirmeisen, published in the special edition of DNA Repair in 2023. This review focuses on how homologous recombination (HR) mediates genome stability and prevents tumour development. In this review, I contributed towards the elucidation of how the repair and restart of broken or dysfunctional replication forks is brought about by the HR machinery.

### **Section II: Publication #2**

Additionally, I worked on a project that focused on uncovering mechanisms by which the NPC compartment provides a critical environment for optimal HR-dependent fork restart. This section is dedicated to the PhD project of a former PhD student in the team, Kamila Schirmeisen. Data in this section is published in the manuscript entitled "*SUMO protease and proteasome recruitment at the nuclear periphery differently affect replication dynamics at arrested forks*" published in Nucleic Acid Research in 2024 that I sign as the 4<sup>th</sup> author. The publication highlights the distinct functional role of the Ulp1-SUMO protease and the proteasome at the nuclear periphery in controlling the dynamics of replication resumption at dysfunctional forks. Ulp1 is associated with the initiation of restart of HR-mediated arrested forks by processing the inhibitory SUMO chains, whereas the proteasome fosters the progression of restarted forks in a SUMO chains independent manner. The two functions were demonstrated to be unable to compensate for each other. In this article, I contributed in performing genetic assays to comprehend how the nuclear periphery affects the efficiency of RFB-induced replication slippage (RS) to promote Recombination-dependent Replication (RDR).

### **Section III: Publication #3**

A major part of my PhD project was devoted to analyse the dynamics of the Slx8 STUbL-regulated SUMOylation. Results for this section are presented in the form of a manuscript entitled "*The fission yeast SUMO-targeted Ubiquitin Ligase Slx8 functionally associates with clustered centromeres and the silent mating type region at the nuclear periphery.*" that I sign as the first author and is ready for submitting. This article is

prepared in collaboration with Prof. Elizabeth Bayne from the University of Edinburgh, UK. In this part, I have characterised the functional relationship between Slx8 and centromeres in fission yeast. I have identified that the Slx8 forms single focus in the nucleus upon being tagged with GFP and specifically marks the SPB, centromere and the mating type region. Additionally, our collaborators provided functional data revealing that Slx8 promotes centromere clustering along with gene silencing at the centromere and mating type region to support my initial results.

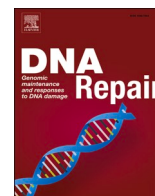
#### **Section IV: Additional data**

Moreover, during my PhD, I explored the hypothesis of two spatially segregated RDR pathways: one at the nuclear periphery while the other occurring in the nucleoplasm. My research focused on understanding the role of HR factors in D-loop formation or resolution, and the impact of SUMOylation on the regulation of the different RDR pathways. The second section includes some unpublished results which require additional investigation to consolidate the previously obtained data, in order to reinforce the overall conclusion.

## **I: Publication #1**

The multifaceted functions of homologous recombination in dealing with replication-associated DNA damages





# The multifaceted functions of homologous recombination in dealing with replication-associated DNA damages

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## ABSTRACT

The perturbation of DNA replication, a phenomena termed “replication stress”, is a driving force of genome instability and a hallmark of cancer cells. Among the DNA repair mechanisms that contribute to tolerating replication stress, the homologous recombination pathway is central to the alteration of replication fork progression. In many organisms, defects in the homologous recombination machinery result in increased cell sensitivity to replication-blocking agents and a higher risk of cancer in humans. Moreover, the status of homologous recombination in cancer cells often correlates with the efficacy of anti-cancer treatment. In this review, we discuss our current understanding of the different functions of homologous recombination in fixing replication-associated DNA damage and contributing to complete genome duplication. We also examine which functions are pivotal in preventing cancer and genome instability.

## 1. Introduction

Homologous recombination (HR) is a DNA repair pathway involved in fixing accidental and programmed double strand break (DSB). Defects in the HR machinery predispose to cancer, in particular breast and ovarian cancers. The HR machinery is also connected to the DNA replication process to ensure complete and accurate DNA replication [1]. The bulk of DNA synthesis occurs in the S-phase, thanks to the firing and progression of thousands of replication forks. Accurate genome duplication is critical for cell division, development and tissue renewal. However, DNA replication is not an easy feat since many events of endogenous or exogenous origin can challenge replisome progression, a phenomena termed “replication stress” (RS) [2]. This includes replication obstacles such as protein-DNA complexes, DNA damage, transcription machinery and secondary DNA structures. The discovery that oncogene expression forces cell proliferation in the context of inadequate cell metabolism, a process called oncogene-induced unbalanced DNA replication, has further highlighted the causal relationship between RS, genome instability and cancer development [3,4]. Moreover, many drugs used in chemotherapies target the process of DNA replication, making it a relevant target to trigger replication catastrophe in cancer cells [5]. Therefore, understanding the molecular mechanisms by

which the HR machinery regulates the accuracy of genome duplication under physiological or pathological RS conditions is of increasing interest.

## 2. Recombinogenic versus non-recombinogenic functions of HR during DNA replication

The HR process makes use of an intact and homologous DNA sequence as a template to repair DNA. The central and universal factor of the HR machinery is the recombinase Rad51 (hereafter named Rad51 for yeast models and RAD51 for mammalian cells) that exhibits multiple and inter-dependent biochemical activities (reviewed in [6]). ATP-bound Rad51 monomers bind to resected single stranded (ss) DNA in a process called nucleation with a stoichiometry of 1 monomer per 3 nucleotides. The cooperative binding mode of Rad51 allows the formation of a nucleoprotein filament coated onto ssDNA, viewed as the active form of HR, capable of homology search to find the appropriate DNA template. The Rad51 filament then allows strand invasion generating a joint molecule called displacement loop (D-loop) in which the complementary strand of the donor duplex is displaced as a ssDNA strand. Although Rad51 does not require a DNA end to promote strand exchange, most HR-based repair models assume that a 3'-end-invading

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<sup>1</sup> Equal contribution.

strand, as opposed to a 5'-end-invading strand, generates a productive D-loop for priming DNA synthesis and thus copies the missing genetic information or resume DNA replication. The loading of Rad51 onto DNA requires the assistance of a loader, known as Rad52 in yeast and BRCA2 in vertebrate, although these two loaders have different operating modes (Fig. 1). Rad51 has also a DNA-dependent ATPase activity that facilitates its dissociation from DNA. Regulating the ATPase activity of Rad51 is therefore critical to control filament formation and strand exchange, and several HR mediators are proposed to play such a function (discussed in [7]). Finally, Rad51 has a second low affinity dsDNA binding site that is critical for homology search and strand exchange [8].

The direct observation of DNA replication structures by electronic microscopy (EM) from cells defective for Rad51 (in *Xenopus* egg and yeast cells) revealed the presence of large ssDNA gaps both at the elongation point of replication forks and behind them [9,10]. Thus, continuous DNA synthesis is ensured by the recombinase Rad51 that

couple lagging and leading strand synthesis and protects newly replicated DNA from nucleolytic degradation. Since then, several studies have revealed multiple functions of the core HR machinery in dealing with replicative DNA damage and fork obstacle, including the repair of broken fork, the restart of dysfunctional fork, fork protection, and the repair of daughter strand gap. Interestingly, some of these molecular transactions do not require the strand exchange activity of Rad51 and thus refer to as non-recombinogenic functions in contrast to strand exchange-based mechanisms that refer to as homology-directed repair (HDR). Below, we summarize the multifaceted functions of the HR machinery during DNA replication (Fig. 1), illustrate how these functions ensure key biological processes to complete genome duplication, and how these HR sub-pathways are regulated.

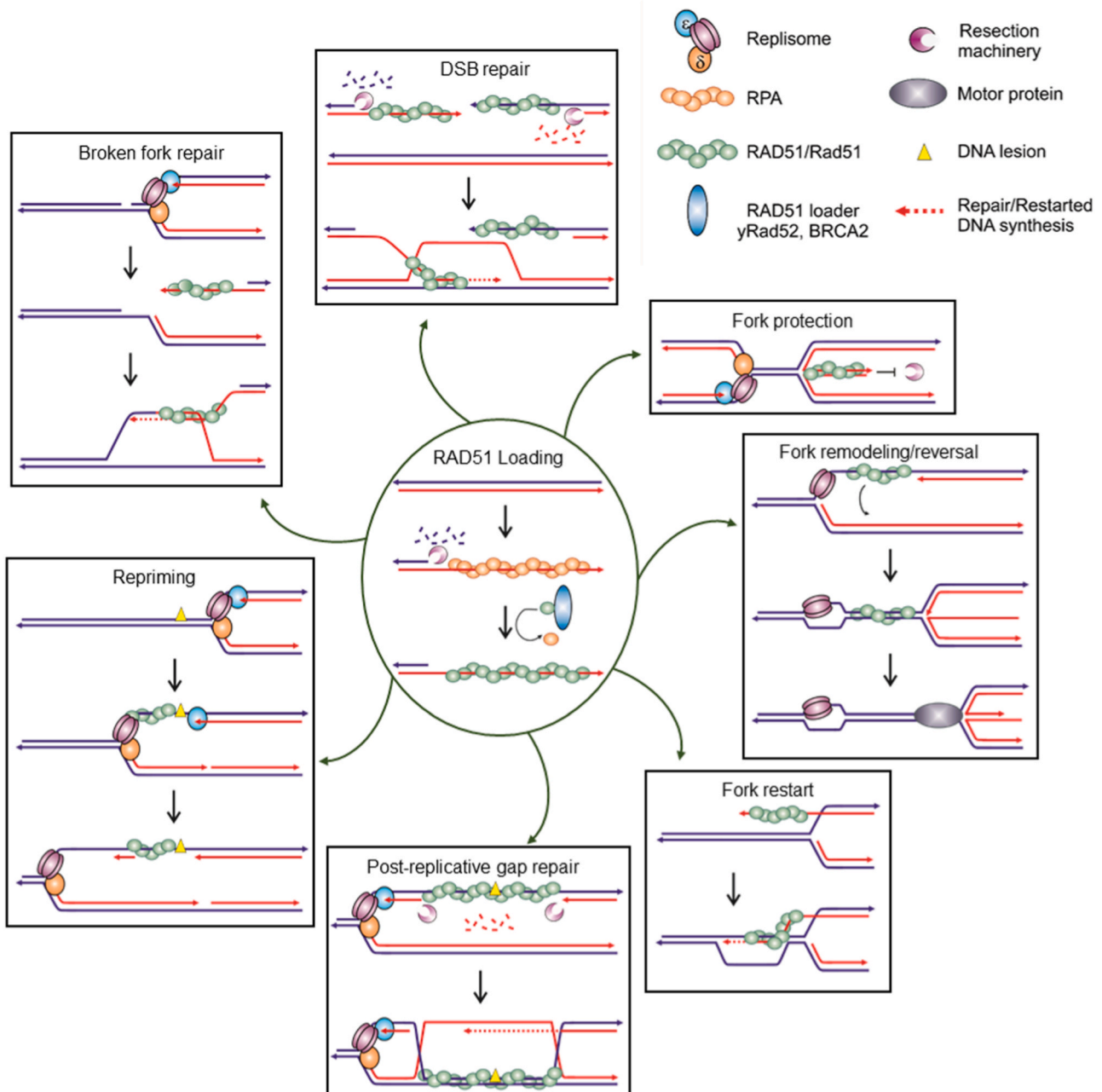


Fig. 1. The multifaceted functions of homologous recombination in DSB and replication-associated DNA damage (See text, Section 3 for details).

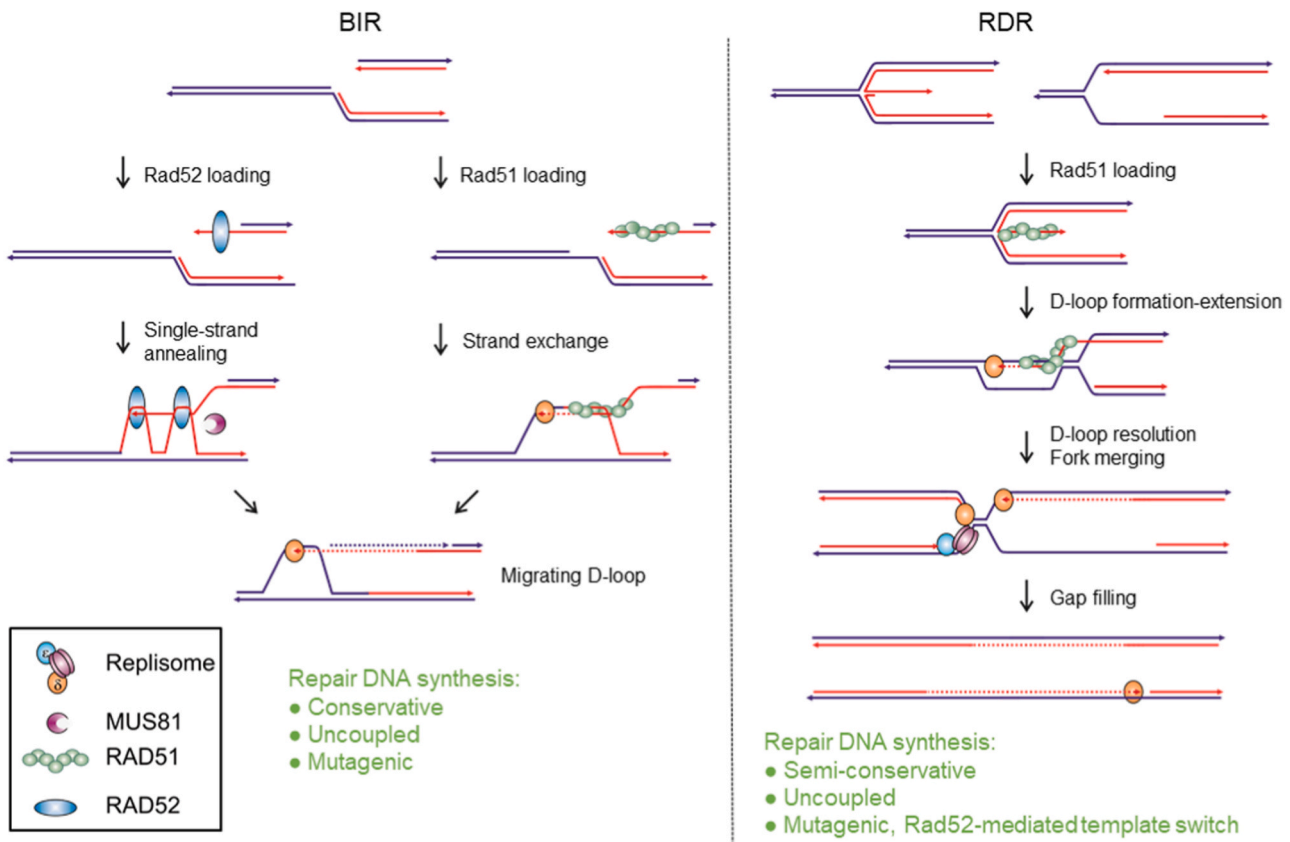
### 3. The homologous recombination machinery engages at replication fork for multiple functions

#### 3.1. The repair and restart of replication fork require a recombinogenic function

When a replication fork encounters a nick or a ssDNA gap, it results in a broken fork exhibiting a one-sided DSB, a typical HDR substrate. The Rad51-coated filament initiates strand invasion within the parental duplex, followed by DNA synthesis, an HR pathway referred to as break-induced replication (BIR) (reviewed in [11]). BIR has been extensively studied in yeast models, upon induction of a DSB in G2 cells with only one DNA end able to search for homology. In this case, BIR can proceed with DNA synthesis over hundreds of kilobases until reaching the end of the chromosome, although this DNA synthesis step is highly mutagenic [12–17]. In *Xenopus* egg extract, broken forks lead to the loss of some components of the replicative helicase CMG (the GINS subunits) and the restoration of a functional replisome requires RAD51 and the nuclease MRE11, showing that an HR-dependent process is necessary to maintain replisome integrity upon fork collapse [18]. By definition, BIR initiates from an invading one-sided DSB end from which DNA synthesis is extended within a migrating D-loop [19–23]. The newly synthesized strand is extruded from the D-loop and is used as a template to copy the second strand. Thus, BIR synthesis is conservative and both strands are synthesized by the DNA polymerase delta with the assistance of the non-essential subunit Pol32 [12,24,25]. Pol alpha is proposed to be required for stabilizing the long leading strand but its exact function remains unclear [12]. This non-canonical form of DNA synthesis results in an 100- to 1000-fold increase in mutation frequency, compared to the bulk of DNA synthesis as well as frequent template switches resulting in

complex genome rearrangement favoring cancer progression [13–17]. BIR events underlie Alternative Lengthening of Telomeres (ALT), a mechanism that allows maintaining telomere length in ~20% of cancer cells (reviewed in [26]). Many ALT pathways were found to be independent of RAD51 but relying on RAD52, which may exploit its strand annealing activity to recombine telomeric repeats, similarly to the RAD51-independent BIR described in budding yeast. BIR also underlies Mitotic DNA Synthesis (MiDAS), a process occurring on condensed chromosomes and viewed as the “last chance” to complete genome duplication before chromosome segregation initiates [27] (and reviewed in [28]). MiDAS is initiated by Mus81-mediated enzymatic cleavage of unresolved and late replication intermediates at “difficult-to-replicate” sites, such as common fragile sites, allowing BIR to resume DNA synthesis. MiDAS was initially described as RAD52 and POLD3 (the human orthologue of budding yeast Pol32) dependent but RAD51 and BRCA2 independent [29,30], consistent with these two last factors being excluded from the chromatin in mitosis. Nonetheless, a recent study established a role for RAD51 in promoting MiDAS, acting upstream of Mus81-dependent cleavage of late replication intermediates to complete DNA replication in mitosis [31].

In the last 10 years, evidences have accumulated to support that DSB is not a prerequisite to initiate HR-dependent replication fork restart [32–35]. This mechanism, called recombination-dependent-replication (RDR) is initiated by the controlled resection of newly synthesized strands to generate ssDNA gaps at the fork, further promoting the loading of the ssDNA binding protein RPA and HR factors (reviewed in [1]). Although there is a tendency to use the terms BIR and RDR interchangeably, BIR could be denoted as a specialized form of RDR, initiated by a DSB instead of an ssDNA gap (Fig. 2). Mutations in the second DNA binding site of RAD51, called RAD51-II3A, impair the strand exchange



**Fig. 2. Models of Break-induced Replication (BIR) and Recombination-dependent-replication (RDR).** BIR: the left part illustrates the Rad52-dependent and Rad51-independent BIR and the right panel illustrates the Rad51 and Rad52-dependent RDR. BIR can initiate from broken replication fork after enzymatic cleavage or not during or outside S-phase. RDR is initiated from ssDNA gap generating by the control degradation of nascent strand initiated at reversed fork or not, resulting in a Rad51-bound extruded leading strand.



activity without affecting nucleoprotein filament formation or binding to DNA [8]. This mutant is defective in restarting dysfunctional forks induced by a site-specific replication fork barrier (RFB) in the fission yeast, *Schizosaccharomyces pombe* and in restarting forks stalled upon depletion of dNTP pool in human cells (*i.e.* upon hydroxyurea (HU) treatment) [36,37]. However, the expression of hRAD51-II3A also resulted in the accumulation of collapsed forks, in a more severe way than in the RAD51 depletion condition, suggesting that a stable but unproductive RAD51 filament inhibits alternative fork restart/repair pathways or leads to enzymatic cleavage of stalled forks [36]. Extensively studied in the fission yeast *S. pombe*, collapsed forks can be restarted by RDR in 15–20 min and can travel over long distances, up to 20 Kb, before fusing with a canonical fork [38–40]. During RDR, the DNA synthesis remains semi-conservative but both strands are synthesized by the DNA polymerase delta, likely in an uncoupled manner [39, 40]. Thus, similarly to BIR, this non-canonical form of DNA synthesis is mutagenic, leading to frequent dissociation of the nascent strand from the template, followed by template switches triggering chromosomal rearrangements, genomic duplications or deletions and replication slippages [33,34,41–44]. Although the initial step of RDR is largely dependent on the strand exchange activity of Rad51, a recent report demonstrated that the strand annealing activity of Rad52 makes significant contribution to template switches during the elongation step of RDR, thus modulating replication errors during the progression of the restarted fork [44]. RDR may contribute to complete DNA replication in human cells. Indeed, upon mild replication stress conditions, DNA synthesis persists during the transition of late S to G2/M phase to minimize unfinished DNA replication and RS-induced mitotic abnormalities. In contrast to MiDAS, this resilient DNA synthesis relies on RAD51 and RAD52 but not MUS81, suggesting that fork breakage is not required to sustain DNA replication in G2 cells [45].

### 3.2. Replication fork remodeling and protection

Reversed replication forks are 4-branched DNA structures in which the newly synthesized strands are annealed together, and the opened parental DNA strands are back into a duplex form (Fig. 1). Fork reversal occurs not only at a fork encountering any type of DNA lesion, but also as an overall response to RS to restrict fork elongation [35,46]. Thus, fork reversal is viewed as a “holding state” during which an active replication fork can undergo cycle of fork reversal and fork restoration, depending on RS signaling conditions. However, a reversed fork provides a one-ended DNA end that is somehow recognized and processed as a DSB end, leading to nascent strand degradation by the resection machinery (reviewed in [5]). This refers to as unprotected forks that are detected using a DNA fiber assay in which nascent strands are labelled by two successive rounds of distinguishable thymidine analogues incorporated into DNA. Unprotected forks are revealed, upon HU treatment, by the shortening of the DNA fiber length labeled during the second round, requiring both strands of the reversed arm to be degraded. Several motor proteins promote fork reversal *in vivo* and *in vitro* (reviewed in [47]) and RAD51 promotes both fork reversal and fork protection [35,48]. Although RAD51-dependent fork reversal is independent of BRCA2 [49–51], whether or not it is a recombinogenic function remains debated. Human cells expressing RAD51-II3A are able to protect the fork against degradation by the nuclease DNA2 [36]. A more recent study, in which the reversed fork was detected directly by EM, investigated the role of several RAD51 mutated forms affecting distinct biochemical activities in promoting fork reversal. The authors proposed that RAD51-mediated fork reversal allows bypassing the replicative helicase CMG by annealing the parental strands behind the stalled fork while the translocase SMARCAL1 further extends this parental duplex, resulting in nascent strands being annealed together. All mutants impaired for the strand exchange activity were found defective in promoting fork reversal [52]. One hypothesis is that RAD51 mediates fork reversal by multiple mechanisms, either by itself or by stimulating the activity of

motor proteins.

In contrast, the strand exchange activity of RAD51 is dispensable for fork protection, which requires RAD51 DNA binding, its loader BRCA2 and nucleoprotein filament formation. RAD51-T131P, a heterozygous mutated allele identified in a Fanconi Anemia patient, acts as a dominant negative form when mixed with wild type RAD51, producing an unstable filament ineffective for fork protection, but sufficient to perform fork reversal [50,53]. Thus, the role of RAD51 in promoting fork reversal and fork protection engages distinct biochemical functions: fork reversal may require a few RAD51 molecules engaged at fork upon uncoupling between lagging and leading strand synthesis, whereas fork protection requires numerous monomers to form a filament onto the reversed arm, which can reach multiple kilobases in length. Surprisingly, RAD51-mediated fork reversal is BRCA2 independent whereas RAD51-mediated fork protection is BRCA2-dependent. Therefore, BRCA2 deficient cells suffers from unprotected forks that are alleviated by downregulating RAD51 [49–51]. The roles of BRCA2 in promoting HDR and fork protection are genetically separable and require distinct modes of interaction with RAD51 and its loading [48]. For example, BRC repeats of BRCA2 are critical to promote HDR but not fork protection whereas a single mutation in the C-terminal TR2 domain (S3291A) impairs fork protection but not HDR. It was recently shown *in vitro* that the protective function of RAD51 largely involves its capacity to bind dsDNA instead of ssDNA [54]. While BRC repeats abrogated RAD51-dependent DNA protection by removing RAD51 from dsDNA, the TR2 fragment restored DNA protection by stabilizing RAD51 onto dsDNA, a property not shared by a TR2-S3291A fragment. These results provide a better framework to understand the biology of separation-of-function mutants of RAD51 and BRCA2. Nonetheless, it remains key to understand how the full-length BRCA2 protein handles *in vivo* these different regulating functions to orchestrate RAD51 activity at the fork. Indeed, the same replication intermediate, a reversed fork, contributes to restrain fork progression while also being an entry point for the degradation of nascent strands, if unprotected. This suggests that RAD51-mediated fork reversal and fork protection must be tightly coupled to avoid genome instability.

### 3.3. The repair of post-replicative gaps and repriming

When the replisome encounters a DNA lesion that the replicative DNA polymerases cannot replicate, the DNA damage tolerance or bypass pathways ensure the completion of genome duplication, without repairing the damage. These mechanisms require priming *de novo* DNA synthesis downstream of the DNA lesion, ensuring continuous fork progression but leaving stretches of ssDNA behind the fork, named daughter-strand gaps. These gaps are then filled in either by the Translesion Synthesis (TLS) DNA polymerases or by template switch (TS), an HR sub-pathway (reviewed in [55]). Technical advances such as single-molecule analysis by EM or DNA fiber, have provided evidence of post-replicative gaps and their accumulation in the absence of a functional HR pathway in several organisms (yeast, *Xenopus*, human cells). In budding yeast, daughter-strand gaps are first enlarged by the nuclease Exo1, followed by a Rad51-mediated invasion of the ssDNA gap into the fully replicated sister chromatid [56,57]. This process is uncoupled from the bulk of DNA synthesis since Rad52 and Rad51 foci are mainly observed in the G2 phase, despite Rad51 being able to associate with unperturbed fork during S-phase [58,59]. The dynamic tracking of RPA foci relative to DNA synthesis sites concluded that the repair of post-replicative gaps is confined to specific territories that are spatially and temporally distant from ongoing replication forks [59]. HR-dependent repair of post-replicative gaps has also been reported in human cells upon treatment with BPDE (benzo(a)pyrene diol epoxide) that induces bulky adducts. In this situation, both MRE11 and EXO1 promote gap expansion to generate RAD51 foci, independently of fork stalling or collapse [60]. The current model of HR-dependent gap repair suggests that the intact sister chromatid is invaded by the ssDNA gap,

without the use of a 3' end extremity, thus a different mode of HR than the ones used to repair broken forks or DSBs (reviewed in [61]). How these distinct types of strand invasion are regulated remains largely unknown. Interestingly, a recent study in budding yeast identified physical interactions between the MCM complex, a component of the replicative DNA helicase, and Rad51-Rad52 to form a nuclease-insoluble nuclear scaffold in the G1 phase, in which MCM is bound to DNA but not Rad51 and Rad52 [62]. The authors uncovered a Rad51 mutant (Rad51m) that was no longer able to interact with MCM *in vivo*, although a direct interaction was not established. This mutant showed defective post-replicative gap repair and sensitivity to damaged replication fork-inducing agents but normal DSB repair, suggesting either a non-recombinogenic function of Rad51 in promoting gap repair or that the Rad51 strand exchange activity operates differently at DSBs versus ssDNA gaps. Physical interactions between human MCM and RAD51 were also reported without clear biological role (reviewed in [61]).

Repriming requires the DNA polymerase Pripol in human cells and the Primase-Pol alpha-Ctf4 complex in budding yeast [63,64]. Evidence has emerged supporting that HR factors ensure non-recombinogenic functions to regulate repriming. In the absence of the TLS polymerase Eta, mutations in which lead to the *Xeroderma pigmentosum* human syndrome, the analysis of replication intermediates by EM after exposure to UV irradiation, revealed a dual requirement for RAD51 at the fork and behind it [65]. First, RAD51 was detected in a limited amount at the fork, whereas its detection was greater behind the fork. Second, using the inhibitor B02 that disorganizes the RAD51 nucleoprotein filament in a way that is no longer functional to promote strand exchange activity, the authors suggested that RAD51 plays a non-recombinogenic function at a fork stalled by DNA lesion to ensure efficient repriming, a function that may involve an interaction with Pol alpha [50,66]. Given that restricting yeast Rad52 expression to G2 phase leads to defect in tolerating replicative DNA lesions [58], an emerging picture is that the recombinase Rad51 binds to unperturbed fork to promote continuous fork progression and DNA synthesis via repriming and then switches to a recombinogenic function to promote post-replicative gap repair. Consistently, mammalian cells defective for BRCA1 or BRCA2 accumulate post-replicative gaps from multiple origins [67,68]. This includes a defect in a FEN1-independent pathway of Okazaki fragment maturation on the lagging strand that remains to be

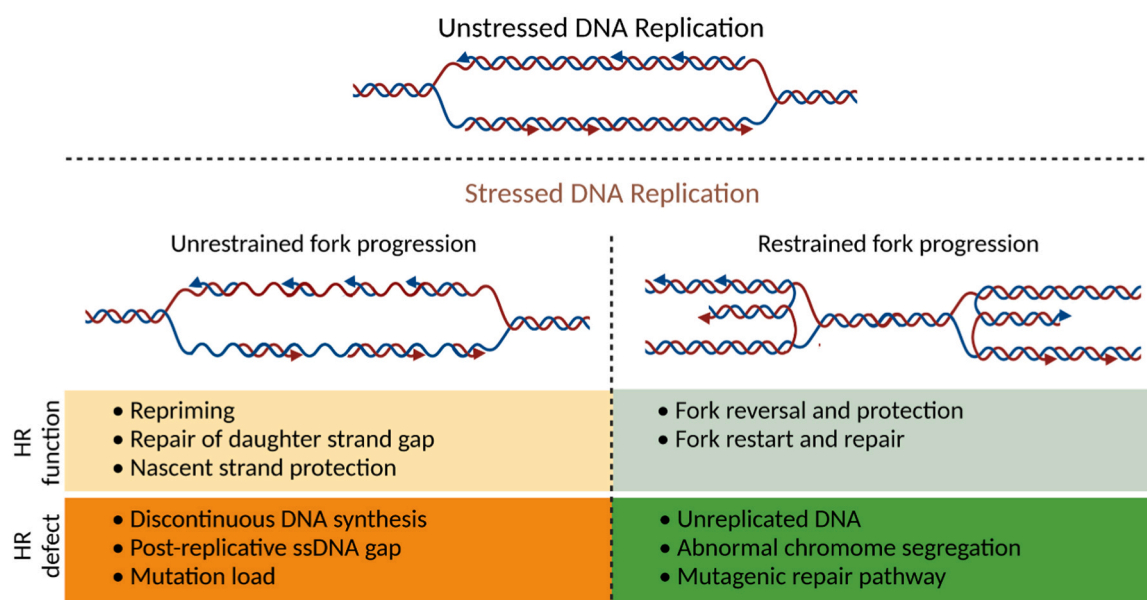
defined, unrepaired post-replicative gaps, and the inability to restrain repriming [68–75]. Indeed, BRCA2 interacts with the replication factor MCM10 to restrain PRIMPOL activity on the leading strand, independently of fork protection [76].

The replicative HR functions help to complete genome duplication by several means (Fig. 3). Two types of “unfinished DNA replication” can be considered: when some parts of the genome are not replicated at all or when the genome is replicated in a discontinuous manner. By ensuring fork restart and repair, especially at transcription-replication collisions, or fork protection to ensure accurate termination, HR prevents an under-replication of the genome and the formation of a particular type of mitotic bridges, named ultra-fine bridges, a hallmark of unreplicated DNA in mitosis [37,77–79]. By limiting the accumulation of post-replicative gaps, HR ensures a continuous DNA synthesis and avoid the excessive use of TLS activity that may contribute to increasing mutation load.

#### 4. Regulation of the distinct replicative HR functions by nuclear positioning

The distinct replicative HR functions exhibit different outcomes on genome stability and must therefore be strictly kept in check. In addition to the above-mentioned cell-cycle regulations, nuclear positioning and chromatin context significantly influence the outcomes of HR on genome stability. We intentionally tend to avoid using the term “pathway choice” because, in our view, it implies an active process of choosing among all possible HR subpathways to deal with replication-associated DNA damage and that all mechanisms are equally available and effective. Instead, it appears that depending on the nuclear compartment, the availability of some repair factors may make one pathway more efficient than another one.

DNA repair occurs in the context of eukaryotic genomes organized into a compartmentalized nucleus. The quest to locate distant homologous regions can be a challenge, which may require advanced mechanisms like chromatin mobility both locally (at the damage site) and globally. Increased chromatin mobility is a phenomenon conserved across different organisms in response to DSBs (reviewed [80,81]). For example, DSBs occurring within repeated sequences or in heterochromatic regions shift away from their compartment to achieve HR repair



**Fig. 3. Two types of “unfinished DNA Replication”.** The top panel illustrate the progression of canonical fork, including semi-discontinuous DNA synthesis on the lagging strand. A hallmark of unrestrained fork progression is discontinuous DNA synthesis on both sister chromatid, generating post-replicative daughter strand gaps. Fork reversal contributes to restrain fork progression upon stress, increasing the risk of “unreplicated DNA” when cells enter mitosis if no restart occurs.

(reviewed in [82,83]). As observed for DSBs, replication stress sites were reported to relocate to the nuclear periphery, and in some cases, to anchor to nuclear pore complexes (NPCs) to regulate and fine-tune HR-based mechanisms. This includes forks stalled by telomere repeats, structures forming sequences, DNA-bound proteins or replication inhibitors [84–89]. Relocation to NPCs and/or the nuclear periphery may create an environment more favorable to some replicative HR sub pathways. Diverse scenarios emerge depending on the type of fork obstacle and the model organism (Fig. 4).

#### 4.1. Fork stalling within telomere repeats

Inactivation of telomerase in yeast leads to telomere erosion and replicative senescence. The relocation of eroded telomeres to NPCs facilitates the emergence of survivors (of type II) by a process similar to mammalian ALT [90]. However, telomerase is also essential to counteract replication-induced damage at telomeres, as its inactivation leads to stochastic replication fork stalling and transient cell-cycle arrests (reviewed in [91]). Thus, in the absence of telomerase, telomere replication becomes dependent on HR factors, as their deletion dramatically boosts senescence. In budding yeast, telomeric stalled forks relocate and associate with NPCs to resume replication. In the absence of anchorage, stalled forks engage in an error-prone Rad51-dependent pathway to maintain telomere length by recombination between sister chromatids [86]. This emphasizes an unsuspected role of NPCs in restricting error-prone HR events at stalled forks.

#### 4.2. Forks stalled by tri-nucleotide repeats

In budding yeast, tri-nucleotide repeats, such as CAG, have the propensity to form secondary DNA structures prone to stall replication

forks (reviewed in [92]). Such stalled forks transiently relocate and anchor to NPCs in late S-phase, in a SUMO-dependent manner [87]. SUMOylated RPA, loaded onto ssDNA, prevents Rad51 loading that is permitted only after NPC anchorage [93]. It was proposed that at the NPC, the Slx5–8 STUBL pathway promotes the degradation of SUMOylated proteins to alleviate the inhibition of Rad51 loading and favor HR-mediated fork restart. Impaired relocation leads to repeat instability in a Rad52-dependent manner [87]. This scenario exemplifies the concept of a spatially segregated mechanism to regulate the sequential loading of HR factors at stalled forks and ensure replication restart in an accurate manner.

#### 4.3. Forks stalled at DNA-bound protein complex

Another study in fission yeast describes how relocation and anchorage to NPCs help to sustain RDR. A fork arrested by a protein-mediated RFB relocates to the nuclear periphery and anchors to NPCs during S-phase, for the time necessary to complete RDR [85]. Relocation depends on the recombinogenic function of Rad51, suggesting that joint molecules are important nuclear positioning signals and that Rad51 loading is not prevented at unique sequences, in contrast to repeated sequences. Relocation also requires the E3 SUMO ligase Pli1 and the STUBL pathway, although the exact SUMOylated targets are still unknown. It was proposed that the accumulation of SUMO chains limits the Rad51-dependent RDR, creating a need for the SUMO protease Ulp1 and the proteasome; two activities enriched at the NPC level, to eliminate SUMO conjugates and enable replication to restart. This exemplifies how a SUMO-based mechanism spatially segregates the subsequent RDR steps from Rad51 loading and activity occurring in the nucleoplasm and the restart of DNA synthesis occurring after anchorage to NPCs. One remaining question is the identification of the Rad51-independent RDR,

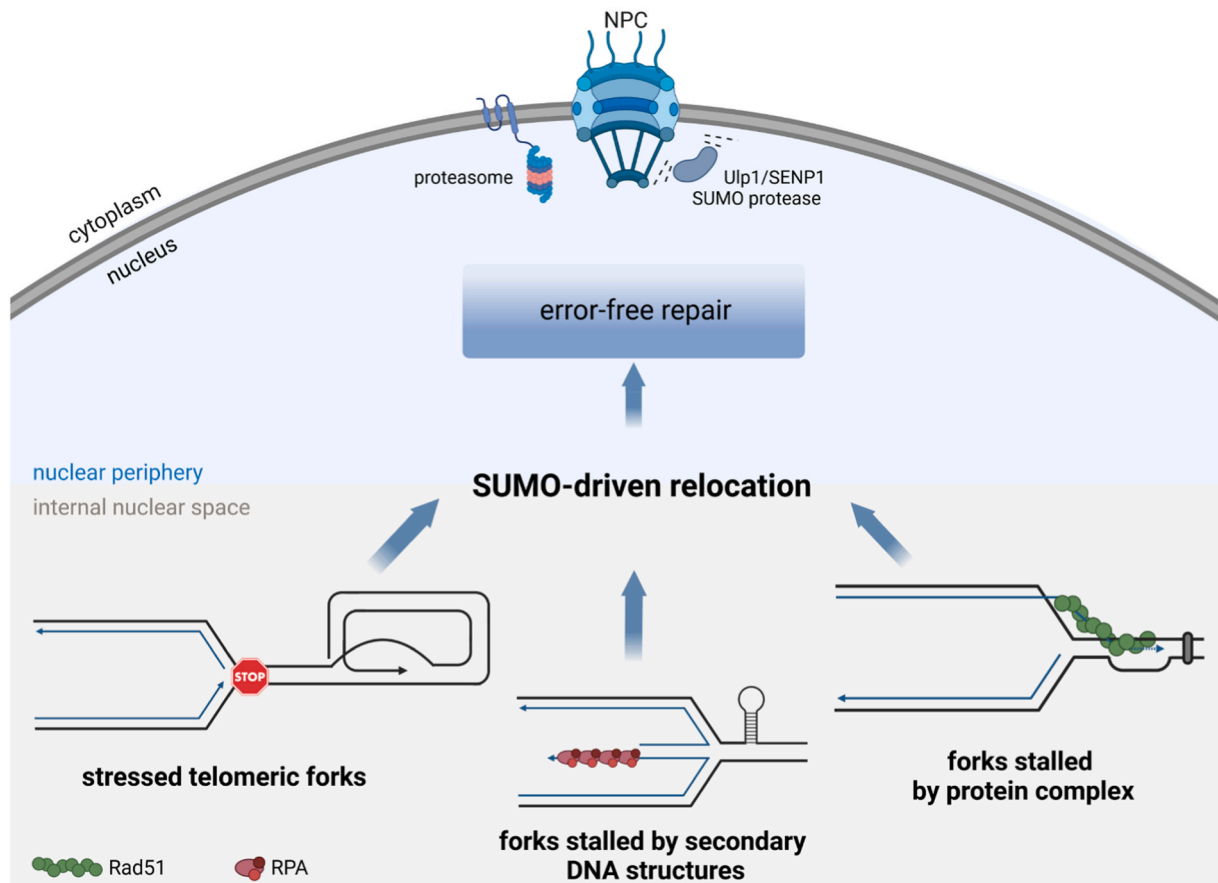


Fig. 4. Several type of stressed replication fork relocate to the nuclear periphery and anchor to nuclear pore complex (NPC) in a SUMO-dependent manner.

which occurs in the nucleoplasm and that is limited by SUMO chains [82,85]. As mentioned above, both RDR and BIR are mutagenic pathways, and one attractive hypothesis is that nuclear positioning may influence their intrinsic mutagenicity, for example by regulating transition from Rad51-dependent to Rad51-independent template switching [44].

##### 5. Concluding remark: what are the key HR functions to prevent genome instability, tumor development and modulate sensitivity to chemotherapies?

Beyond our fundamental understanding of the most prevalent functions of the HR machinery to prevent genome instability, scientists and clinicians face two distinct challenges. The first one is to assign the cancer risk associated to specific BRCA patient mutations. The second is to predict the response to treatment of a tumor defective in BRCA1/2, or more generally in the HR pathway. In this context, multiple cellular (Rad51 foci), molecular (repair efficiency) and genomic assays (mutational signature) have been developed to assign HDR defects in cancer cells. The first challenge is linked to defining which HR defect (unprotected fork, HDR defect, gap suppression and repair) is more prone to generating genome instability fueling cancer development. The second one depends on understanding which replicative function is defective in a certain type of cancer to delineate the best genotoxic-based treatment. It must be taken into account that unrepaired and persistent gaps are converted into collapsed and broken forks at the next round of DNA replication, creating a higher need for HR-mediated fork restart and repair [73]. Both unprotected forks and suppression of gaps have been shown to correlate with chemo-resistance, in particular to cisplatin and PARP inhibitor (reviewed and discussed in [94]). The use of separation-of-function mutated forms of BRCA2 in mice showed that HDR defect is prone to tumorigenesis, unlike fork protection and gap suppression defects [95]. Interestingly, defective fork protection and gap suppression, but not HDR, was observed in BRCA2 heterozygous mice, a situation highly relevant to patients. It is conceivable that defective fork protection and/or gap accumulation favor a “background noise” of genome instability sufficient to accumulate genetic damages. Interestingly, *BRCA* genes are hot spots of fork stalling, undergoing error-prone fork repair in *BRCA* haplo-insufficient cells, a mechanism that may favor loss of heterozygosity [96]. Upon loss of the second allele, cells switch into an HDR defect mode that amplifies genome instability and tumor development. In support of this, DSB repair in *BRCA2*-deficient cells relies on the use of alternative and error-prone DNA repair pathways, named Alt NHEJ, mediated by the DNA polymerase Theta [97]. Thus, a part of the genome instability that fuels cancer development may result from such mutagenic repair, as discussed in [98].

##### Declaration of Competing Interest

The authors declare no competing interests.

##### Data Availability

No data was used for the research described in the article.

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## **II: Publication #2**

SUMO protease and proteasome recruitment at the nuclear periphery differently affect replication dynamics at arrested forks.





# SUMO protease and proteasome recruitment at the nuclear periphery differently affect replication dynamics at arrested forks

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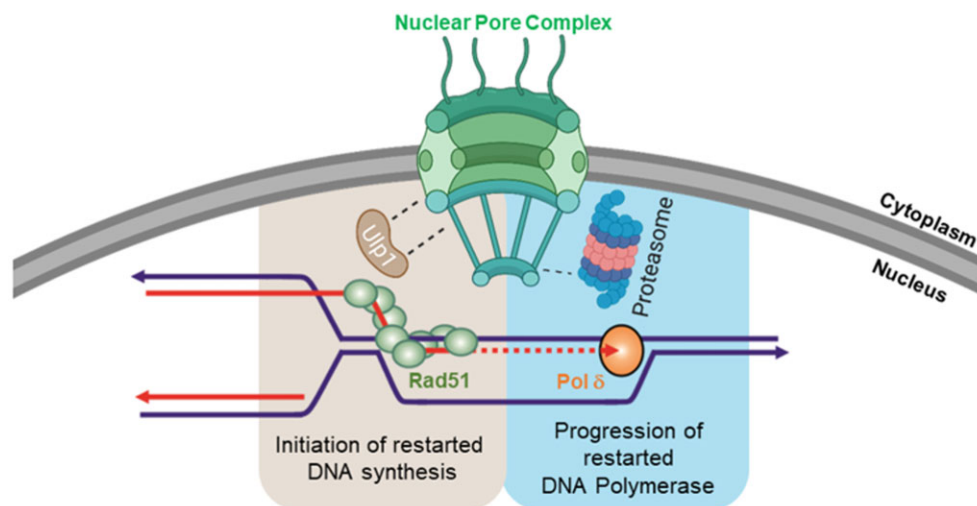
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## Abstract

Nuclear pore complexes (NPCs) have emerged as genome organizers, defining a particular nuclear compartment enriched for SUMO protease and proteasome activities, and act as docking sites for the repair of DNA damage. In fission yeast, the anchorage of perturbed replication forks to NPCs is an integral part of the recombination-dependent replication restart mechanism (RDR) that resumes DNA synthesis at terminally dysfunctional forks. By mapping DNA polymerase usage, we report that SUMO protease Ulp1-associated NPCs ensure efficient initiation of restarted DNA synthesis, whereas proteasome-associated NPCs sustain the progression of restarted DNA polymerase. In contrast to Ulp1-dependent events, this last function is not alleviated by preventing SUMO chain formation. By analyzing the role of the nuclear basket, the nucleoplasmic extension of the NPC, we reveal that the activities of Ulp1 and the proteasome cannot compensate for each other and affect the dynamics of RDR in distinct ways. Our work probes two distinct mechanisms by which the NPC environment ensures optimal RDR, both controlled by different NPC components.

## Graphical abstract



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## Introduction

The eukaryotic genome is folded in 3D within a compartmentalized nucleus. Nuclear organization constitutes a critical layer of regulation of DNA-associated transactions and an important determinant of genome integrity (1). The stability of the genome is jeopardized during DNA replication; the progression of the replisome being recurrently threatened by a broad spectrum of obstacles that cause replication fork slowing, temporary fork stalling or terminal fork collapse (2). Such alterations of fork progression are a defining hallmark of replication stress. Failure to safeguard genome stability upon replication stress is a potent driving force behind the onset and progression of human diseases including cancer (3). While multiple replication fork repair pathways can be engaged at stressed forks to promote the completion of genome duplication, they result in variable outcomes for genome stability and thus must be carefully controlled and regulated. Our current knowledge of the regulatory functions played by nuclear organization in the usage of fork repair pathways remains in its infancy.

Among the fork repair pathways, homologous recombination (HR) is particularly active in protecting, repairing and restarting stressed forks, making HR an efficient tumor suppressor mechanism (4). The central factor of the HR machinery is the Rad51 recombinase that forms a nucleoprotein filament on single-stranded DNA (ssDNA) with the assistance of a loader, known as Rad52 in yeast models. In a non-recombinogenic mode, the Rad51 filament limits the degradation of ssDNA by various nucleases, thus ensuring the protection and integrity of stressed forks. In a recombinogenic mode, HR repairs broken forks with a single-ended double-strand break (DSB) by a mechanism called break-induced replication (BIR) and promotes replication resumption at DSB-free collapsed forks by a mechanism called recombination-dependent replication (RDR) (5). Both BIR and RDR are associated with non-canonical DNA synthesis, which is approximately 100 times more mutagenic than canonical replication. Furthermore, during BIR and RDR, both DNA strands are synthesized by DNA polymerase delta (Pol  $\delta$ ) (6,7). These features allow experimental differentiation between DNA replicated by a repaired/restarted fork and DNA replicated by a canonical origin-born fork. Although stressed forks have the potential to relocate to the nuclear periphery (NP), little is known about the contribution of such changes in nuclear positioning in regulating the replicative functions of the HR machinery.

3D genome folding within the complex nuclear environment is a critical layer of DNA repair regulation. A striking example is the DNA damage response-dependent fate of DSBs that relocate to the NP or shift away from heterochromatic compartments to achieve error-free repair (8,9). This led to the concept that the membrane-less nuclear compartment exhibits distinct DNA repair capacities and that DNA repair machineries are spatially segregated. Nuclear pore complexes (NPCs) are macromolecular structures embedded in the nuclear envelope (NE) that act as nuclear scaffolds to regulate cellular processes via a wide range of mechanisms (10). The overall structure of NPCs is conserved among eukaryote kingdom, being composed of multiple copies of 30 different nucleoporins that associate in stable sub-complexes. The core NPC defines a central channel composed of transmembrane and channel nucleoporins. This core complex assembles with the outer and inner rings at the cytoplasmic and nuclear sides,

respectively. A Y-shaped structure, located both at the cytoplasmic and nuclear side of NPCs, called in fission yeast the Nup107–Nup160 complex, is crucial for NPCs organization and proper segregation of chromosomes in eukaryotes (11–13). The final composition of individual NPCs is variable, depending on their position within the NE, suggesting that the NPC structure is dynamic. In particular, the nuclear basket, a nucleoplasmic extension of the core NPC, is the most dynamic part and NPCs localized in the nucleolar part of the NE are more frequently devoid of a nuclear basket (12). The primary functions of NPCs are the transport of macromolecules from the cytoplasm to the nucleus and mRNA export. NPCs also define a particular nuclear compartment enriched for the SUMO SENP protease and the proteasome and act as docking sites for DSBs and perturbed replication forks (8).

Stressed forks can relocate to the NP and, in some cases, anchor to NPCs (14). These include forks stalled by structure-forming DNA sequences, telomeric repeats, DNA-bound proteins and replication inhibitors (15–21). Although distinct scenarios arise depending on the source of replication stress and the model organism, the common emerging concept is that nuclear positioning of replication stress sites influences the usage of fork repair pathways. For example, in *Saccharomyces cerevisiae* (Sc), forks stalled within telomeric repeats associate with NPCs to restrict error-prone HR events and maintain telomere length (18). Forks stalled by CAG repeats, prone to form secondary DNA structure, also anchor to NPCs in a SUMO-dependent manner (16). In this instance, SUMOylated RPA on ssDNA at the stalled fork inhibits Rad51 loading, which is permitted only after NPC anchorage that subsequently favors error-free fork restart (17). Changes in nuclear positioning are far from being a yeast-specific phenomenon: upon DNA polymerase inhibition, stalled forks in human cells relocate to the NP to minimize chromosomal instability and ensure timely fork restart (20). Additionally, stressed forks at human telomeres relocate to NPCs to maintain telomere integrity (19).

We previously reported that, in the yeast *Schizosaccharomyces pombe* (Sp), dysfunctional forks relocate and anchor to NPCs in a SUMO-dependent manner, for the time necessary to achieve RDR (15). This change in nuclear positioning is critical to spatially segregate the subsequent steps of RDR. Dysfunctional forks are processed and remodeled in the nucleoplasm to load Rad51. SUMO chain, generated by the E3 SUMO ligase Pli1, then triggers relocation to NPCs. Relocation of dysfunctional forks to NPCs allows SUMO conjugates to be cleared by the SUMO deconjugating enzyme, Ulp1, which is sequestered at the NP (22). Therefore, NPCs are an integral part of RDR regulation to promote HR-dependent DNA synthesis at dysfunctional forks. However, the dynamics underlying this process remain unexplored. In particular, the contribution of NPCs to non-canonical Pol  $\delta$ /Pol  $\delta$  DNA synthesis, a hallmark of HR-restarted forks, has not been addressed. Here, by mapping DNA polymerase usage during HR-mediated fork restart, we reveal that the SUMO protease, Ulp1, and the proteasome differentially affect the dynamics of HR-dependent fork restart by ensuring efficient resumption of DNA synthesis and by sustaining the dynamic progression of the restarted fork, respectively. Moreover, by studying the role of the nuclear basket in RDR, we show that Ulp1 and the proteasome do not compensate for each other. In particular, the defect in RDR caused by defective Ulp1-associated NPCs, but not defective proteasome-enriched NPCs, is alleviated by

preventing SUMO chain formation. Our study uncovers mechanisms by which the NPC compartment acts as a critical environment for optimal HR-dependent fork restart.

## Materials and methods

### Standard yeast genetics and biological resources

Yeast strains and primers used in this work are listed in [Supplementary Table S1](#) and [S2](#), respectively. Gene deletion and tagging were performed by classical genetic techniques. To assess the sensitivity of chosen mutants to genotoxic agents, mid log-phase cells were serially diluted and spotted onto yeast extract agar plates containing hydroxyurea (HU), methyl methanesulfonate (MMS), camptothecin (CPT) or bleomycin (bleo). Strains carrying the *RTS1* replication fork block sequence were grown in minimal medium EMMg (with glutamate as a nitrogen source) with addition of appropriate supplements and 60  $\mu$ M thiamine (barrier inactive, OFF). The induction of replication fork block was obtained by washing away the thiamine and further incubation in a fresh medium for 24 hours (barrier active, ON).

### Live cell imaging

For snapshot microscopy, cells were grown in filtered EMMg with or without 60  $\mu$ M thiamine for 24 h to exponential phase (RFB OFF and RFB ON), then centrifuged and re-suspended in 500  $\mu$ l of fresh EMMg. 1  $\mu$ l from the resulting solution was dropped onto Thermo Scientific slide (ER-201B-CE24) covered with a thin layer of 1.4% agarose in filtered EMMg (15). 21 z-stack pictures (each z step of 200 nm) were captured using a Nipkow Spinning Disk confocal system (Yokogawa CSU-X1-A1) mounted on a Nikon Eclipse Ti E inverted microscope, equipped with a 100 $\times$  Apochromat TIRF oil-immersion objective (NA: 1.49) and captured on sCMOS Prime 95B camera (Photometrics) operated through MetaMorph<sup>®</sup> software (Molecular Devices). GFP and mCherry proteins were excited with a 488 nm (Stradus<sup>®</sup> - Vortran Laser Technology, 150mW) and a 561 nm (Jive<sup>™</sup>-Cobolt, 100 mW) lasers, respectively. A quad band dichroic mirror (405/488/568/647 nm, Semrock) was used in combination with single band-pass filters of 525/50 or 630/75 for the detection of GFP and mCherry, respectively. Fluorescence and bright-field 3D images were taken at every 0.3 $\mu$ m over a total of 4.5 $\mu$ m thickness. Exposure time for the GFP channel was 500 ms and for the mCherry channel was 1000 ms. During the imaging, the microscope was set up at 25°C. For the experiment on Ulp1 and Cut11, the Gataca Live SR module (Gataca Systems), implemented on the Spinning Disk confocal system, was used to generate super-resolution images with lateral image resolution improvement (around 120 nm).

### Image analysis

Images were mounted and analyzed with Fiji software (23). First, the 3D Z series are converted into 2D projection based on maximum intensity values. The quantification of Ulp1 and Cut11 was performed using a homemade macro. The user draws manually all nuclear rings on the merge images as a first step. Then automatically, three types of regions were created from the manual annotation:

- the nucleus was obtained by enlarging the manual annotation to 3 pixels.

- the nucleoplasm was obtained by shrinking the nucleus to 8 pixels.
- the nuclear periphery has been extracted from the previous two regions by selecting only those pixels that are not common.

Several measurements were exported for all regions, such as perimeter of nuclei in pixels, area in pixels<sup>2</sup>, the fluorescence density of a protein (annotated as 'Mean fluorescence intensity' in Fiji: this value represents the Raw Integrated Density measured in the selection and normalized by the area of the same selection) and the total fluorescence intensity of the protein (annotated as 'RawIntDen'(Raw Integrated Density) in Fiji: this value represents the sum of all pixels intensities in the selection). To assess the co-localization of Ulp1 and Cut11 proteins, the JACoP plugin (24) was used to study the correlation between the intensities of these two proteins in different NPC mutant strains. Pearson and Manders' coefficients were calculated for each nucleus obtained. Before running the analysis, pre-processing was applied (background subtraction using the rolling ball algorithm with a radius of 20 pixels and a Gaussian filter (sigma 1)) to reduce image noise and facilitate detection of the Ulp1 and Cut11 proteins needed to calculate Manders' coefficients. The 'Default' thresholding method was used for the detection of Ulp1-GFP and Cut11-mCherry positive signals.

### 2DGE analysis of replication intermediates

Exponential cells ( $2.5 \times 10^9$ ) were treated with 0.1% sodium azide and subsequently mixed with frozen EDTA (of final concentration at 80 mM). Genomic DNA was crosslinked with trimethyl psoralen (0.01 mg/mL, TMP, Sigma, T6137) which was added to cell suspensions and incubated for 5 min in the dark. Next, cells were irradiated with UV-A (365 nm) for 90 s at a constant flow of 50 mW/cm<sup>2</sup>. Subsequently, cell lysis was performed by adding lysing enzymes (Sigma, L1412) at a concentration of 0.625 mg/ml and zymolyase 100 T (Amsbio, 120493-1) at 0.5 mg/ml. Obtained spheroplasts were next embedded into 1% low melting agarose (In-Cert Agarose 50123, Lonza) plugs and incubated overnight at 55°C in a digestion buffer with 1 mg/ml of proteinase K (Euromedex EU0090). Plugs were then washed with TE buffer (50 mM Tris, 10 mM EDTA) and stored at 4°C. Digestion of DNA was performed using 30 units of restriction enzyme *AseI* (NEB, R0526M) per plug. Samples were then treated with RNase (Roche, 11119915001) and beta-agarase (NEB, M0392L). Melted plugs were equilibrated to 0.3 M NaCl. Replication intermediates were purified using BND cellulose (Sigma, B6385) poured into columns (Biorad, 731-1550) (25). RIs were enriched in the presence of 1M NaCl 1.8% caffeine (Sigma, C-8960), precipitated with glycogen (Roche, 1090139001) and migrated in 0.35% agarose gel (1 $\times$  TBE) for the first dimension. The second dimension was cast in 0.9% agarose (1 $\times$  TBE) supplemented with 0.5  $\mu$ g/ml of EtBr. Next, DNA was transferred to a nylon membrane (Perkin-Elmer, NEF988001PK) in 10 $\times$  SSC. Finally, membranes were incubated with <sup>32</sup>P-radiolabeled *ura4* probe (TaKaRa *BcaBEST*<sup>™</sup> Labeling Kit, #6046 and alpha-<sup>32</sup>P dCTP, Perkin-Elmer, BLU013Z250UC) in Ultra-Hyb buffer (Invitrogen, AM8669) at 42°C. The signal of replication intermediates was collected in phosphor-imager software (Typhoon-trio) and quantified by densitometric analysis

with ImageQuantTL software (GE healthcare). The ‘tail signal’ was normalized to the overall signal corresponding to arrested forks.

### Replication slippage assay

The frequency of *ura4* + revertants arising from the *ura4-sd20* allele was determined as follows. 5-FOA (EUROMEDEX, 1555) resistant colonies were grown on plates containing uracil with or without thiamine for 2 days at 30°C and subsequently inoculated into EMMg supplemented with uracil for 24 h. Then cultures were diluted and plated on EMMg complete (for cell survival) and on EMMg without uracil, both supplemented with 60 μM thiamine. After 5–7 days of incubation at 30°C colonies were counted to determine the frequency of *ura4*+ reversion.

### Flow cytometry

Flow cytometry analysis of DNA content was performed as follows (26): cells were fixed in 70% ethanol and washed with 50 mM sodium citrate, digested with RNase A (Sigma, R5503) for 2 h, stained with 1 μM Sytox Green nucleic acid stain (Invitrogen, S7020) and subjected to flow cytometry using FACSCANTO II (BD Biosciences).

### Whole protein extract analysis

Aliquots of  $1 \times 10^8$  cells were collected and disrupted by bead beating in 1 ml of 20% TCA (Sigma, T9159). Pellets of denatured proteins were washed with 1 M Tris pH 8 and resuspended in 2× Laemmli buffer (62.5 mM Tris pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol with bromophenol blue). Samples were boiled before being subjected to SDS-PAGE on Mini-PROTEAN TGX Precast Gel 4–15% (Biorad, 4561086). Western blot using either anti-GFP (Roche, 11814460001), anti-HA (Santa Cruz Biotechnology, sc-57592), anti-TIR1 (MBL, PD048), anti-PCNA (Santa Cruz, sc-56) or anti-tubulin (Abcam, Ab6160) antibodies was subsequently performed. For the analysis of cellular patterns of global SUMOylation, whole protein extraction was performed as follows: aliquots of  $2 \times 10^8$  cells were collected and resuspended in 400 μl of water. The cell suspensions were mixed with 350 μl of freshly prepared lysis buffer (2M NaOH, 7% β-mercaptoethanol) and 350 μl of 50% TCA (Sigma, T9159). After centrifugation, pellets were further washed with 1 M Tris pH 8 and resuspended in 2× Laemmli buffer (62.5 mM Tris pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol with bromophenol blue). Samples were boiled before being subjected to SDS-PAGE on Mini-PROTEAN TGX Precast Gel 4–15% (Biorad, 4561086). Western blot using anti-SUMO antibody (non-commercial, produced in rabbit by Agro-Bio) was subsequently performed.

### Pulse field gel electrophoresis

Yeast cultures were grown to logarithmic phase in rich YES medium to a concentration of  $5 \times 10^6$ /ml, synchronized in 20 mM HU for 4 h, and subsequently released to fresh YES medium. At each time point, 20 ml of cell culture was harvested, washed with cold 50 mM EDTA pH 8 and digested with lyticase (Sigma, L4025) in CSE buffer (20 mM citrate/phosphate pH 5.6, 1.2 M sorbitol, 40 mM EDTA pH 8). Next cells were embedded into 1% UltraPure™ Agarose (Invitrogen, 16500) and distributed into 5 identical agarose

plugs for each time point. Plugs were then digested with Lysis Buffer 1, LB1 (50 mM Tris-HCl pH 7.5, 250 mM EDTA pH 8, 1% SDS) for 1.5 h at 55°C and transferred to Lysis Buffer 2, LB2 (1% N-lauryl sarcosine, 0.5 M EDTA pH 9.5, 0.5 mg/ml proteinase K) o/n at 55°C. The next day, LB2 was exchanged to for fresh LB2 and digestion was continued o/n at 55°C. After this, plugs were kept at 4°C. To visualize intact chromosomes, one set of plugs was run on a Biorad CHEF-DR-III pulse field gel electrophoresis (PFGE) system for 60 h at 2.0 V/cm, angle 120°, 14°C, 1800 s single switch time, pump speed 70 in 1× TAE buffer. Separated chromosomes were stained in ethidium bromide (10 μg/ml) for 30 min, washed briefly in 1× TAE and visualized with a UV transilluminator.

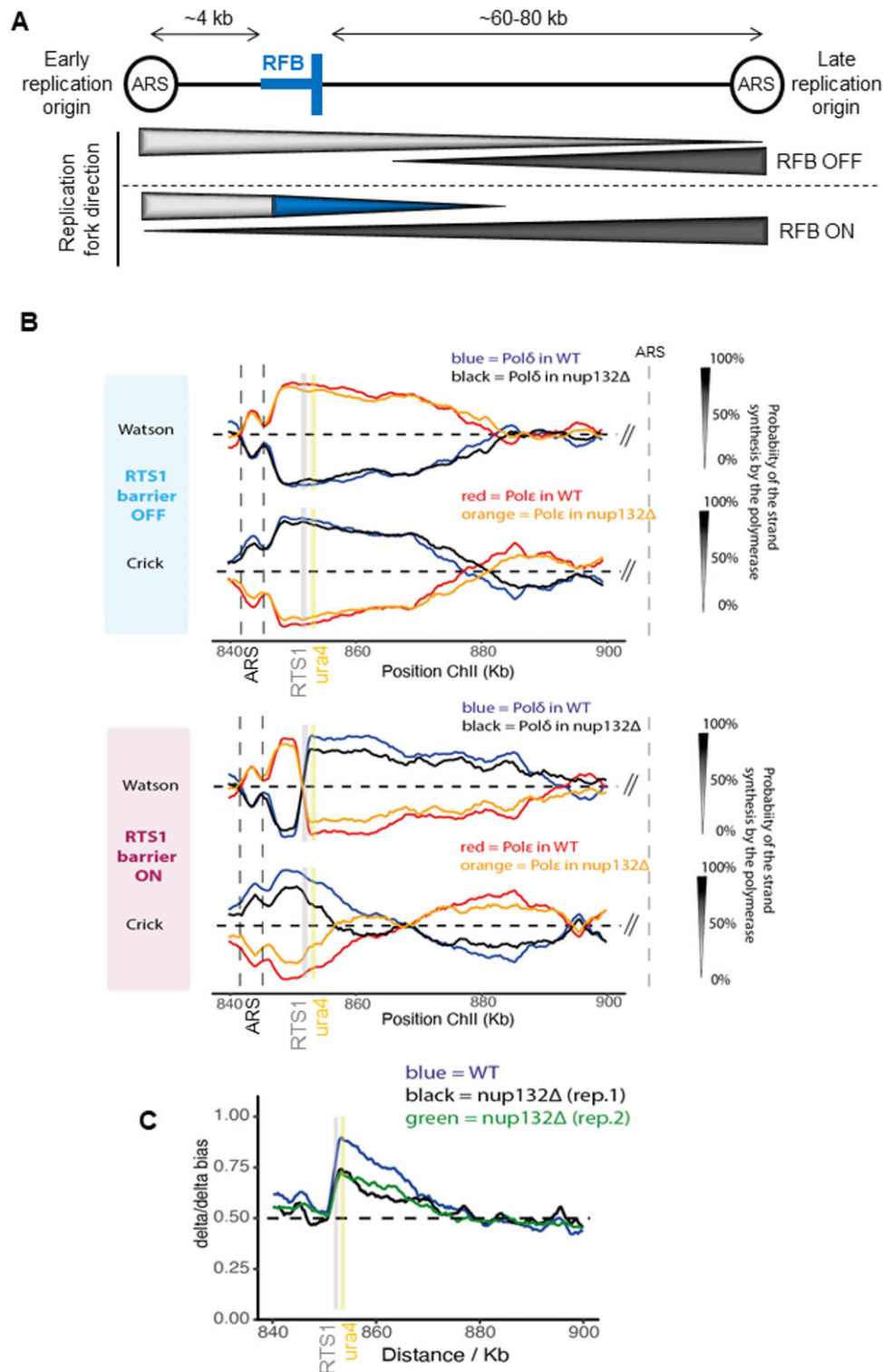
### Npp106-GFP chromatin immuno-precipitation

Chromatin immunoprecipitation against Npp106-GFP was done as described earlier (15) with the following modifications: 200 ml of logarithmic yeast culture ( $OD_{600} \sim 1$ ) for each strain and condition (*RTS1*-RFB OFF and ON) was divided into two 100ml aliquots and double-cross-linked first with 10 mM dimethyl adipimidate (DMA, Thermo scientific, 20660) and then with 1% formaldehyde (Sigma, F-8775). Next, cells were frozen in liquid nitrogen. Cell lysis was performed by bead beating in 400 μl of lysis buffer (50 mM HEPES pH7.5, 140 mM NaCl, 1% Triton X100, 0.1% Nadeoxycholate, 1 mM EDTA plus 1 mM PMSF and protease inhibitors, Sigma-Aldrich, P8215). Sonication of chromatin was done in a Diagenode Bioruptor Pico in Easy Mode, 10 cycles, 30s ON and 30s OFF at 4°C. Sonicated chromatin fractions were pooled for each condition. 5 μl of sonicated chromatin was preserved as the input fraction. Immunoprecipitation was carried out over night as follows: 300 μl of sonicated chromatin extract was incubated with anti-GFP antibody (Invitrogen, A11122) at 1:150 concentration and another 300 μl was incubated with Normal Rabbit IgG antibody (Cell Signaling Technology, #2729S) at dilution 1:75. The next morning, protein G Dynabeads (Invitrogen, 10003D) were added for 1 h. Immunoprecipitated proteins and inputs were decrosslinked for 2 h at 65°C. Inputs and the DNA associated with Npp106-GFP or control Rabbit antibody were purified using Qiaquick PCR purification kit (QIAGEN, 28104) and eluted in 400 μl of water for molecular biology (Sigma-Aldrich, 95284). Quantitative PCR was performed using SsoAdvanced Universal SYBR® Green Supermix (#1725274, Biorad). Primers used for qPCR are listed in [Supplementary Table S2](#). The relative amounts of DNA, starting quantities based on standard curves for each pair of primers, were obtained using Biorad CFX Maestro 1.1.

### Pu-Seq

The published protocol (27) was used with minor modifications: size selection was performed using a Blue Pippin (Sage Science). We used *rnh201*-RED instead of *rnh201::kan* strain (28). Sequence files were aligned with Bowtie2 and alignment data converted to counts with custom Perl script (27). Analysis of polymerase usage was performed with custom R script (27). Sequence data is available under GEO dataset GSE247371.

The percentage of forks that restart at the barrier is estimated from the delta/delta bias plots (Figures 1C and 6C). Immediately downstream of the site of the barrier the level of increase in delta/delta bias is indicative of percentage of



**Figure 1.** Ulp1-associated NPC promotes the dynamics of recombination-mediated fork restart. **(A)** Schematic of the *RTS1*-RFB locus on chromosome II. The position of the *RTS1*-RFB is indicated as thick blue bars. The directional RFB blocks the progression of right-moving forks that initiate from the left autonomously replicating sequence (ARS). The direction of unperturbed (RFB OFF) and perturbed replication (RFB ON) forks is indicated by the thickness of the arrows underneath. Light and dark gray bars indicate the progression of canonical rightward and leftward-moving forks, respectively. The blue bar indicates the progression of restarted replication forks mediated by homologous recombination. **(B)** Pu-Seq traces of the ChrII locus in *RTS1*-RFB OFF (top panel) and ON (bottom panel) conditions in WT and *nup132* $\Delta$  strains. The usage of Pol delta (in blue and black for WT and *nup132* $\Delta$  cells, respectively) are shown on the Watson and Crick strands. Note the switch from Pol epsilon (in red and orange for WT and *nup132* $\Delta$  cells, respectively) are shown on the Watson and Crick strands. The usage of Pol epsilon (in red and orange for WT and *nup132* $\Delta$  cells, respectively) are shown on the Watson and Crick strands. Note the switch from Pol epsilon to Pol delta on the Watson strand at the RFB site (gray bar), which is indicative of a change in polymerase usage on the leading strand in RFB ON condition. The genomic location of the ARS, the *RTS1*-RFB and the *ura4* marker are indicated by dashed lines, a gray line and a yellow line, respectively. **(C)** Graph of Pol delta/delta bias over both strands (Watson and Crick) around the RFB site in WT and two independent replicates of *nup132* $\Delta$  strains. The gray and yellow bars indicate the position of the *RTS1*-RFB and of the *ura4* marker, respectively.

restarted forks (1 = 100% restarted; 0.5 = 0% restarted). The relative progression of the restarted forks is estimated from the relative change in slope of the delta/delta bias score as the forks progress from the barrier towards the right. This reflects the termination of the restarted forks as they meet canonical forks progressing leftwards: slow restarted forks will terminate closer to the barrier.

## Statistical analysis

Quantitative densitometric analysis of Southern blots after 2DGE was carried out using ImageQuant software. The 'tail signal' of resected forks was normalized to the overall signal corresponding to arrested forks.

Quantification of PFGE was performed using ImageJ and presented as % of migrating chromosomes relative to asynchronous profile. Cell imaging was performed using META-MORPH software and processed and analyzed using ImageJ software (23). The explanation and definition of values and error bars are mentioned within the figure legends. In most experiments, the number of samples is >3 and obtained from independent experiments to ensure biological reproducibility. For all experiments based on the analysis of cell imaging, the number of nuclei analyzed is mentioned in the figure legends. Statistical analysis was carried out using Mann–Whitney *U* test, Student's *t*-test and Fischer's exact test.

## Results

To investigate the contribution of the NP to the dynamics of HR-mediated fork restart, we exploited the *RTS1* replication fork barrier (RFB) that promotes the polar arrest of a single replisome at a specific genomic location (Figure 1A) (25). The activity of the RFB is fully dependent on the Rtf1 protein that binds to the *RTS1* sequence. The expression of Rtf1 can be artificially regulated by the *nmt41* promoter, which allows Rtf1 repression in thiamine-containing media (RFB OFF condition) and its expression upon thiamine removal (RFB ON condition). Alternatively, the *rtf1* gene can be deleted and the results compared with an *rtf1* + strain. Forks arrested at the RFB become fully dysfunctional and undergo controlled degradation of the nascent strand by the end-resection machinery to generate ssDNA gap of ~1 kb in length (29,30). RPA, Rad52 and Rad51 are loaded onto these ssDNA gaps, ensuring fork protection until the arrested fork is either fused with a converging fork or actively restarted by RDR, which occurs approximately 20 minutes after the arrest (6,28,31,32). The restarted fork is associated with a non-canonical, mutagenic DNA synthesis in which both strands are synthesized by Pol  $\delta$ , making it insensitive to the RFB (28 31,33,34).

### Ulp1-associated NPCs ensure the efficient priming of recombination-mediated DNA synthesis

We previously reported that the nucleoporin Nup132, part of the Y complex of NPCs core, promotes RDR in a post-anchoring manner and acts downstream of Rad51 loading (15). The RDR defect observed in *nup132* null cells is caused by the delocalization of the Ulp1 SUMO protease from the NP since the artificial tethering of Ulp1 to the RFB, anchored to NPCs, restored RDR efficiency. Thus, Ulp1-associated NPCs prime HR-dependent DNA synthesis to ensure efficient RDR, but the dynamics of this process is unknown. To address this,

we employed the polymerase usage sequencing (Pu-Seq) approach that allows the genome-wide mapping of the usage of Pol  $\delta$  and polymerase epsilon (Pol  $\epsilon$ ) during DNA replication (35). Pu-Seq makes use of a pair of yeast strains mutated in either Pol  $\delta$  or Pol  $\epsilon$  that incorporate higher levels of ribonucleotides during DNA synthesis. The mapping of ribonucleotides in a strand-specific manner in strains mutated either for Pol  $\delta$  or Pol  $\epsilon$  allows the genome-wide tracking of polymerase usage. Combined with the *RTS1*-RFB, the Pu-Seq method allows monitoring the usage frequency of each polymerase separately on both the Watson and Crick strand when the RFB is either inactive (RFB OFF, in an *rtf1*  $\Delta$  genetic background) or constitutively active (RFB ON, Rtf1 expressed from the *adb1* promoter to maximize fork arrest efficiency) (28).

At an inactive barrier site (RFB OFF), replication is canonical (leading strand synthesized by Pol  $\epsilon$  and lagging strand synthesized by Pol  $\delta$ ) and proceeds from left to right in the majority of cells, initiating from an early replication origin (Figure 1A-B, top panel). This division of labor between Pol  $\delta$  and  $\epsilon$  changed sharply in an RFB ON strain: at the barrier site, Pol  $\epsilon$  in the leading strand is switched to Pol  $\delta$  during the restart of the blocked fork (Figure 1B, bottom panel). This sharp transition characterizes the efficiency of the restart itself. It means that this creates a bias towards Pol  $\delta$  when considering both strands (Watson and Crick) downstream of the *RTS1*-RFB site due to the restart. The Pol  $\delta/\delta$  bias reflects the time needed for the restart as well as the progression of the restarted fork relative to the canonical convergent fork coming from a late replication origin (28). Based on the Pol  $\delta/\delta$  bias (Figure 1C), we estimated that, when compared to WT (*nup132*+) cells, only 60% of the expected number of forks were arrested and restarted in *nup132*  $\Delta$  cells, while the remaining 40% were either not arrested or were arrested and did not restart before being rescued by an incoming leftward moving canonical fork. The increase in Pol  $\epsilon$  usage on the Crick strand for ~10 Kb downstream of the *RTS1* barrier is indicative of this latter scenario (Figure 1B). Remarkably, this fork-restart defect is consistent with our previous estimation using a proxy-restart assay that exploits the mutagenic nature of restarted DNA synthesis to provide a genetic readout of RDR efficiency. Using this proxy-restart assay, we previously reported a nearly two-fold reduction in RDR efficiency in *nup132*  $\Delta$  cells compared to WT (15). Finally, the relative change in slope of the Pol  $\delta/\delta$  bias reduction over distance was similar between the two replicates from *nup132*  $\Delta$  cells and the WT strain, indicating that the forks that succeeded to restart progress with similar speed (Figure 1C).

### The nuclear basket promotes RDR in a pre- and post-anchoring manner

We next investigated the role of the nuclear basket in dealing with replication stress. The *S. pombe* nuclear basket is composed of 4 non-essential nucleoporins: Nup60 (ScNup60), Nup61 (ScNup2, HsNup50), Nup124 (ScNup1, HsNup153) and Alm1 (ScMlp1/2, HsTPR) (12, 13). A fifth component is the essential nucleoporin Nup211, a second orthologue of ScMlp1/2 and HsTPR. Some of these components are known to contribute to resistance to DNA damage (36,37). We confirmed that *alm1*  $\Delta$  cells were highly sensitive to a wide range of replication-blocking agents and bleomycin-induced DSBs, whereas *nup60*  $\Delta$  and *nup61*  $\Delta$  cells exhibited mild sensitivity

only to hydroxyurea (HU), a replication inhibitor that depletes dNTP pool (Supplementary Figure S1A).

To establish if this HU sensitivity correlates with a defect in resuming replication following HU treatment, we arrested cells for 4 h in 20mM HU and then followed DNA content by flow cytometry upon release into HU-free media. Among nuclear basket mutants, only *nup61Δ* cells displayed a defect in the recovery from HU-stalled forks, a defect similar to the one previously reported for *nup132Δ* cells (15) (Supplementary Figure S1B): the WT strain reached a G2 DNA content 45 minutes after release, whereas both *nup132Δ* and *nup61Δ* cells exhibited an additional 15 minutes delay. This observation is supported by the analysis of chromosomes by Pulse Field Gel Electrophoresis (PFGE). HU treatment prevented chromosomes from migrating into the gel because of the accumulation of replication intermediates (Supplementary Figure S1C). WT chromosomes migrated into the gel with twice intensity of an asynchronous culture 90 minutes after release, indicating a complete recovery from HU-stalling forks and genome duplication of the WT genome (Supplementary Figure S1D). Consistent with the flow cytometry data, only chromosomes from *nup132Δ* and *nup61Δ* cells showed a clear delay in their ability to migrate into the gel following release from HU, confirming a role for Nup61 in promoting DNA replication upon transient fork stalling by HU.

To establish the role of the nuclear basket in promoting replication resumption at the RFB, we first measured replication slippage (RS) downstream of *RTS1*, a proxy measure of non-canonical replication resulting from RDR (34) (Figure 2A). The absence of Nup60 and Alm1, but not Nup124 or Nup61, led to a ~2-fold reduction in the frequency of RFB-induced RS, indicating a reduced RDR efficiency (Figure 2B). The analysis of replication intermediates by bi-dimensional gel electrophoresis (2DGE) showed that fork arrest and the formation of large ssDNA gaps (>100 bp) at the RFB (which are visualized as a specific 'tail' DNA structure emanating from the fork arrest signal and descending toward the linear arc; see red arrow on Figure 2C) (32) were unaltered in all four non-essential nucleoporin mutants (Figure 2C, D). This indicates that the controlled degradation of the nascent strand and Rad51-dependent fork protection are unaffected. Thus, the RDR defect observed in *nup60Δ* and *alm1Δ* is not related to defect in the early steps of RDR, from ssDNA gap formation to Rad51 loading.

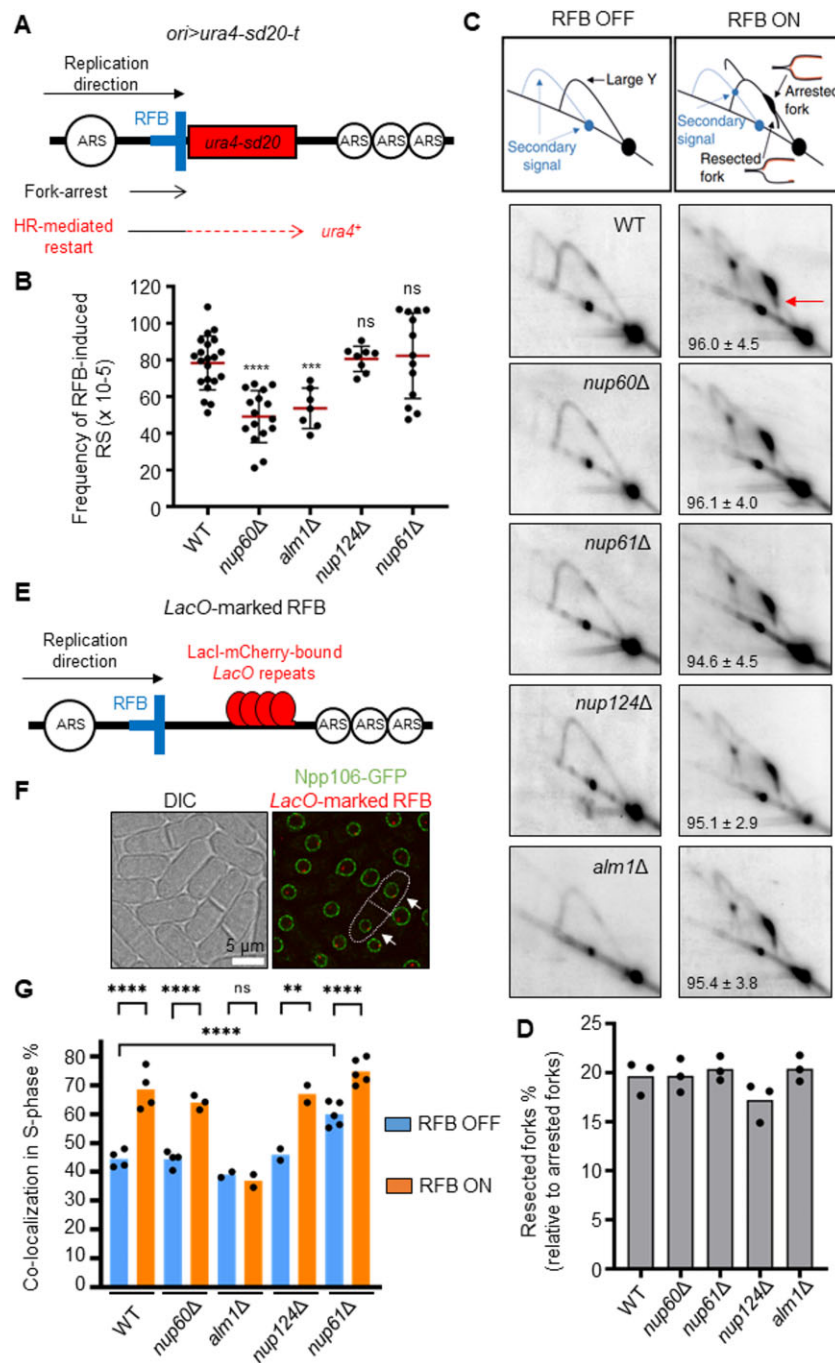
We next investigated the ability of the RFB to relocate to the NP. We employed a strain harboring a *LacO*-marked RFB expressing LacI-mCherry (Figure 2E) and an endogenously GFP-tagged Npp106, a NPC component, to mark the NP (Figure 2F). We counted co-localization events between the NP and the *LacO*-marked RFB, visualized by a LacI-mCherry focus (see white arrows on Figure 2F), as previously reported (15). When the RFB was inactive (RFB OFF), LacI foci co-localized with the NP in ~45% of S-phase cells (Figure 2G). Upon activation of the RFB (RFB ON), the *LacO*-marked RFB was more often (~70%) localized at the NP in WT cells (15). This shift of the active RFB to the NP was observed in all nuclear basket mutants, except *alm1Δ* (Figure 2G). The *nup61Δ* cells exhibited a slight increase in the frequency of co-localization events in RFB OFF condition but reached a similar enrichment at the NP to WT cells in RFB ON condition. Thus, Alm1 and Nup60 promote RDR in a pre- and post-anchoring manner, respectively.

## The nuclear basket promotes the sequestration of Ulp1 at the nuclear periphery

In budding yeast, several components of the nuclear basket are critical for peripheral Ulp1 localization, including ScNup60 and the synergistic action of ScMlp1 and ScMlp2 (38,39). We thus investigated the expression and the nuclear sub-localization of Ulp1 upon loss of nuclear basket functionality. Ulp1 was C-terminally tagged with GFP and Ulp1-GFP functionality was established using resistance to genotoxic stress (Supplementary Figure S2A). We observed that, in *nup60Δ* and *nup132Δ* mutants, Ulp1-GFP levels were largely abrogated whereas a ~75% and ~60% reduction was observed in *nup124Δ* and *alm1Δ* backgrounds, respectively (Figure 3A). Cell microscopy analysis showed that Ulp1-GFP was no longer sequestered at the NP in *nup60Δ* and *nup132Δ* mutants (Figure 3B). Treating cells with bortezomib, a proteasome inhibitor (40), partly restored Ulp1-GFP protein level in *nup132Δ* and *nup60Δ* cells, similar to previous findings in budding yeast (38). However, the sequestration of Ulp1-GFP at the NP was far from being fully restored (Supplementary Figure S2B, C).

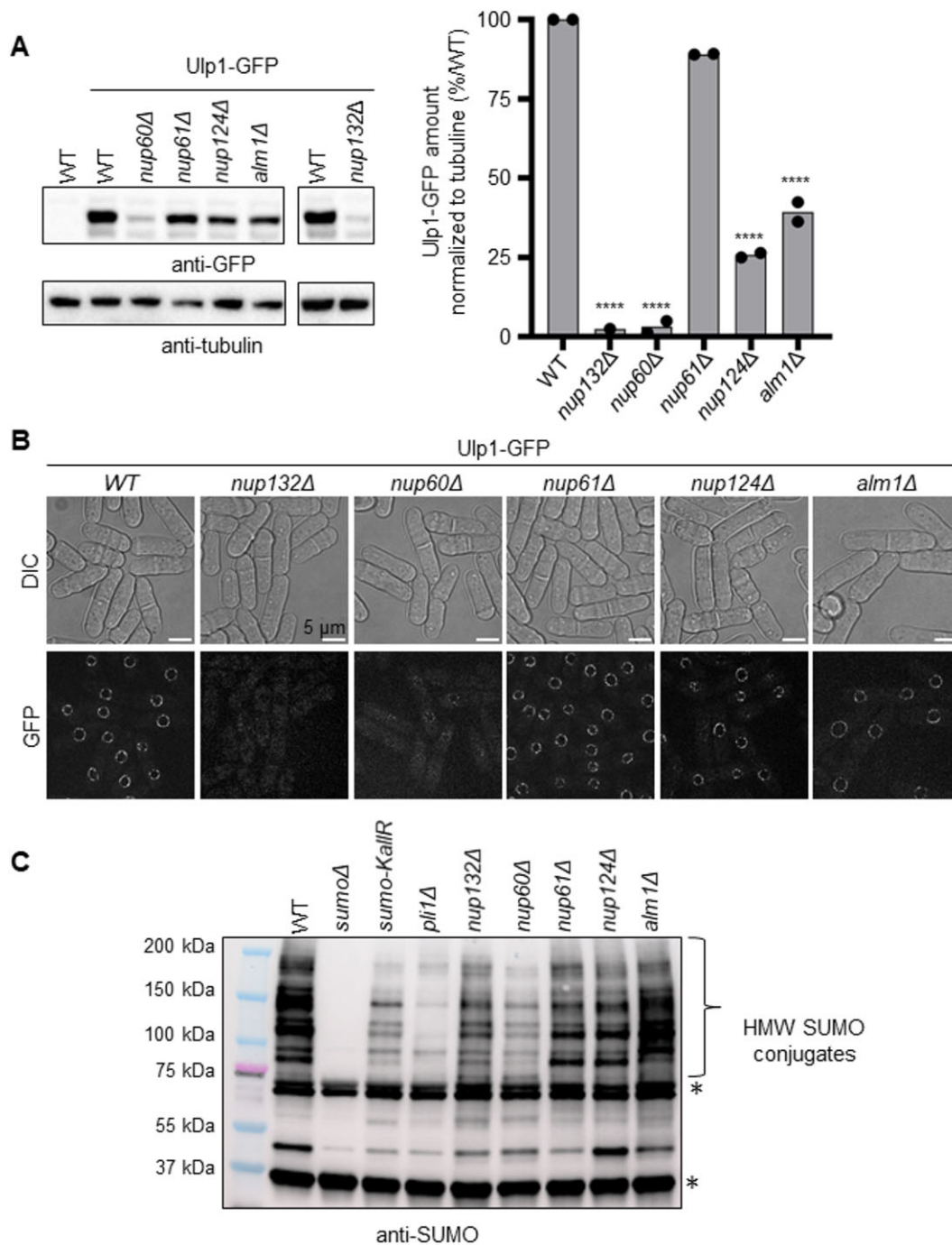
In *S. pombe*, Ulp1 delocalization leads to the degradation of the SUMO chain-modified Pli1, an E3 SUMO ligase, resulting in a global decrease of SUMO conjugates (41). Consistent with Ulp1 expression being severely lowered and delocalized from the NP in *nup132Δ* and *nup60Δ* (Figure 3A and B), we observed a global reduction in the accumulation of SUMO conjugates when compared to WT (Figure 3C). The pattern of SUMO conjugates in *nup132Δ* and *nup60Δ* backgrounds was similar to that observed in a strain expressing SUMO-KallR, in which all internal lysine are mutated to arginine to prevent SUMO chain formation (15). The accumulation of SUMO conjugates was more adversely affected by the absence of Pli1 than in *nup132Δ* and *nup60Δ* cells, suggesting that Pli1 maintains some activity in these genetic backgrounds, as reported for *nup132Δ* cells (15). Despite the reduced Ulp1 expression in *nup124Δ* and *alm1Δ* cells, the pattern of SUMO conjugates was less affected, suggesting that the remaining Ulp1 is sequestered properly at the NP in these genetic backgrounds (Figure 3B and C).

To better characterize the nuclear basket function in sequestering Ulp1 at the NP, we employed live cell imaging to detect simultaneously Ulp1 in WT and mutant backgrounds and quantify Ulp1 density at the NP. To ensure accuracy, we mixed an equal amount of exponentially growing WT cells expressing Ulp1-GFP with WT or nuclear basket mutants co-expressing Ulp1-GFP and Cut11-mCherry (Figure 4A). This approach allowed us to distinguish WT cells from the mutated strains within the same microscopy field, and thus accurately quantify peripheral Ulp1 irrespective of exposure and acquisition parameters. In addition, as Cut11 is a transmembrane core NPC nucleoporin, we also could quantify the total amount and density of NPCs. As previously reported (42), the nuclear morphology of *alm1Δ* cells was different from WT, with an increase in nuclear perimeter and size (Supplementary Figure S3A and S3B). The total amount of peripheral Ulp1 (i.e. total Ulp1 intensity) decreased in *nup132Δ*, *nup60Δ* and *nup124Δ* cells when compared to WT (Supplementary Figure S3C), resulting in a reduced peripheral Ulp1 density (i.e. intensity normalized by nuclear size, see materials and methods section) (Figure 4A and B). Although the total peripheral Ulp1 intensity was



**Figure 2.** The nuclear basket promotes recombination-dependent replication in a pre- and post-anchoring manner. **(A)** Diagram of the *ori > ura4-sd20-t* construct on chromosome III (*ori*: replication origin, *>*: *RTS1*-RFB orientation that block right-moving forks, *t*: telomere). The non-functional *ura4-sd20* allele (red box), containing a 20 nt duplication flanked by micro-homology, is located downstream of the RFB (blue bar). During HR-mediated fork restart, the *ura4-sd20* allele is replicated by an HR-associated DNA synthesis that is liable to replication slippage (RS) resulting in the deletion of the duplication and the restoration of a functional *ura4<sup>+</sup>* gene (34). ARS: autonomously replicating sequence. **(B)** Frequency of RFB-induced RS in indicated strains. Each dot represents one sample from independent biological replicate. Red bars indicate mean values ± standard deviation (SD). *P* value was calculated by two-sided *t*-test (\*\*\*\* *P* ≤ 0.0001; \*\*\* *P* ≤ 0.001; ns: non-significant). **(C)** Top panel: scheme of replication intermediates (RI) analyzed by neutral-neutral 2DGE of the *AseI* restriction fragment in RFB OFF and ON conditions. Partial restriction digestion caused by psoralen-crosslinks results in a secondary arc indicated on scheme by blue dashed lines. Bottom panels: representative RI analysis in indicated strains and conditions. The *ura4* gene was used as a probe. Numbers indicate the % of forks blocked by the RFB ± SD. The red arrow indicates the tail signal resulting from resected forks. **(D)** Quantification of resected forks in indicated strains. Dots represent values obtained from independent biological experiments. No statistical difference was detected between the samples using the two-sided *t*-test. **(E)** Diagram of the LacO-marked RFB. LacO arrays bound by mCherry-LacI (red ellipses) are integrated ~7 kb away from the *RTS1*-RFB (blue bar). **(F)** Example of fluorescence microscopy (right panel) and bright-field images (left panel, DIC) cells expressing the endogenous Npp106-GFP fusion protein and harboring the LacO-marked RFB. Mono-nucleated cells and septated bi-nucleated cells correspond to G2 and S-phase cells, respectively. White arrows indicate co-localization events in S-phase cells. Scale bar: 5 μm. **(G)** Quantification of co-localization events, shown in *f*, in S-phase cells in indicated conditions and strains. Dots represent values obtained from independent biological experiments. At least 100 nuclei were analyzed for each strain and condition. Fisher's exact test was used for group comparison to determine the *P* value (\*\*\*\* *P* ≤ 0.0001; \*\* *P* ≤ 0.01; ns: non-significant).

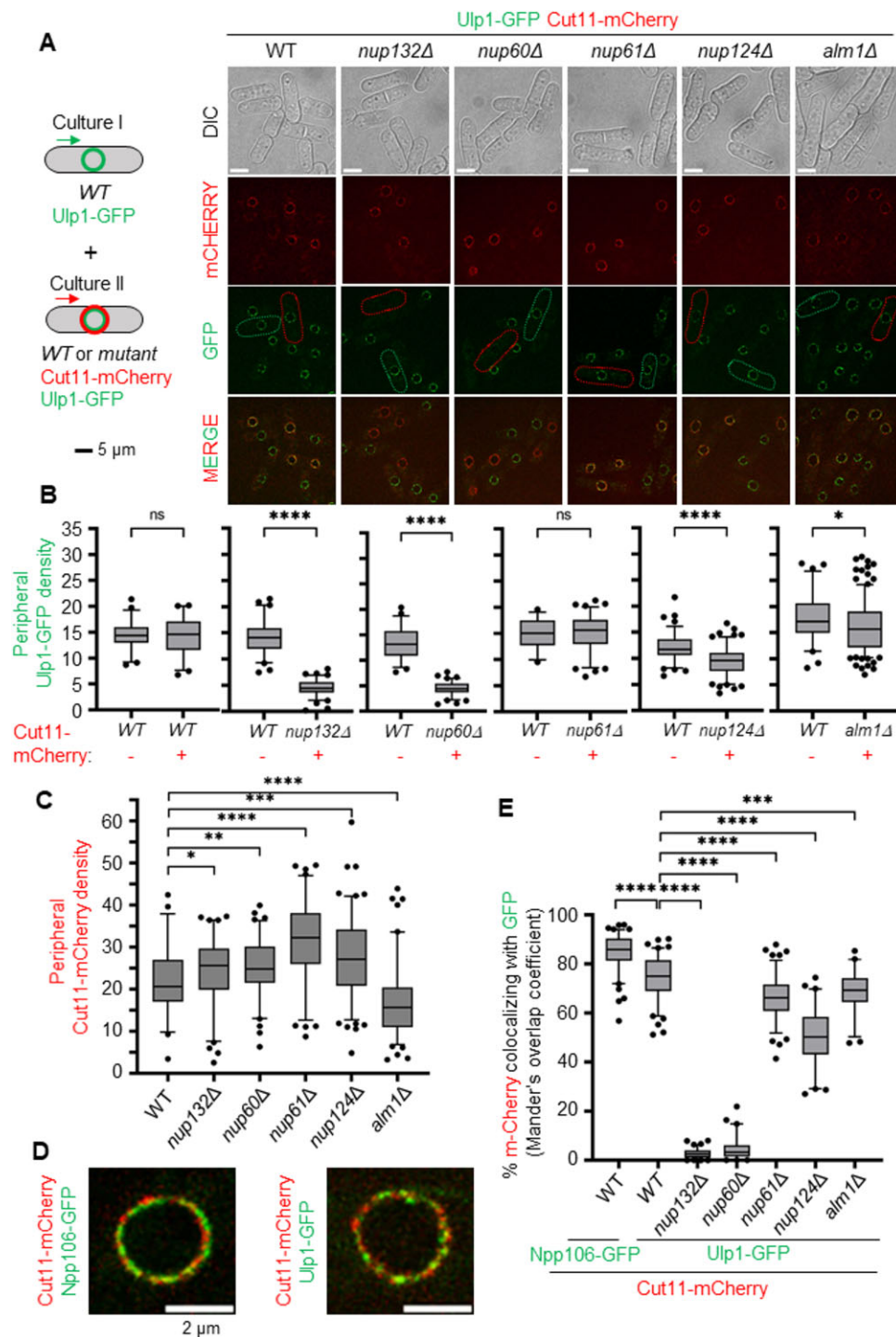




**Figure 3.** The nuclear basket regulates the expression of the SUMO SENP protease Ulp1. **(A)** Left panel: expression of Ulp1-GFP in indicated strains. An untagged WT strain was included as control for antibody specificity. Tubulin was used as a loading control. Right panel: quantification. The normalized amount of Ulp1 was calculated by dividing the GFP signal by tubulin signal. The normalized amount of Ulp1-GFP in the mutants is indicated as a percentage of the WT. Dots represent values obtained from independent biological experiments.  $P$  value was calculated by two-sided  $t$ -test (\*\*\*\*  $P \leq 0.0001$ ). **(B)** Example of fluorescence (bottom panel) and bright-field images (top panel, DIC) of cells expressing the endogenous Ulp1-GFP fusion protein in indicated strains. Scale bar 5  $\mu$ m. **(C)** Expression of SUMO conjugates in indicated strains. A strain deleted for *pmt3* gene that encodes the SUMO particle (*sumoΔ*) was added as control for antibody specificity. \* indicates unspecific signal.

slightly increased in *alm1Δ* cells (Supplementary Figure S3C), the increased nuclear size led to a significant reduction in peripheral Ulp1 density (Figure 4B). The total amount of Cut11 was variable in all strains when compared to WT (Supplementary Figure S3D) but we observed a clear reduction in peripheral Cut11 density in *alm1Δ* cells because of an increased nucleus size (Figure 4C). Finally, we quanti-

fied co-localization between Cut11-mCherry and Ulp1-GFP signals as a read-out of Ulp1-associated NPCs, using Manders overlap coefficient (Figure 4D-E) and Pearson correlation coefficient (Supplementary Figure S3E). As a control we first assigned co-localization between Cut11-mCherry and Npp106-GFP, two core components of NPC. Between 80 and 90% of Cut11 signal was associated with Npp106 under our



**Figure 4.** The nuclear basket contributes to sequester the SUMO SENP protease Ulp1 at the nuclear periphery. **(A)** Left panel: scheme of the strategy employed by equally mixing two genetically distinct cell cultures. Right panel: representative cell images of Cut11-mCherry and Ulp1-GFP in indicated strains. Green and red cell borders indicate cells from culture I (expressing Ulp1-GFP) and culture II (expressing Ulp1-GFP Cut11-mCherry), respectively. Scale bar 5 μm. **(B)** Box-and-whisker plots of Ulp1-GFP density (mean fluorescence intensity) at the nuclear periphery in indicated strains and conditions. Boxes represent the 25/75 percentile, black lines indicate the median, the whiskers indicate the 5/95 percentile and dots correspond to minimum and maximum values. *P* value was calculated by Mann-Whitney *U* test (\*\*\*\* *P* ≤ 0.0001; \*\*\* *P* ≤ 0.001; \*\* *P* ≤ 0.01; \* *P* ≤ 0.05; ns: non-significant). At least 50 nuclei were analyzed for each strain. **(C)** Box-and-whisker plots of Cut11-mCherry density (mean fluorescence intensity) at the nuclear periphery in indicated strains. Boxes represent the 25/75 percentile, black lines indicate the median, the whiskers indicate the 5/95 percentile and dots correspond to minimum and maximum values. *P* value was calculated by Mann-Whitney *U* test (\*\*\*\* *P* ≤ 0.0001; \*\*\* *P* ≤ 0.001; \*\* *P* ≤ 0.01; \* *P* ≤ 0.05; ns: non-significant). At least 50 nuclei were analyzed for each strain. **(D)** Example of the localization of Npp106-GFP and Cut11-mCherry (left panel) or Ulp1-GFP and Cut11-mCherry (right panel) on overlay images. Scale bar: 2 μm. **(E)** Box-and-whisker plots of co-localization between Cut11-mCherry and Ulp1-GFP (Mander's overlap coefficient) in indicated strains. The co-localization between the Npp106-GFP, an inner ring nucleoporin of NPC, and Cut11-mCherry, was performed as a control to show maximum correlation between intensities of those both proteins at the resolution achieved on the images. Boxes represent the 25/75 percentile, black lines indicate the median, the whiskers indicate the 5/95 percentile and dots correspond to minimum and maximum values. *P* value was calculated by Mann-Whitney *U* test (\*\*\*\* *P* ≤ 0.0001; \*\*\* *P* ≤ 0.001).

microscopy conditions, validating our methodological approach (Figure 4D-E and Supplementary Figure S3E). In the absence of either Nup132 or Nup60, the low level of Ulp1 appeared to not overlap with Cut11 at the resolution achieved on the images, indicating that Ulp1-associated NPCs are abolished. Despite a lower NPCs density and a reduced Ulp1 expression in the absence of Alm1, Ulp1-associated NPCs were only moderately affected (~70% compared to ~75% in the WT background). In contrast, only ~50% of Cut11 signal was correlated with Ulp1 in *nup124Δ* cells (Figure 4E and Supplementary Figure S3E), indicating that Ulp1-associated NPCs are less abundant. We concluded that Nup60, and to a lesser extent Nup124, are two key components of the nuclear basket that sequester Ulp1 at the NP.

In budding yeast, Mlp1 and Mlp2 act synergistically to sequester Ulp1 to the NP (39). We therefore addressed the role of the second TPR orthologue Nup211, an essential nucleoporin in *S. pombe*. We employed an auxin-inducible degron (AID) approach using the recently developed AID2 version that makes use of OsTIR1-F74A to target AID-tagged proteins for degradation (43). Nup211-HA-mAID was efficiently degraded 30 minutes after the addition of 5-adamantyl-IAA and no degradation was observed in the absence of TIR1-F74A (Supplementary Figure S4A). We observed a ~40% reduction in Ulp1-GFP expression 60 minutes after 5-adamantyl-IAA addition, compared to the control strain in which TIR1-F74A is not expressed (compare lines 3 and 4 in Supplementary Figure S4B). However, Ulp1-GFP expression was slightly decreased in the strain expressing TIR1-F74A in the absence of 5-adamantyl-IAA (compare lines 1 and 2 in Supplementary Figure S4B). Consistently, these strains showed a significant growth defect when plated on media free of drug (Supplementary Figure S4C), indicating that either the AID2 system applied to Nup211 is leaky or that the C-terminal degron tag partially compromised Nup211 function. When we quantified peripheral Ulp1-GFP by live-cell imaging, we observed that the addition of 5-adamantyl-IAA led to an increased in peripheral Ulp1 density in WT cells and no changes were observed upon degradation of Nup211 (Supplementary Figure S4D). We concluded that Nup211 makes little contribution to Ulp1 expression and peripheral sequestration. We wanted to test the possibility that Alm1 and Nup211 act synergistically to regulate Ulp1 expression and localization, but we failed in generating viable spores combining *alm1* deletion with the *nup211-HA-mAID* allele.

### Tethering of the RFB to Ulp1-associated NPCs rescues RDR defect in *nup60Δ* but not in *alm1Δ* cells

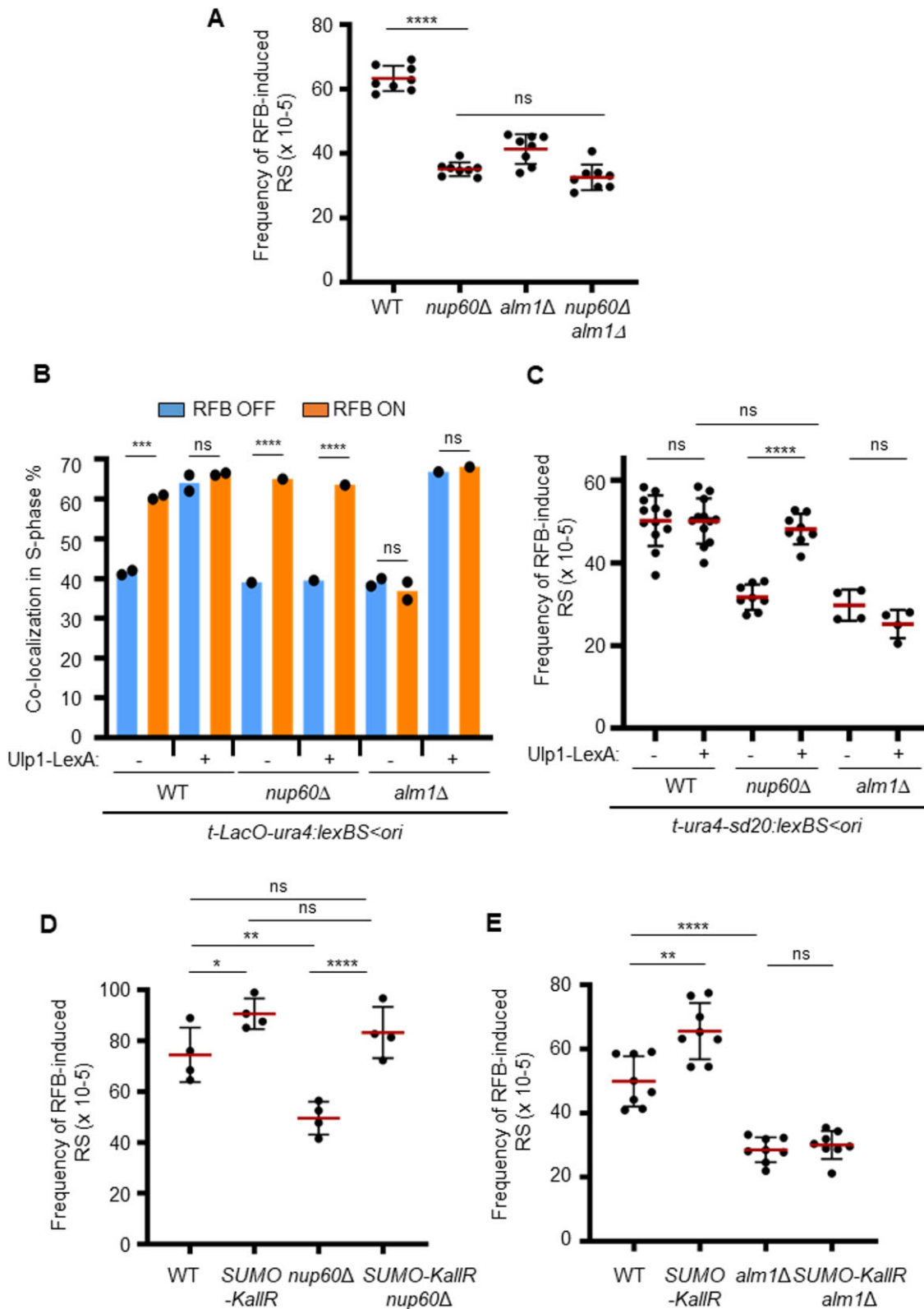
Since Ulp1 is no longer sequestered at the NP in the absence of Nup60 and the RFB is no longer enriched at the NP in the absence of Alm1, we analyzed the double mutant and found that the level of RFB-induced RS was similar to that observed in *nup60Δ* cells (Figure 5A). This result would be consistent with arrested forks having no access to Ulp1-associated NPCs in the absence of Alm1, resulting in an RDR defect. To directly test this, we employed a previously successful approach to tether Ulp1-LexA to the RFB harboring 8 LexA binding sites (either *t-LacO-ura4:LexBS < ori* for nuclear positioning (Figure 5B) or *t-ura4-sd20:lexA < ori* for RFB-induced RS (Figure 5C)) (15). In WT cells, the *LacO*-marked RFB was constitutively enriched at the NP upon ex-

pression of Ulp1-LexA, whatever its activity (OFF or ON), showing that Ulp1 is successfully tethered to the RFB (Figure 5B). Consistent with the role of Nup60 in sequestering Ulp1 at the NP, the inactive RFB did not shift to the NP in *nup60Δ* cells, but was efficiently enriched at the NP in RFB ON condition. This confirms that Ulp1 is dispensable for the NPC anchorage of the RFB (Figure 5B). By performing chromatin immuno-precipitation (ChIP), we confirmed that Npp106-GFP was similarly enriched at the RFB upon Ulp1-LexA tethering in the RFB ON condition in both WT and *nup60Δ* cells (Supplementary Figure S5). Remarkably, tethering Ulp1-LexA to the active RFB, anchored to NPC, resulted in an increased frequency of RFB-induced RS in *nup60Δ* cells, indicating that the lack of Ulp1-associated NPCs is a limiting step in promoting HR-mediated DNA synthesis (Figure 5C). In addition, we combined the *nup60* deletion with SUMO-KallR, which allows only mono-SUMOylation to occur (cf. Figure 3C). As previously reported (15), we observed a slight increase in RFB-induced RS in SUMO-KallR strain (Figure 5D). As expected, preventing SUMO chain in *nup60Δ* cells restored RFB-induced RS to the WT level, further confirming that the reduction in RDR efficiency caused by defective Ulp1-associated NPCs is alleviated by preventing SUMO chain formation (Figure 5D).

Surprisingly, applying similar approaches to *alm1Δ* cells resulted in different outcomes, indicating a distinct flavor of RDR defect. Preventing SUMO chain formation did not rescue the RDR defect observed in the absence of Alm1 (compare *alm1Δ* and *alm1Δ* SUMO-KallR on Figure 5E), indicating that the RDR defect observed in this mutant is not alleviated by preventing SUMO chain formation. Moreover, tethering Ulp1 to the RFB did not rescue the RDR defect (Figure 5B and C). The analysis of the nuclear positioning of the *LacO*-marked RFB showed that the RFB was efficiently shifted to the NP in *alm1Δ* cells regardless its activity, thus allowing bypassing the role of Alm1 in locating the active RFB at the NP (compare RFB ON condition with or without Ulp1-LexA in *alm1Δ* on Figure 5B). In other words, the artificial anchorage of the RFB to Ulp1-associated NPCs is not sufficient to rescue the RDR defect of *alm1Δ* cells. This indicates that the lack of RFB relocation to the NP is not the underlying cause of the RDR defect and that Alm1 is probably required at NPCs to promote RDR independently of Ulp1. Interestingly, Daga *et al.* have reported that Alm1 is required for proper localization of the proteasome to the NE: several proteasome subunits and anchors, such as Mts2 (also known as Rpt2), Mts4 and Cut8, are not properly localized at the NP in *alm1Δ* cells (42). Furthermore, we previously proposed that the proteasome activity is necessary to promote RDR but that this might not be under Nup132 regulation (15). Given the technical difficulty of restoring a stoichiometric proteasome at the NP in *alm1Δ* cells, we turned our attention to a viable proteasome mutant to address its role in the dynamic of RDR. This decision was also motivated by the fact that the deletion of both *alm1* and *rtf1*, a genetic background needed for Pu-Seq analysis, has been reported to be synthetic lethal (42).

### Proteasome-associated NPCs sustain the dynamics of HR-restarted fork

Rpn10 is a regulatory subunit of the 19S proteasome that physically interacts with Mts4/Rpn1 and that is enriched at the NP (44–46) and promotes cell resistance to replication



**Figure 5.** Tethering of Ulp1 to the RFB rescues RDR defect in *nup60*Δ but not in *alm1*Δ cells. **(A)** Frequency of RFB-induced RS in indicated strains and conditions. Dots represent values obtained from independent biological experiments. Red bars indicate mean values ± SD. *P* value was calculated by two-sided *t*-test (\*\*\*\* *P* ≤ 0.0001; ns: non-significant). **(B)** Quantification of co-localization events in S-phase cells in indicated conditions and strains. Dots represent values obtained from independent biological experiments. At least 100 nuclei were analyzed for each strain and condition. *P* value was calculated by two-sided Fisher's exact test (\*\*\*\* *P* ≤ 0.0001; \*\*\* *P* ≤ 0.001; ns: non-significant). **(C–E)** Frequency of RFB-induced RS in indicated strains and conditions. Dots represent values obtained from independent biological experiments. Red bars indicate mean values ± SD. *P* value was calculated by two-sided *t*-test (\*\*\*\* *P* ≤ 0.0001; \*\* *P* ≤ 0.01, \* *P* ≤ 0.05 ns: non-significant).

blocking agents (Supplementary Figure S6A). We previously reported that, in the absence of Rpn10, the active RFB shifts to the NP but RDR efficiency was severely decreased (15), as evidenced by the strong reduction in RFB-induced RS (Figure 6A). Rpn10 acts as a ubiquitin receptor for the proteasome and its absence results in the accumulation of ubiquitin conjugates. Despite an accumulation of SUMO conjugates in *rpn10Δ* cells (Supplementary Figure S6B), we observed that the defect in RFB-induced RS was not rescued by preventing SUMO chain formation (Figure 6A), a situation similar to the *alm1Δ* mutant. We were unable to investigate whether Alm1 and Rpn10 act in the same pathway to promote RDR as we found that the double mutant is not viable.

To probe the function of Rpn10 in RDR, we applied the Pu-Seq approach to the *rpn10* mutant to compare DNA polymerase usage at, and downstream of, the barrier site. We first noticed that polymerase usage was altered upstream of the RFB specifically in the RFB ON condition in *rpn10Δ* cells when compared to WT (Figure 6B), a phenotype suggestive of loss of integrity of the newly synthesized strand. To test this, we analyzed replication intermediates by 2DGE (as described in Figure 2C and D) and observed that nascent strand degradation at arrested forks was slightly increased in the absence of Rpn10 (Supplementary Figure S6C and D), indicating a role for the proteasome in the negative regulation of fork resection. Based on the Pol  $\delta/\delta$  bias immediately downstream of the RFB, we estimated that, when compared to *rpn10+* cells, approximately 85% of the expected number of forks were arrested and restarted in *rpn10Δ* cells (Figure 6B and C). Remarkably, the relative change in slope of the Pol  $\delta/\delta$  bias reduction over distance was much steeper in the two replicates from *rpn10Δ* cells when compared to the WT strain, indicating a lower speed or increased instability of the restarted forks (Figure 6C). This slow/unstable replication accounts for the increased number of leftward moving canonical forks evident in the Pu-Seq traces (Figure 6B). We estimated that half of restarted forks progress approximately one third of the distance of WT restarted forks. This scenario contrasts with that observed in the *nup132Δ* cells, in which fewer forks were restarted but the progression of those that did was unaffected. We confirmed that Ulp1 expression and sequestration at the NP were unaffected by the loss of Rpn10 (Supplementary Figure S6E and F). We therefore tested a scenario in which arrested forks are enriched in the NPC environment but devoid of Ulp1 and functional proteasome by analyzing RDR efficiency in *nup60Δ rpn10Δ* cells. We observed an additive effect in RFB-induced RS in this double mutant (Figure 6D), consistent with Rpn10 and Nup60 acting in separate pathways to promote RDR. We concluded that both Ulp1 and the proteasome are required at the NP to foster the dynamics of HR-mediated DNA synthesis by sustaining the efficient initiation of restarted DNA synthesis and the progression of the restarted fork, respectively.

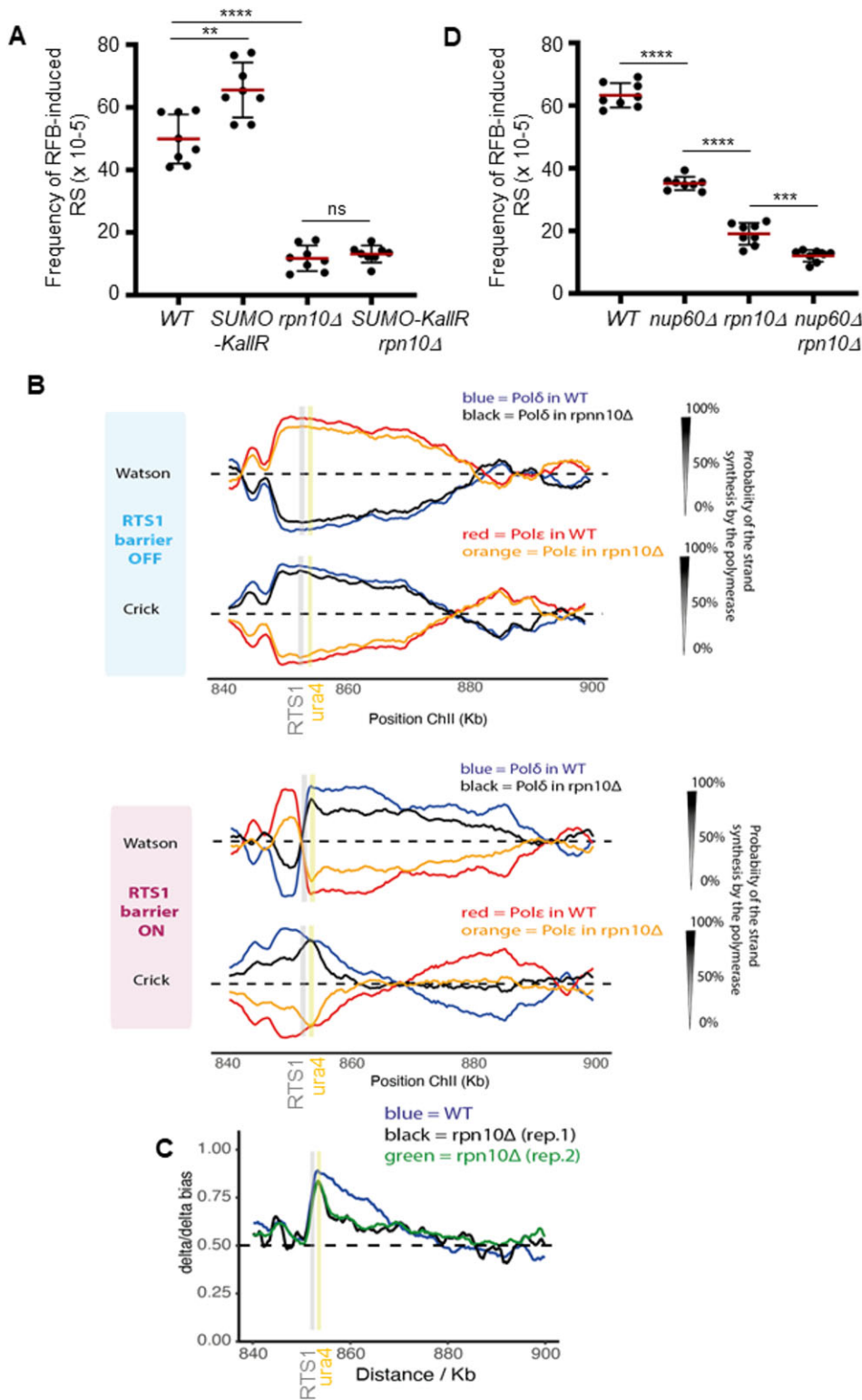
## Discussion

Halted replication forks are diverted to the NP and can associate with NPC components to engage error-free DNA repair pathways (8,14–21). How the NPC environment acts mechanistically to foster the dynamics of DNA repair pathways remains unclear. Here, we reveal that NPCs define a particular nuclear compartment that favors the dynamic of HR-dependent DNA synthesis at dysfunctional forks by two dis-

tinct mechanisms. The Ulp1 SUMO protease ensures an efficient initiation of restarted DNA synthesis. This mechanism requires the sequestration of Ulp1 at the NP which is coordinated by the Y complex and the nuclear basket nucleoporin Nup60. The second mechanism relies on the ability of the nuclear basket to enrich proteasome components at the NP (42,46) to foster the progress of restarted DNA polymerases. Surprisingly, preventing SUMO chain formation mitigates the defects in HR-mediated fork restart caused by defective Ulp1-associated NPCs but not the defects caused by defective proteasome-associated NPCs. We thus establish that Ulp1 and the proteasome differentially affect the dynamics of HR-mediated DNA synthesis without compensating for each other.

We previously reported that the Y complex nucleoporin Nup132 promotes RDR in a post-anchoring manner, downstream of Rad51 loading at dysfunctional forks, by sequestering Ulp1 at the NP (15). Here, we reveal that the nuclear basket contributes to this pathway. Akin to budding yeast (38), the sequestration of Ulp1 at the NP in *S. pombe* requires the nuclear basket nucleoporin Nup60. Despite our efforts, we cannot rule out a synergistic function of TPR homologs, Alm1 and Nup211, in the spatial segregation of Ulp1 at the NP. By mapping DNA polymerase usage during HR-dependent fork restart (28), we establish that Ulp1-associated NPCs are necessary to ensure efficient initiation of restarted DNA synthesis. This function may be linked to the fact that Ulp1 counterbalances the inhibitory effect of SUMO chain on unknown targets. In budding yeast, a similar inhibitory effect of SUMO chain on DNA replication initiation at origins has been reported (47). The MCM helicase and other replication factors were identified as SUMO chain-modified substrates targeted by the SUMO protease Ulp2 and ultimately proteasomal degradation. Although we did not formally address the function of SpUlp2 in RDR, our data clearly highlight a role for Ulp1-associated NPCs in promoting efficient initiation of restarted DNA synthesis. Protein-protein docking studies predicted a higher affinity of SpUlp1 towards SUMO particles compared to ScUlp1, suggesting a more specific role of SpUlp1 in targeting SUMO chain than Ulp2 (48). Alternatively, preventing SUMO chain formation may act on other mechanisms that favor the frequency of replication slippage during the progression of restarted forks. Further investigations are needed to clarify the role played by Pli1-dependent SUMO chain in the dynamics of RDR. Notwithstanding this, our work establishes that the abundance of Ulp1-associated NPCs is not a limiting factor in promoting RDR, as their reduction by 40% in *nup124Δ* cells leads to no noticeable RDR defect.

Our work also establishes that Nup132, a component of the Y complex, and Nup61, a component of the nuclear basket, contribute to the cellular response to replication stress and recovery from transient fork stalling. However, the lack of Ulp1-associated NPCs does not correlate with cell sensitivity to replication-blocking agents or with a defective recovery from HU-stalled forks, since *nup132Δ* and *nup60Δ* cells exhibit distinct phenotype. In addition, no defect in Ulp1-associated NPCs was observed in the absence of Nup61. Therefore, the integrity of the nuclear basket and the Y complex is required to promote cells resistance to replication blocking agents and recovery from HU-stalled forks, beyond the formation of Ulp1-associated NPCs and SUMO homeostasis. This conclusion is consistent with a previous report establishing that NPCs contribute to the DNA damage response, beyond



**Figure 6.** Proteasome-associated NPCs promote the progression of restarted fork. **(A)** Frequency of RFB-induced RS in indicated strains and conditions. Dots represent values obtained from independent biological experiments. Red bars indicate mean values  $\pm$  SD. *P* value was calculated by two-sided *t*-test (\*\*\*\*  $P \leq 0.0001$ ; \*\*\*  $P \leq 0.001$ ; \*\*  $P \leq 0.01$ ; ns: non-significant). **(B)** Pu-Seq traces of the ChrII locus in *RTS1*-RFB OFF (top panel) and ON (bottom panel) conditions in WT and *rpn10*Δ strains. The usage of Pol delta (in blue and black for WT and *rpn10*Δ cells, respectively) are shown on the Watson and Crick strands. The usage of Pol epsilon (in red and orange for WT and *rpn10*Δ cells, respectively) are shown on the Watson and Crick strands. Note that the switch from Pol epsilon to Pol delta on the Watson strand at the RFB site (gray bar) is indicative of a change in polymerase usage on the leading strand in RFB ON condition. The genomic location of the ARS, the *RTS1*-RFB and the *ura4* marker are indicated by dashed lines, a gray line and a yellow line, respectively. **(C)** Graph of Pol delta/delta bias in RFB ON condition according to chromosome coordinates in WT and two independent replicates of *rpn10*Δ strains. The gray and yellow bars indicate the position of the *RTS1*-RFB and of the *ura4* marker, respectively. **(D)** Frequency of RFB-induced RS in indicated strains and conditions. Dots represent values obtained from independent biological experiments. Red bars indicate mean values  $\pm$  SD. *P* value was calculated by two-sided *t*-test (\*\*\*\*  $P \leq 0.0001$ ; \*\*\*  $P \leq 0.001$ ).

a role in sequestering Ulp1 at the NP (49). Further investigations are needed to establish, for example, the contribution of macromolecular transport by NPC in the DNA damage response.

We previously reported that the proteasome, whose activity is enriched at the NP (46), promotes RDR in a post-anchoring manner (15). The mapping of DNA polymerase usage during HR-dependent fork restart reveals that a proteasome defect more severely affects the progression of restarted DNA polymerases, with a reduction of forward movement by up to 70%, than the initiation of restarted DNA synthesis. This contrasts with Ulp1 function in contributing primarily to the initiation of DNA synthesis with no apparent contribution to the dynamics progression of restarted DNA polymerases. This division of labour between the proteasome and the SUMO protease in ensuring the dynamics of HR-dependent fork restart is reinforced by the fact that these activities cannot compensate for each other. Indeed, the artificial tethering of the RFB to NPCs in the *alm1*  $\Delta$  mutant shows that Ulp1-associated NPCs are insufficient to promote efficient RDR without a functional proteasome at the NP. We noticed that RFB-induced RS are more severely decreased in the absence of Rpn10 than in the absence of Alm1. This might be due to a more drastic effect of the loss of Rpn10 on proteasome functionality than the loss of Mts2/Rpt2 and Mts4, as observed in *alm1*  $\Delta$  cells. We were unable to test RDR efficiency in the double *rpn10*  $\Delta$  *alm1*  $\Delta$  mutant to address epistatic interaction since this genetic background is not viable. This finding is consistent with a previous report, showing that the deletion of both *rpn10* and *mts4* results in synthetic lethality (44). Alternatively, the extensive degradation of nascent strand at arrested forks observed in *rpn10*  $\Delta$  cells, but not in *alm1*  $\Delta$  cells, may contribute to the more severe defect in RDR efficiency. Additionally, the fact that only the NP-enriched fraction of the proteasome is defective in the absence of Alm1 could contribute to a less severe defect in RDR efficiency.

Our genetic analysis establishes that defect in fostering the progress of restarted DNA synthesis caused by defective proteasome-associated NPCs cannot be alleviated by preventing SUMO chain, contrasting with the defect caused by Ulp1-associated NPCs. This suggests distinct specificities between the proteasome and Ulp1 towards SUMOylated targets which affect differentially the dynamics resumption of DNA synthesis at dysfunctional forks. We do not exclude that SUMO-independent poly-ubiquitination, targeted by Rpn10 for proteasomal degradation, plays a role in promoting RDR. However, we previously identified that the SUMO Targeted Ubiquitin Ligase (STuBL) Slx8-Rfp1-Rfp2, a family of E3 ubiquitin ligases, that targets SUMOylated proteins for degradation (50), promotes both the relocation of dysfunctional forks to NPCs and RDR efficiency in a Pli1-dependent manner (15). This supports the notion that mono-SUMOylated or chain-free multi-SUMOylated factors are potential targets of a proteasome and Slx8-dependent pathway that ensures the progress of restarted DNA polymerases. SUMO chain-independent functions of STuBLs are documented, including the relocation of forks collapsed at CAG repeats via mono-SUMOylation recognized by the SUMO interacting motif of ScSlx5 (17). The human STuBL RNF4 can also bind the substrate ETV4 mono-SUMOylated on multiple lysines, in a process requiring the multiple SIM domains of RFN4 (51).

Our work also identified that, in the absence of the nuclear basket nucleoporin Alm1, the RFB was no longer enriched

at the NP. To our knowledge, TPR homologs have not been previously implicated in anchoring DNA lesions to NPCs in yeast models. Upon telomeric replication stress, human telomeres relocate to the NP and associate with NPC components, including TPR, to resolve replication defects (19). Depletion of human TPR is associated with a variety of replication defects and TPR was proposed to coordinate at NPCs a network of factors involved in RNA metabolism to protect cells from RNA-mediated replication stress (52). Given the nuclear morphology alterations in the absence of Alm1, we cannot exclude that the lack of anchorage is an indirect effect. In human cells, the mobility of stressed forks towards the NP requires F-nuclear actin polymerization (8,20), but such a mechanism has not been reported in yeast. We estimated that, in the absence of Alm1, the RFB must explore a nuclear volume 40% larger to reach the NP and associate with NPCs whose abundance is reduced by one quarter.

Overall, this work uncovers two mechanisms by which the NPC environment ensures the dynamic of HR-dependent replication restart, streamlining the need for dysfunctional forks to change nuclear positioning. Ulp1-associated NPCs contribute to the efficient initiation of restarted DNA synthesis to engage a Pol $\delta$ /Pol $\delta$  DNA synthesis, whereas proteasome-associated NPCs foster the progression of restarted DNA synthesis. These two functions cannot compensate for each other, are differently required and ensured by distinct components of the NPC.

## Data availability

The data underlying this article are available in Mendeley data and are available at 'Schirmeisen, Naiman et al 2023', Mendeley Data, V1, at doi: 10.17632/2kgnb9d66r.1. RAW data from Pu-Seq experiments are available under GEO dataset GSE247371. All relevant data are available and further information and requests for reagents and resources should be directed to and will be fulfilled by Dr. Sarah A.E. Lambert (sarah.lambert@curie.fr).

## Supplementary data

[Supplementary Data](#) are available at NAR Online.

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## Conflict of interest statement

None declared.

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### **III: Publication #3**

The fission yeast SUMO-targeted Ubiquitin Ligase Slx8 functionally associates with clustered centromeres and the silent mating type region at the nuclear periphery.

# **The fission yeast SUMO-targeted Ubiquitin Ligase Slx8 functionally associates with clustered centromeres and the silent mating type region at the nuclear periphery**

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## **Running title:**

Slx8 promotes gene silencing and centromere clustering at nuclear peripheral regions

**Keywords:** SUMO Targeted Ubiquitin Ligase, Slx8, SUMO, centromere, heterochromatin, gene silencing

## Abstract

The SUMO-targeted Ubiquitin ligase (STUbL) family is involved in multiple cellular processes via a wide range of mechanisms to maintain genome stability. One of the evolutionarily conserved functions of STUbL is to promote changes in the nuclear positioning of DNA lesions, targeting them to the nuclear periphery. In *Schizosaccharomyces pombe*, the STUbL Slx8 is a regulator of SUMOylated proteins and promotes replication stress tolerance by counteracting the toxicity of SUMO conjugates. In order to study the dynamic dialectic between Ubiquitylation and SUMOylation in the nuclear space of the *S. pombe* genome, we analyzed Slx8 localization. Unexpectedly, we did not detect replication stress-induced Slx8 foci. However, we discovered that Slx8 forms a single nuclear focus, enriched at the nuclear periphery, which marks both clustered centromeres at the spindle pole body and the silent mating type region. The formation of this single Slx8 focus requires the E3 SUMO ligase Pli1, poly-SUMOylation and the histone methyl transferase Crl4 that is responsible for the heterochromatin histone mark H3-K9 methylation. Finally, we established that Slx8 promotes centromere clustering and gene silencing at heterochromatin domains. Altogether, our data highlight evolutionarily conserved and functional relationships between STUbL and heterochromatin domains to promote gene silencing and nuclear organization.

## Highlights:

- The *S. pombe* STUbL Slx8 forms a single nuclear focus enriched at the nuclear periphery in a SUMO-chain-dependent manner.
- Slx8 foci mark clustered centromeres and the silenced mating type region but not telomeres.
- H3-K9 methylation by Crl4 promotes the single nuclear Slx8 focus
- Slx8 promotes centromere clustering and gene silencing.

## Introduction

The nuclear architecture and the 3D genome organization have emerged as important regulation layers of genome maintenance, contributing to numerous DNA-associated transactions such as chromosome segregation, transcription and DNA repair (Misteli & Soutoglou, 2009). Chromatin displays functional compartmentalization: while gene-rich, transcriptionally active chromatin tends to localize to the interior of the nucleus, gene-poor, transcriptionally repressed heterochromatin is typically enriched at the nuclear periphery (NP), which is believed to provide a microenvironment favoring association of factors required for silencing (reviewed in (Towbin et al., 2009)). In many organisms, centromeres also cluster together at the NP, and this spatial organisation has been shown to be important for promoting loading of centromeric proteins (W. Wu et al., 2022b), silencing of repetitive elements (Padeken et al., 2013), and the prevention of micronuclei formation (Jagannathan et al., 2018). The stability of the genome is particularly vulnerable during the process of DNA replication since a broad spectrum of obstacles can jeopardize the progression of the replication machinery, resulting in fork stalling, collapse or breakage (Zeman & Cimprich, 2014b). In several organisms, from yeast to flies and mammalian cells, DNA lesions, including double strand break (DSB) and replication stress site, shift away from their initial nuclear compartment to associate with the NP. Such mobility of DNA lesions allows a spatial regulation of DNA repair processes to ensure optimal error-free repair outcome (reviewed in (Lamm et al., 2021b; Whalen & Freudenreich, 2020b)).

The NP is composed of a double membrane nuclear envelop (NE) and multiple nuclear pore complexes (NPCs) embedded in the NE. In yeast, the spindle pole body (SPB), the functional macromolecular structure equivalent to centrosome, is also embedded in the NE. Components of both the NE and the NPC have been reported as factors allowing anchorage of DNA lesions to the NP (reviewed in (Whalen & Freudenreich, 2020b)). Although the mechanisms of relocation and anchorage differ depending on the type of DNA lesion and the cell cycle stage, an emerging common feature is the requirement for SUMOylation which homeostasis is critical to maintain genome integrity (Schirmeisen et al., 2021b). SUMO (small ubiquitin-like modifier) is a post-translational modification present in all eukaryotic systems. SUMO is covalently attached to a target thanks to the coordinated activity of E2 and E3 SUMO ligases (reviewed in (Y. C. Chang et al., 2021)). Target proteins can be either mono-SUMOylated on a single lysine residue, or harbor multiple single SUMO modifications on several lysine residues, a type of poly-SUMOylation. Moreover, additional SUMO molecule can be covalently attached to the internal lysine of SUMO to form SUMO chains, another

type of poly-SUMOylation. SUMOylation affects the activity, the localization and stability of modified targets, with SUMO chains often favoring protein degradation.

A key determinant of the fate of SUMOylated proteins is the SUMO-targeted E3 Ubiquitin ligase (STUbL) family that recognizes SUMOylated proteins and attaches ubiquitin to them. STUbLs are involved in diverse molecular processes, including DNA repair and replication, both during unchallenged conditions and in response to genotoxic stresses (reviewed in (Y. C. Chang et al., 2021)). STUbLs are characterized by a RING-type E3 ubiquitin ligase domain and one or several SUMO-interacting motifs (SIMs) to recognize SUMOylated substrates. Modification by STUbLs can target substrates for proteosomal degradation or mediate non-proteolytic functions. STUbLs act in specific environments, such as the NE, centromere, kinetochore or PML nuclear bodies in human cells. STUbLs have also been implicated in localizing DSBs and replication stress sites to the NP to promote DNA repair and fork restart (reviewed in (Lamm et al., 2021b; Whalen & Freudenreich, 2020b)). A seminal study in *Saccharomyces cerevisiae* (Sc) first showed that difficult-to repair DSBs and collapsed forks anchor to the NPC in a process requiring the ScSlx5-Slx8 STUbL that physically associates with the Nup84 complex, a component of the NPC (Nagai et al., 2008b). Further studies established that the SUMOylation status of proteins bound to DSBs influences the target destination. For example, mono-SUMOylation allows S-phase DSBs to relocate to Mps3, a NE component, whereas poly-SUMOylation allows DSBs in G1 to associate with the NPC in STUbL-dependent manner, suggesting a specificity of STUbL for poly-SUMO chains (Horigome et al., 2014, 2016b).

The target destination of replication stress sites described so far is the NPC. This includes forks stalled within telomeres sequences, at tri-nucleotides repeats, at a replication fork barrier (RFB) mediated by DNA-bound protein and forks stalled by global replication stress in human cells (Aguilera et al., 2020; Kramarz et al., 2020b; Nagai et al., 2008b; Pinzaru, Kareh, et al., 2020b; Rivard et al., 2024; X. A. Su et al., 2015b). In *S. cerevisiae*, forks stalled at expanded CAG repeats, anchor to the NPC in a process that requires the SIMs of Slx5 and mono-SUMOylation, since preventing poly-SUMOylation does not affect relocation to the NP (X. A. Su et al., 2015b). The SUMOylation of the repair factors RPA, Rad52 and Rad59 is sufficient to trigger Slx5-dependent relocation to the NP, suggesting that Slx5 may recognize several SUMO particles covalently attached to distinct targets (Whalen et al., 2020b). Targeting forks stalled at CAG repeats to the NPC allows the loading of the recombinase Rad51 and prevents the chromosomal fragility of CAG repeats.

In *Schizosaccharomyces pombe*, we have revealed a SUMO-based mechanism that allows the spatial regulation of the recombination-dependent replication (RDR)

process, a mechanism that ensures the restart of arrested forks by homologous recombination (Kramarz et al., 2020b). Forks arrested by the *RTS1*-RFB relocate to the NP to associate with the NPC in a process requiring SUMO chain formation and the SpSTUbL. In *S. pombe*, Rfp1 and Rfp2 are functional homologs of ScSlx5 but lack E3 activity. They recruit Slx8 through a RING-RING domain interaction to form a functional E3 Ubiquitin ligase (Prudden et al., 2007b, 2011a). The absence of a functional spSlx8 STUbL results in the accumulation of high molecular weight (HMW) SUMO conjugates and sensitivity to genotoxic drugs that can be alleviated by the inactivation of the E3 SUMO ligase Pli1 and by preventing SUMO chain formation, suggesting that SpSTUbL has a specificity in targeting poly-SUMOylated substrates (Kosoy et al., 2007a; Nie et al., 2017; Prudden et al., 2007b; Steinacher et al., 2013b). We further established that the relocation of the RFB to the NP promotes RDR via two activities that are enriched in the NPC environment, namely the SUMO protease Ulp1 and the proteasome (Schirmeisen et al., 2024).

One of the unresolved questions in the field is to understand the dynamic crosstalk between SUMOylation and Ubiquitination during the process of relocation of stressed forks and how such crosstalk is spatially segregated in the nuclear space. For example, both SUMOylation and STUbL activity are expected to occur at the site of replication stress before relocation to the NP. Indeed, the drosophila STUbL Dgrn (for degradingolade) is recruited at heterochromatic DSBs prior to relocation and after the action of E3 SUMO ligases (Ryu et al., 2015b, 2016b). To investigate the temporal and spatial dynamics of SpSlx8 by live cell imaging in response to global replication stress, we generated a functional fusion protein Slx8-GFP, in a similar approach to the one employed to characterize damage-induced ScSlx5 foci (Cook et al., 2009a) and SpUfd1 (for ubiquitin-fusion degradation protein) that physically interacts with STUbL (Køhler et al., 2013). We observed that Slx8-GFP did not form replication-stress induced foci but a single discrete focus enriched at the NP in unstressed condition. Both SUMO chains and the E3 SUMO ligase Pli1 are necessary to sustain Slx8-GFP focus formation. Further cellular analysis established that Slx8-GFP focus marks heterochromatin domains positioned at the NP and in the SPB environment, including centromeres and the mating type (*mat*) region. Both heterochromatin and anchoring of centromeres to SPB promotes Slx8-GFP focus. Finally, we provide functional evidence that Slx8 is actively involved in gene silencing and in the clustering of centromeres. Our results highlight functional and physical crosstalk between STUbL and heterochromatin to orchestrate the nuclear organization of specific domains.

## **Materials and methods**

### **Standard yeast genetics and biological resources**

Yeast strains used in this work are listed in Table S1. Gene deletion and tagging were performed by classical genetic techniques. To assess the sensitivity of chosen mutants to genotoxic agents, mid log-phase cells were serially diluted and spotted onto yeast extract agar plates containing hydroxyurea (HU), methyl methanesulfonate (MMS), camptotecin (CPT).

### **Live cell imaging**

For snapshot microscopy, cells were grown in filtered EMMg to exponential phase, then centrifuged and resuspended in 500  $\mu$ L of fresh EMMg. 1  $\mu$ L from the resulting solution was dropped onto Thermo Scientific slide (ER-201B-CE24) covered with a thin layer of 1.4 % agarose in filtered EMMg (Kramarz et al., 2020b). 11 z-stack pictures (each z step of 200 nm) were captured using a Nipkow Spinning Disk confocal system (Yokogawa CSU-X1-A1) mounted on a Nikon Eclipse Ti E inverted microscope, equipped with a 100x Apochromat TIRF oil-immersion objective (NA: 1.49) and captured on sCMOS Prime 95B camera (Photometrics) operated through MetaMorph<sup>®</sup> software (Molecular Devices). The GFP proteins were excited with a 488 nm (Stradus<sup>®</sup> - Vortran Laser Technology, 150mW) laser, while RFP and m-Cherry proteins were excited with a 561 nm (Jive<sup>™</sup> - Cobolt, 100mW) laser. A quad band dichroic mirror (405/488/568/647 nm, Semrock) was used in combination with single band-pass filters of 525/50 or 630/75 for the detection of GFP, RFP and m-Cherry, respectively. Fluorescence and bright-field 3D images were taken at every 0.2 $\mu$ m by acquiring one wavelength at a time. Exposure time for GFP channel was 500 ms, for RFP was 300 ms and for mCherry was 600 ms. During the imaging, the microscope was set up at 25°C. For all the experiments the Gataca Live SR module (Müller et al., 2016, Gataca Systems), implemented on the Spinning Disk confocal system, was used to generate super-resolution images. All image acquisition was performed on the PICT-IBiSA Orsay Imaging facility of Institut Curie.

### **Image analysis**

Images were mounted and analyzed with Fiji software (Schindelin et al., 2012). First, the 3D Z series were converted into 2D projection based on maximum intensity values to produce the image with merged stacks. Since, Slx8 is a low abundant protein, with a high nuclear background, the quantification of Slx8 foci were performed using a noise tolerance threshold value of 50 (Maxima) from Fiji. This was decided after comparing different Maxima values in order to detect foci vs random background noise. Once the threshold was applied, the foci could be manually counted by selecting them as



detected by the software. All experiments have been analysed with the same Maxima value in this report. For quantification of the percentage of co-localization between Slx8 and other markers, the same as above was done onto the GFP channel to first annotate the Slx8 foci above the "set" threshold. In a separate window, the GFP and RFP/mCherry channels with different stacks were merged together followed by manually analysing the co-localization of the green and red foci signal at each stack. Maxima was not applied to RFP/mCherry channel because the foci detection was clear and obvious with no nuclear background noise. The probability of a random event for the co-localisation experiments were performed by using the 180° transform tool in Fiji for the RFP/mCherry marker, followed by merge with the normal Slx8 GFP stacks (without the 180° transform). Consequently, analysis of co-localization between the green and red foci signal at each stack in this setting provided the number of random co-localisation events possible in each given field. This value is referred to as the "random event" that provides a threshold to calculate the possibility of significant co-localisation events as compared to random events.

### **Centromere clustering analysis**

For clustering analysis, cells expressing GFP–Cnp1 and Sid4–RFP were grown in YES to exponential phase, then centrifuged and resuspended in 30  $\mu$ L YES. 4  $\mu$ L of the resulting cell suspension was mixed with 6  $\mu$ L of 1% low-melting point agarose and imaging was performed at 25°C using a Nikon Ti2 inverted microscope, equipped with a 100 $\times$ 1.49 NA Apo TIRF objective and a Teledyne Photometrics Prime 95B camera. Images were acquired with NIS-elements (version 5.1), with Z-stacks taken at 250 nm intervals. Maximum intensity Z-projections were made in ImageJ. Manual quantification of the number of GFP foci per cell was performed to determine the proportions of cells displaying centromeres 'clustered' (one GFP–Cnp1 focus) versus 'unclustered' (two or three GFP–Cnp1 foci).

### **Whole protein extract analysis**

Aliquots of  $1 \times 10^8$  cells were collected and disrupted by bead beating in 1 mL of 20 % TCA (Sigma, T9159). Pellets of denatured proteins were washed with 1M Tris pH 8 and resuspended in 2x Laemmli buffer (62.5 mM Tris pH 6.8, 20 % glycerol, 2 % SDS, 5 %  $\beta$ -mercaptoethanol with bromophenol blue). Samples were boiled before being subjected to SDS-PAGE on Mini-PROTEAN TGX Precast Gel 4-15 % (Biorad, 4561086). Western blot using anti-GFP (Roche, 11814460001) and anti-PCNA (Santa Cruz, sc-56) antibodies was performed. For the analysis of cellular patterns of global SUMOylation, whole protein extraction was performed as follows: aliquots of  $2 \times 10^8$  cells were collected and resuspended in 400  $\mu$ L of water. The cell suspensions were mixed with 350

μl of freshly prepared lysis buffer (2M NaOH, 7% β-mercaptoethanol) and 350μl of 50% TCA (Sigma, T9159). After spin, pellets were further washed with 1M Tris pH 8 and resuspended in 2x Laemmli buffer (62.5 mM Tris pH 6.8, 20 % glycerol, 2 % SDS, 5 % β-mercaptoethanol with bromophenol blue). Samples were boiled before being subjected to SDS-PAGE on Mini-PROTEAN TGX Precast Gel 4-15 % (Biorad, 4561086). Western blot using anti-SUMO antibody (non-commercial, produced in rabbit by Agro-Bio) was performed (dilution of 1:1000).

### **RT-qPCR**

Total RNA was extracted from  $1 \times 10^7$  mid-log phase cells using the Masterpure Yeast RNA Purification Kit (Epicentre), according to the manufacturer's instructions. 1 μg of extracted RNA was treated with TURBO DNase (Ambion) for 1 h at 37°C, and reverse transcription was performed using random hexamers (Roche) and Superscript III reverse transcriptase (Invitrogen). Lightcycler 480 SYBR Green (Roche) and primers ( $q_{cen[dg]}_F$ : 5'-AATTGTGGTGGTGTGGTAATAC-3' and  $q_{cen[dg]}_R$ : 5'-GGGTTTCATCGTTTCCATTCAG-3';  $q_{mat[D]}_F$ : 5'-GTCCGAGGCAATACAACCTTTGG-3'; and  $q_{mat[D]}_R$ : 5'-GGTTGACAGTAGGAGATATTTACAG-3';  $q_{act1}_F$ : 5'-GTTTCGCTGGAGATGATG-3' and  $q_{act1}_R$ : 5'-ATACCACGCTTGCTTTGAG-3') were used for qPCR quantification of *pericentromere* (*dg*) and *mating type locus* (*mat*) transcript levels, relative to *act1*<sup>+</sup>.

### **STATISTICAL ANALYSIS**

Quantitative analysis of western blots were carried out using Fiji software. The ratio from the Raw Integrated Density value of the protein of interest to housekeeping control was calculated for estimating the amount of protein.

Cell imaging was performed using METAMORPH software and processed and analyzed using ImageJ software (ref). The explanation and definitions of values and error bars are mentioned within the figure legends. In most experiments, the number of samples is > 2 and obtained from independent experiments to ensure biological reproducibility. For all experiments based on the analysis of cell imaging, the number of nuclei analyzed is mentioned in the figure legends. Statistical analysis was carried out using Mann-Whitney U tests, Brown-Forsythe and Welch Anova test, chi-squared test and Student's *t*-test. ns:  $P \geq 0.05$ , \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ .

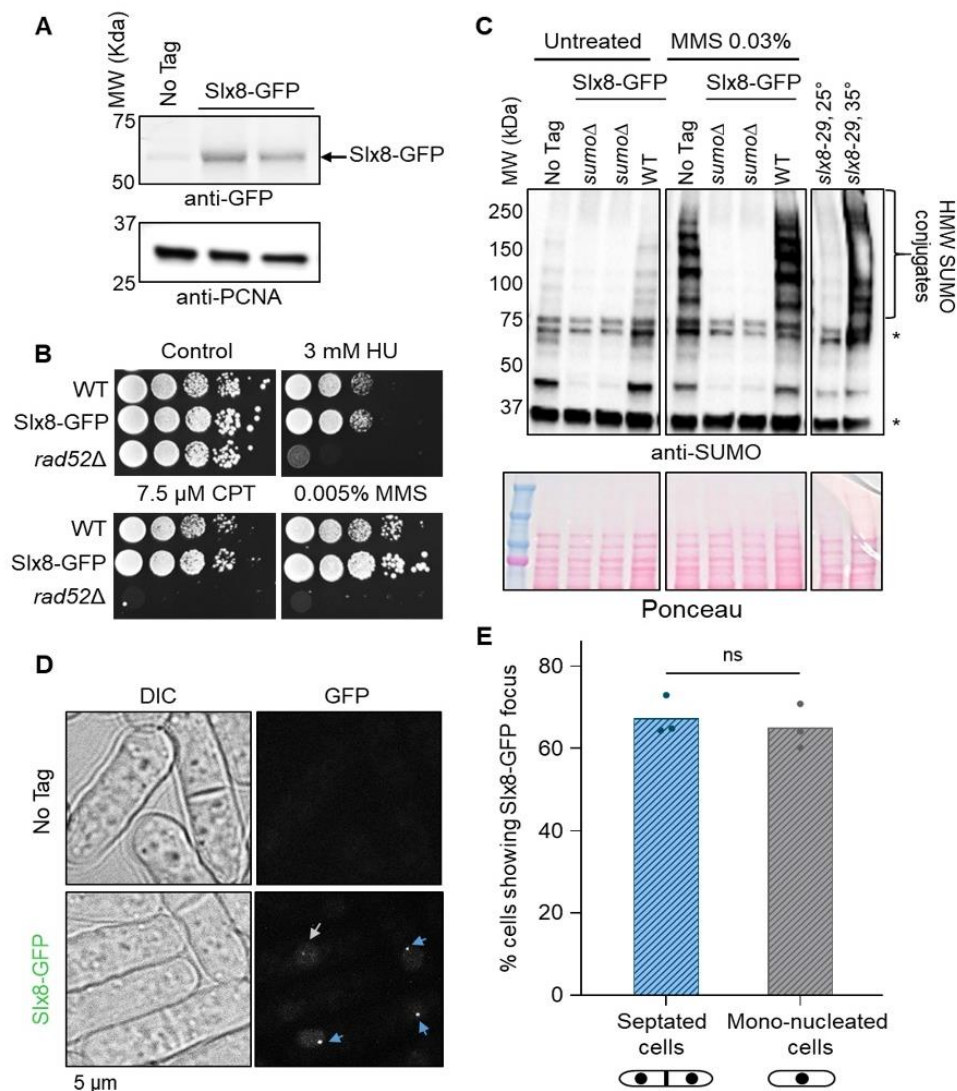
### **DATA AVAILABILITY**

All relevant data are available and further information and requests for reagents and resources should be directed to and will be fulfilled by Dr. Sarah A.E. Lambert ([sarah.lambert@curie.fr](mailto:sarah.lambert@curie.fr)).

## Results

### Slx8-GFP forms a single nuclear focus in a SUMO chain dependent manner

To investigate the spatial dynamics of SUMO conjugates prone to STUbL-dependent processing, Slx8 was C-terminally tagged with GFP, and Slx8-GFP functionality was established based on resistance to genotoxic stress (Figure 1A-B). To further confirm that the GFP tag did not interfere with Slx8 function, we analyzed global SUMO conjugates by immuno-blot. We observed an accumulation of high molecular weight (HMW) SUMO conjugates in the strain bearing the temperature-sensitive *slx8-29* allele when grown at the restrictive temperature (35°C), but not at the permissive temperature (25°C), indicating defective processing of SUMO conjugates in the absence of a functional Slx8 pathway, as expected (Figure 1C) (Nie et al., 2017). None of these HMW SUMO conjugates were detected in WT or Slx8-GFP expressing strains in untreated conditions, whereas they accumulated similarly in both strains upon cells



**Figure 1: Slx8-GFP forms a single focus in unstressed conditions.**

**A.** Expression of the endogenously GFP-tagged Slx8 fusion protein. An untagged WT strain (No Tag) was included as control for antibody specificity. PCNA was used as a loading control. Slx8-GFP has a molecular weight (MW) of 58 KDa. **B.** Sensitivity of indicated strains to indicated genotoxic drugs. Ten-fold serial dilution of exponential cultures were dropped on appropriate plates. HU: hydroxyurea; CPT: camptothecin and MMS: methyl methane sulfonate. **C.** Expression of SUMO conjugates in indicated strains and conditions. A strain deleted for *pmt3* gene that encodes the SUMO particle (*sumoΔ*) was added as control for antibody specificity. \* indicates unspecific signal. A strain bearing the temperature-sensitive allele *slx8-29* was grown at permissive (25°C) and restrictive (32°C) temperature. **D.** Example of bright-field (left panel, DIC) and GFP fluorescence (right panel) images of cells expressing the endogenous Slx8-GFP fusion protein in indicated strains. Blue and white arrows indicate Slx8-GFP foci in septated and mono-nucleated cells, respectively. Scale bar is 5 μm. **E.** Histogram plots showing the percentage of septated and mono-nucleated cells with nuclear Slx8-GFP foci. *p* value was calculated by two-tailed t test (ns: non-significant). Dots represent values obtained from three independent biological experiments. At least 200 nuclei were analyzed for each strain and cell type.

exposure to methyl methane sulfonate (MMS), an alkylating agent known to induce global SUMOylation (Figure 1C) (Nie et al., 2017). These results confirm that the Slx8-GFP fusion protein is functional. Then, we performed live cell imaging and observed that Slx8-GFP formed a single bright focus in most septated cells, which correspond to the bulk of S-phase, and mono-nucleated cells, which mainly correspond to G2 cells (Figure 1 D-E).

To address the link between this single Slx8-GFP focus and SUMO metabolism, we investigated the role of the two E3 SUMO ligases known in *S. pombe*: the SUMO chain-modified Pli1 and Nse2 (Andrews et al., 2005b; Steinacher et al., 2013b). We made use of point mutations in the RING domain of each protein to abolish the E3 SUMO ligase activity. Global SUMOylation was considerably reduced in cells expressing the mutated form Pli1-RING<sup>mut</sup>, compared to WT, and no MMS-induced SUMO conjugates were detected (Figure 2A), consistent with Pli1 being responsible for most of global SUMOylation. In contrast, the global level of SUMO-conjugates was unaffected in cells expressing the mutated form Nse2-RING<sup>mut</sup>, despite this mutation rendering cells sensitive to genotoxic agents (Figure 2A and Figure S1A). Of note, the combination of Slx8-GFP with either Pli1-RING<sup>mut</sup> or Nse2-RING<sup>mut</sup> did not aggravate the cell sensitivity to genotoxic agents, further confirming the functionality of Slx8-GFP (Figure S1A). Interestingly, the Slx8-GFP focus was less frequently observed in S and G2-phase of *pli1-RING<sup>mut</sup>* cells, whereas no differences were detected in *nse2-RING<sup>mut</sup>* cells, compared to WT (Figure 2B-C). Of note, the expression level of Slx8-GFP in *pli1-RING<sup>mut</sup>* and *nse2-RING<sup>mut</sup>* was similar to WT, excluding that the lack of Slx8-GFP focus resulted from an expression defect (Figure S1B-C). We were unable to address the potential overlapping role of Nse2 and Pli1 in promoting Slx8-GFP focus formation, since spores harboring both *pli1-RING<sup>mut</sup>* and *nse2-RING<sup>mut</sup>* alleles were unviable. We concluded

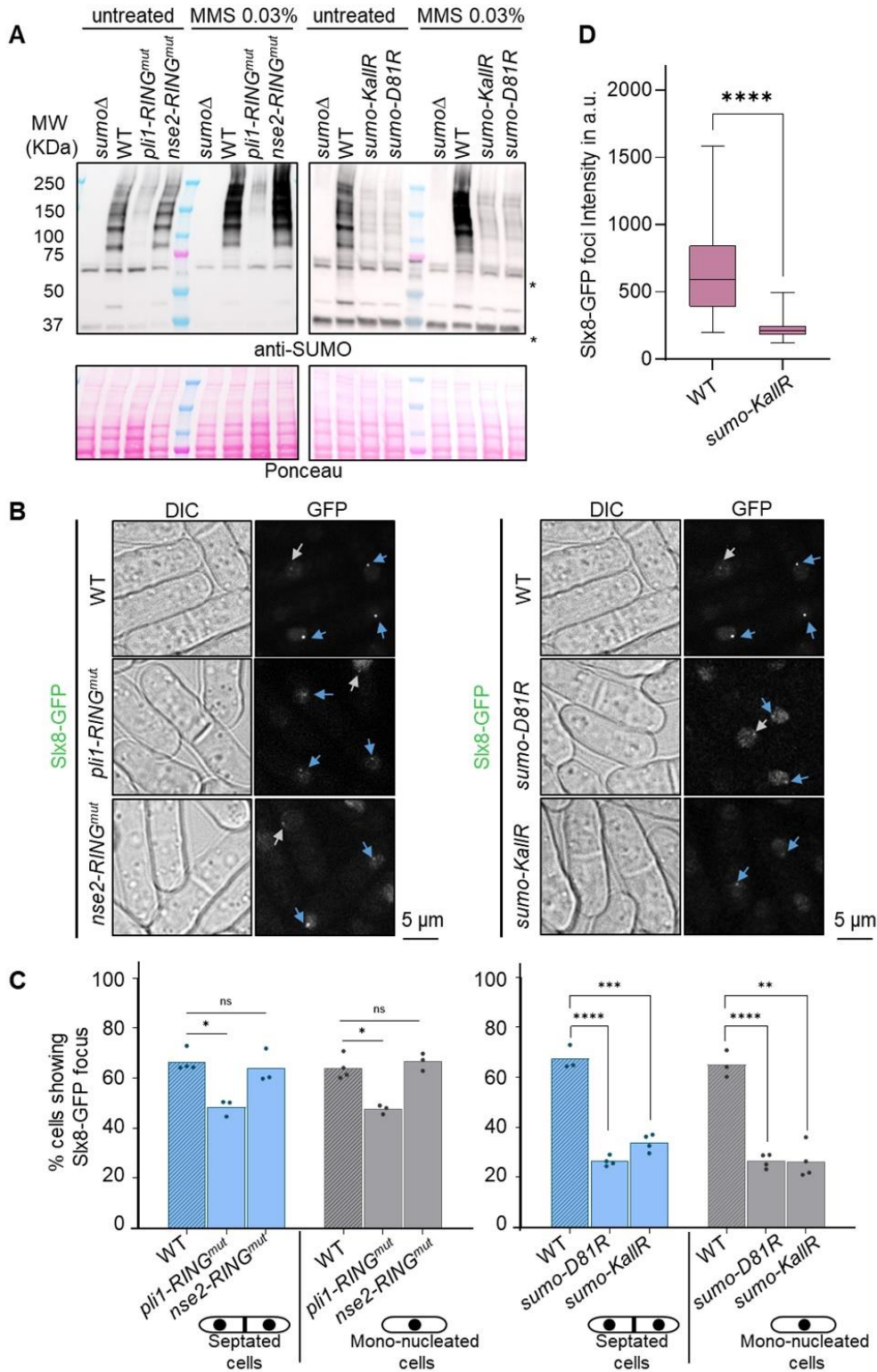
that the SUMO E3 ligase Pli1, that is responsible for global SUMOylation, sustains the formation of the single nuclear Slx8-GFP focus.

Next, we asked which type of SUMOylation contributes to the formation of the Slx8-GFP focus. We could not employ the strain harboring the deletion of the SUMO particle (*pmt3Δ*, here after SUMO $\Delta$ ), since this strain is extremely sick, showing frequent nuclear deformation. Instead, we employed a strain expressing SUMO-KallR, in which all internal lysine are mutated to arginine to prevent SUMO chain formation (Kramarz et al., 2020b) and a strain expressing SUMO-D81R that allows mono and di-SUMOylation to occur but impairs the chain-propagating role of Pli1 (Prudden et al., 2011a). As expected, global SUMOylation was massively reduced in strains expressing SUMO-KallR and SUMO-D81R, even upon MMS treatment, compared to WT (Figure 2A).

Consistently, the frequency of cells showing a single nuclear Slx8-GFP focus was reduced by almost two-thirds in SUMO-KallR and SUMO-D81R cells, compared to WT (Figure 2B-C), indicating that SUMO-chains are critical determinants of Slx8-GFP focus formation. Of note, Slx8-GFP expression level was only slightly reduced (by ~ 20%) in SUMO-D81R, an insufficient reduction to explain the lack of two-thirds of the foci (Figure S2). In addition to being less frequently formed, Slx8-GFP foci were three to four times less intense in SUMO-KallR cells, compared to WT (Figure 2D). We concluded that the formation of the single nuclear Slx8-GFP focus requires SUMO chain formation and the SUMO-chain modified E3 ligase Pli1, suggesting that it marks SUMO conjugates at specific nuclear regions.

### **Slx8-GFP does not form supernumerary foci in response to replication stress**

Having established that Slx8-GFP marks specific nuclear regions in a SUMO-dependent manner, we investigated if Slx8-GFP forms DNA damage-induced foci, as reported for ScSlx5 (Cook et al., 2009b). Treatment with MMS, but not with hydroxyurea (HU, an inhibitor of the ribonucleotide reductase leading to a depletion of the dNTP pool and stalled replication fork), or camptothecin (CPT, an inhibitor of the topoisomerase I leading to collapsed replication fork), resulted in a marked accumulation of SUMO conjugates (Figure 3A). Whatever the replication-blocking agent used, no additional DNA damage-induced Slx8 foci could be detected in our microscopy setup on living cells, even in condition of MMS-induced accumulation of SUMO conjugates (Figure 3A-C).



**Figure 2: SUMOylation promotes the formation of Slx8-GFP foci.**

**A.** Expression of SUMO conjugates in indicated strains (expressing Slx8-GFP) and conditions. A strain deleted for *pmt3* gene that encodes the SUMO particle (*sumoΔ*) was added as control for antibody specificity. \* indicates unspecific signal. **B.** Example of bright-field (DIC) and GFP fluorescence images in indicated strains expressing Slx8-GFP. Blue and white arrows indicate Slx8-GFP foci in septated and

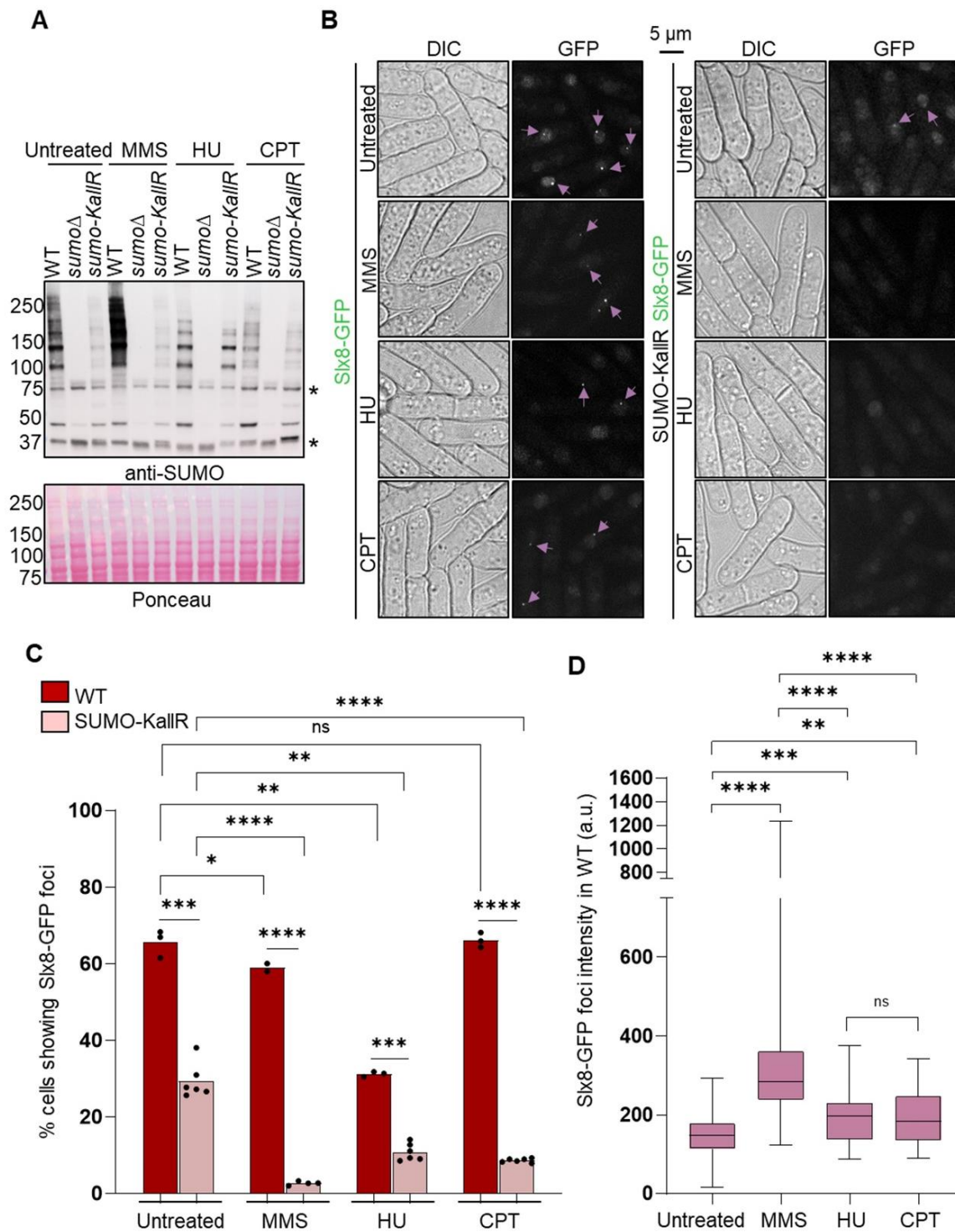
mono-nucleated cells, respectively. Scale bar is 5  $\mu\text{m}$ . **C.** Histogram plots showing the percentage of septated and mono-nucleated cells with nuclear Slx8-GFP foci in indicated strains.  $p$  value was calculated by two-tailed t test (\*\*\*\*  $p \leq 0.0001$ ; \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; ns: non-significant). Dots represent values obtained from independent biological experiments. At least 200 nuclei were analyzed for each strain and cell type. **D.** Box-and-whisker plots of Slx8-GFP intensity (mean fluorescence intensity) in indicated strains. Boxes represent the 25/75 percentile, black lines indicate the median, the whiskers indicate the 5/95 percentile.  $p$  value was calculated by Mann-Whitney U test (\*\*\*\*  $p \leq 0.0001$ ). Values were obtained from at least two independent biological experiments. At least 60 nuclei were analyzed for each strain.

Surprisingly, HU treatment resulted in a 50% reduction in cells showing a single Slx8 focus in WT cells. It's worth noting that, despite the absence of supernumerary Slx8-GFP foci, the intensity of the single Slx8-GFP focus increased significantly upon exposure to genotoxic stresses, particularly after MMS treatment, compared with the untreated condition (Figure 3D). Furthermore, the frequency of cells showing a single Slx8-GFP focus was severely reduced in SUMO-KallR cells after treatment with replication blocking agents (Figure 3B-C) suggesting that SUMO chains become more critical for maintaining the Slx8 GFP focus under replication stress conditions. Although we observed a slight decrease in Slx8-GFP expression in WT and SUMO-KallR cells in response to treatments (Figure S3), the extent of variation seems insufficient to explain the disappearance of Slx8-GFP foci. We concluded that Slx8-GFP cannot serve as a readout of damage-induced SUMO chain formation but that the behavior of the single Slx8-GFP focus is modulated by replication stress in a SUMO-chain dependent manner.

### **The single nuclear Slx8-GFP focus marks centromere and the *mat* region at the nuclear periphery**

The analysis of cell images revealed that the single Slx8-GFP focus in untreated condition was often positioned at the periphery of the nucleus. To confirm this, we asked how frequently Slx8-GFP foci co-localize with Cut11-mCherry, a component of the NPC that marks the NP. We found that the nuclear Slx8-GFP focus, where visible, was positioned at the NP in  $\sim 65\%$  of WT S-phase cells (septated cells) and this frequency dropped to the peripheral Slx8-GFP foci 35% in WT G2 cells (mono-nucleated cells) (Figure 4A-B). Interestingly, this peripheral nuclear positioning in S-phase dropped to  $\sim 35\%$  in cells expressing SUMO-KallR. We concluded that most Slx8 foci are enriched at the NP and that SUMO chains contribute both to Slx8-GFP focus formation and positioning at the NP during S-phase.

The peripheral nuclear location of the single Slx8-GFP focus suggests that Slx8 associates with specific components and/or chromosomal regions known to be at the



**Figure 3: Genotoxic stress does not lead to supernumerary Slx8-GFP foci.**

**A.** Expression of SUMO conjugates in indicated strains (expressing Slx8-GFP) and conditions. A strain deleted for *pmt3* gene that encodes the SUMO particle (*sumo* $\Delta$ ) was added as control for antibody specificity. \* indicates unspecific signal. Strains were treated with genotoxic drugs before the extraction of proteins. HU: hydroxyurea (20 mM, 4 hours); CPT: camptothecin (40  $\mu$ M, 4 hours) and MMS: methyl methane sulfonate (0.03%, 3 hours). **B.** Example of bright-field (DIC) and GFP fluorescence (panel) images in indicated strains and conditions. Genotoxic stresses were generated as in A. Pink arrows indicate cells harboring nuclear Slx8-GFP foci. Scale bar is 5  $\mu$ m. **C.** Histogram plots showing the percentage of cells



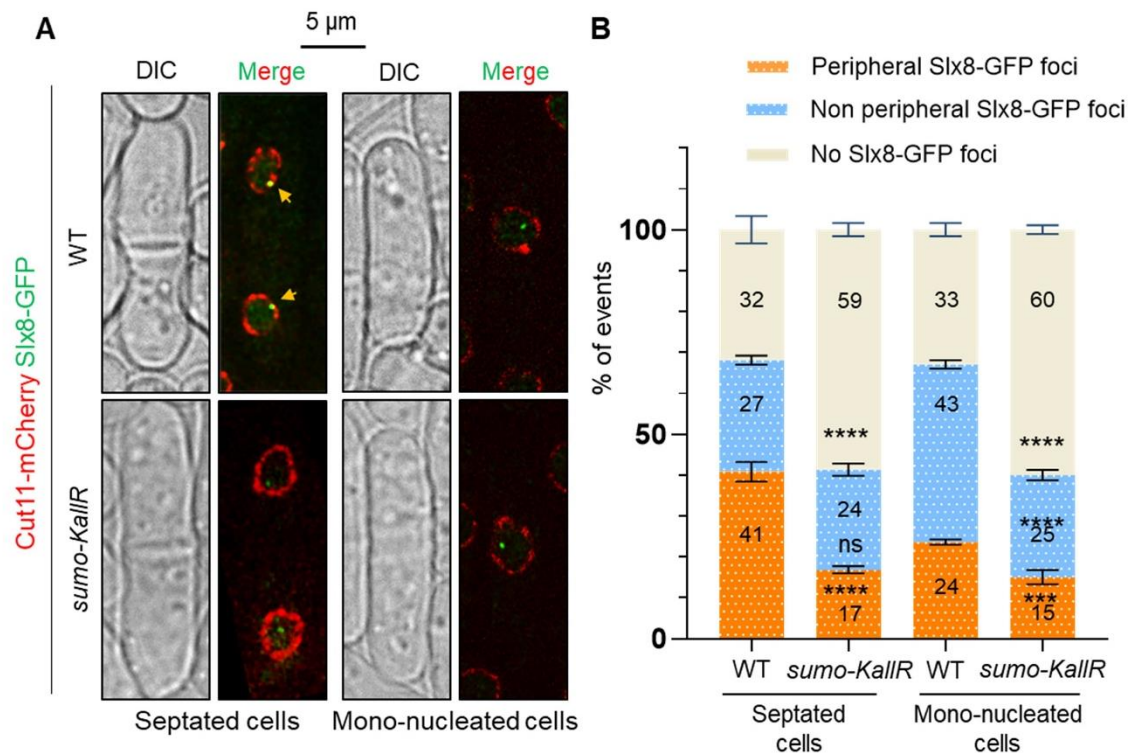
with nuclear Slx8-GFP foci in indicated strains and conditions.  $p$  value was calculated by two-tailed t test (\*\*\*\*  $p \leq 0.0001$ ; \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; ns: non-significant). Dots represent values obtained from two independent biological experiments. At least 200 nuclei were analyzed for each strain and treatment condition. **D.** Box-and-whisker plots of Slx8-GFP intensity (mean fluorescence intensity) in indicated strains and conditions. Boxes represent the 25/75 percentile, black lines indicate the median, the whiskers indicate the 5/95 percentile.  $p$  value was calculated by Mann-Whitney U test (\*\*\*\*  $p \leq 0.0001$ ; \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ). Values were obtained from two independent biological experiments. At least 60 nuclei were analyzed for each strain and treatment condition.

NP. During interphase, the *S. pombe* chromosomes are arranged in a Rab1-like configuration in which the three centromeres are clustered adjacent to the SPB embedded in the NE, while telomeres form discrete foci clustered at the NP at the opposing hemisphere of the nucleus (Mizuguchi et al., 2015b). In addition, the heterochromatin domain of the sexual mating locus (hereafter *mat* region), that contains the silent *mat2* and *mat3* loci, is also positioned at the NP nearby the SPB. We thus addressed if Slx8-GFP localizes with markers of centromere (Mis6-RFP, a kinetochore component), SPB (Sid4-RFP) and telomere (Taz1-RFP) and the *mat* region (using a strain harboring a *LacO* array integrated nearby the *mat* locus, bound by the fluorescent repressor LacI-mCherry) (Figure 5A). During S-phase (in septated cells), the nuclear Slx8-GFP focus co-localized with Sid4-RFP and Mis6-RFP in ~ 60 % of cells showing a Slx8-GFP focus, whereas a co-localization event with the *mat* region was observed in ~ 20 % of the cells (Figure 5B). Such nuclear positioning appeared highly significant compared to random co-localization events. Although less pronounced, the Slx8-GFP focus significantly overlapped with centromere, SPB and *mat* region in G2 cells (mono-nucleated cells). In contrast to the Slx8-GFP focus, all cells exhibited a single Sid4-RFP and Mis6-RFP focus, or a single LacI-mcherry dot marking the *mat* region (Figure 5A). We found that centromere and SPB are positively associated with Slx8 in 40 % of S-phase cells and in 20% of G2 cells, whereas the *mat* region associated with Slx8 in ~ 15-18 % of S and G2-phase cells (Figure 5C). In contrast, no co-localization above random events were detected between Slx8-GFP and Taz1-marked telomeres foci. We concluded that for the most part, the Slx8-GFP focus positioned at the NP marks clustered centromeres, the SPB and the *mat* region.

### **Heterochromatin and centromeres clustering at SPB sustain Slx8-GFP focus formation.**

Slx8-GFP marks the SPB environment and associated chromosomal regions such as centromeres and *mat* region, both being enriched for heterochromatin that ensures gene silencing. Therefore, we asked if heterochromatin formation and centromere

clustering are required to ensure the formation of a single Slx8-GFP focus. We observed that in the absence of *Clr4*, the histone methyl-transferase that promotes H3-K9 methylation, a hallmark of heterochromatin and gene silencing (Nakayama et al., 2001b; Rea et al., 2000), the frequency of Slx8-GFP focus formation was reduced by two fold (Figure 6A-B). In contrast, no effect was observed in the absence of Dicer (*Dcr1*), a component of the RNAi machinery promoting the establishment of heterochromatin, but with only a partial role in maintenance. These results indicate that H3K9 methylation, but not RNAi, is required to promote the formation of the nuclear Slx8-GFP focus. We also investigated the role of centromere clustering. *Csi1* is a key factor that provides a physical link between kinetochores and SPB associated proteins. The lack of *Csi1* leads to a severe defect in centromere clustering (Hou et al., 2012b) and resulted in a 2 fold reduction in the frequency of the Slx8-GFP focus (Figure 6A-B). Of note, the expression of Slx8-GFP was not affected in the absence of *Csi1*, *Clr4* or *Dcr1*, indicating that the decreased in the frequency of Slx8-GFP foci is not caused by variation in expression level (Figure S4).



**Figure 4: Slx8-GFP focus is enriched at the nuclear periphery.**

**A.** Representative cell images of cells expressing Cut11-mCherry (red) and Slx8-GFP (green) in septated and mono-nucleated cells of indicated strains. The nuclear periphery is visualized via Cut11-mCherry. Yellow arrows indicate co-localization events. Scale bar is 5  $\mu$ m. **B.** Stacked bar charts showing the frequency of co-localization between Slx8-GFP and Cut11-mCherry in septated and mono-nucleated cells of indicated strains. Individual bars represent 100% of events and numbers indicate the % of each category (peripheral Slx8-GFP foci co-localizing with Cut11-mCherry in orange, non-peripheral Slx8-GFP

foci in blue, absence of Slx8-GFP foci in cream-white).  $p$  value was calculated by two-tailed t-test (\*\*\*\*  $p \leq 0.0001$ ; \*\*\*  $p \leq 0.001$ ; ns: non-significant). Bars indicate mean values  $\pm$  Standard deviation (SD). Values were obtained from two independent biological experiments. At least 200 nuclei were analyzed for each strain and cell type.

Thus, both heterochromatin formation and centromere clustering contribute to Slx8-GFP focus formation.

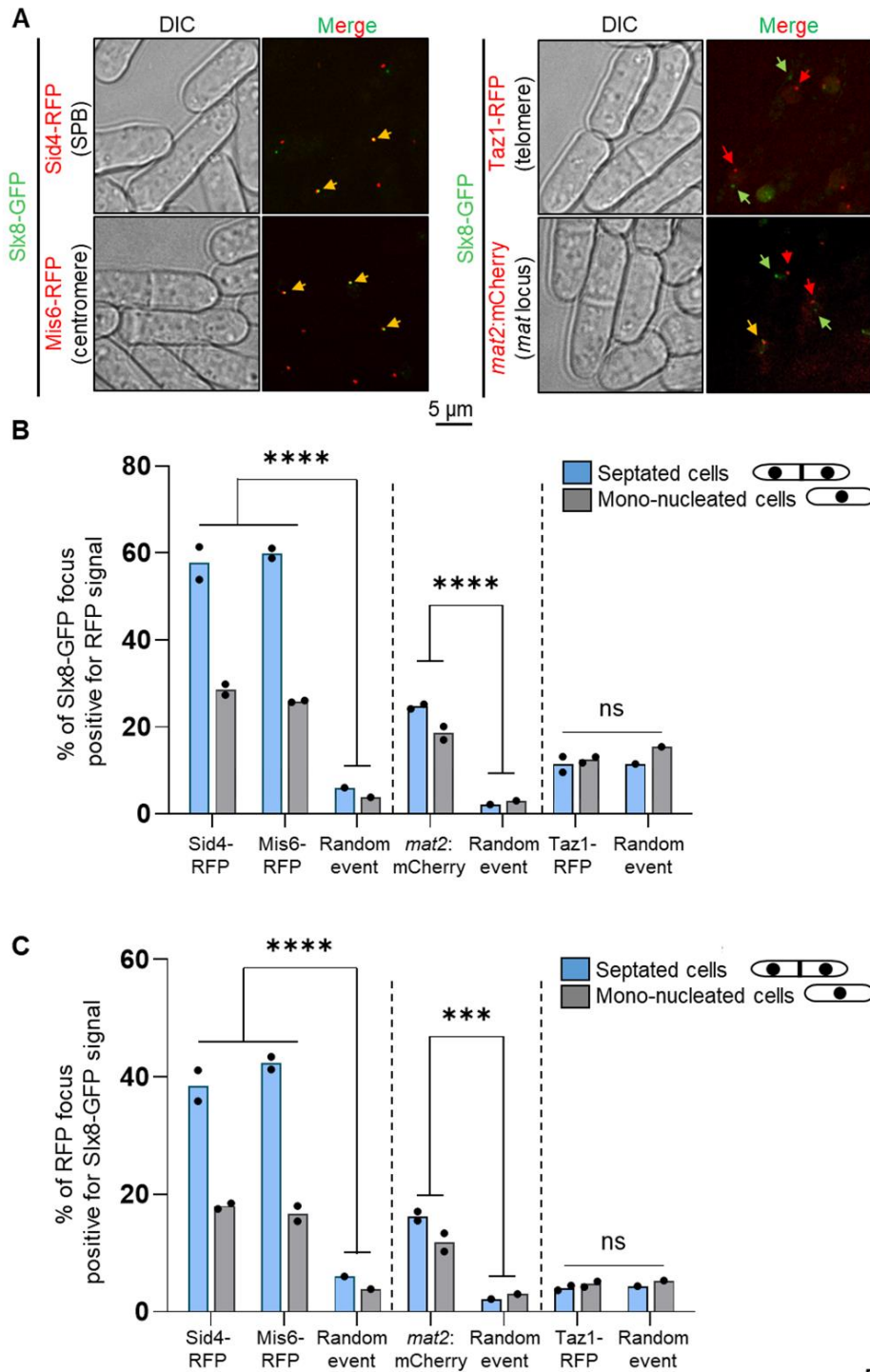
### **Slx8 promotes centromere clustering and gene silencing**

Finally, we tested whether Slx8 functions to promote heterochromatic silencing and centromere clustering. To assess silencing, we performed RT-qPCR analysis of transcripts from the heterochromatic pericentromere (*cen[*dg*]*) and silent mating-type (*mat*) regions. Such transcripts accumulate at very low levels in wild-type cells, but much higher levels in absence of factors such as Clr4 required for heterochromatin assembly. Interestingly, we also observed a small but significant increase in accumulation of transcripts from both the pericentromere and the *mat* locus in cells lacking Slx8, consistent with Slx8 functionally contributing to silencing in these regions (Figure 7A). To assess centromere clustering, we performed live-cell imaging on cells expressing GFP-Cnp1 (*S. pombe* CENP-A, the centromere-specific histone variant) to visualise centromeres, together with Sid4-RFP as a marker of the SPB.

Whereas wild-type cells consistently display a single GFP-Cnp1 focus, representing three clustered centromeres, adjacent to the SPB, absence of Csi1 results in ~35% of cells showing more than one GFP-Cnp1 focus, indicative of defective clustering. Strikingly, the lack of Slx8 also resulted in a significant clustering defect, with ~12% of cells displaying more than one GFP-Cnp1 focus (Figure 7B-C). An epistatic phenotype was seen for *slx8* $\Delta$  *csi1* $\Delta$  double mutant cells, which displayed clustering defects comparable to those in the *csi1* $\Delta$  single mutant, suggesting that Slx8 may function in the same pathway as Csi1. Deletion of the SUMO ligase Pli1 largely suppressed the defect associated with absence of Slx8, consistent with it arising as a result of excess SUMOylation. We conclude that localization of Slx8 in the vicinity of the SPB both depends on, and contributes to, heterochromatin integrity and centromere clustering.

### **Discussion**

STuBL proteins play diverse roles throughout the cell cycle to protect against genome instability. Here, we revealed that the fission yeast STuBL Slx8 functions mainly in the SPB environment in a SUMO-dependent manner to help ensure centromere clustering and gene silencing at heterochromatic domains. These data are consistent with



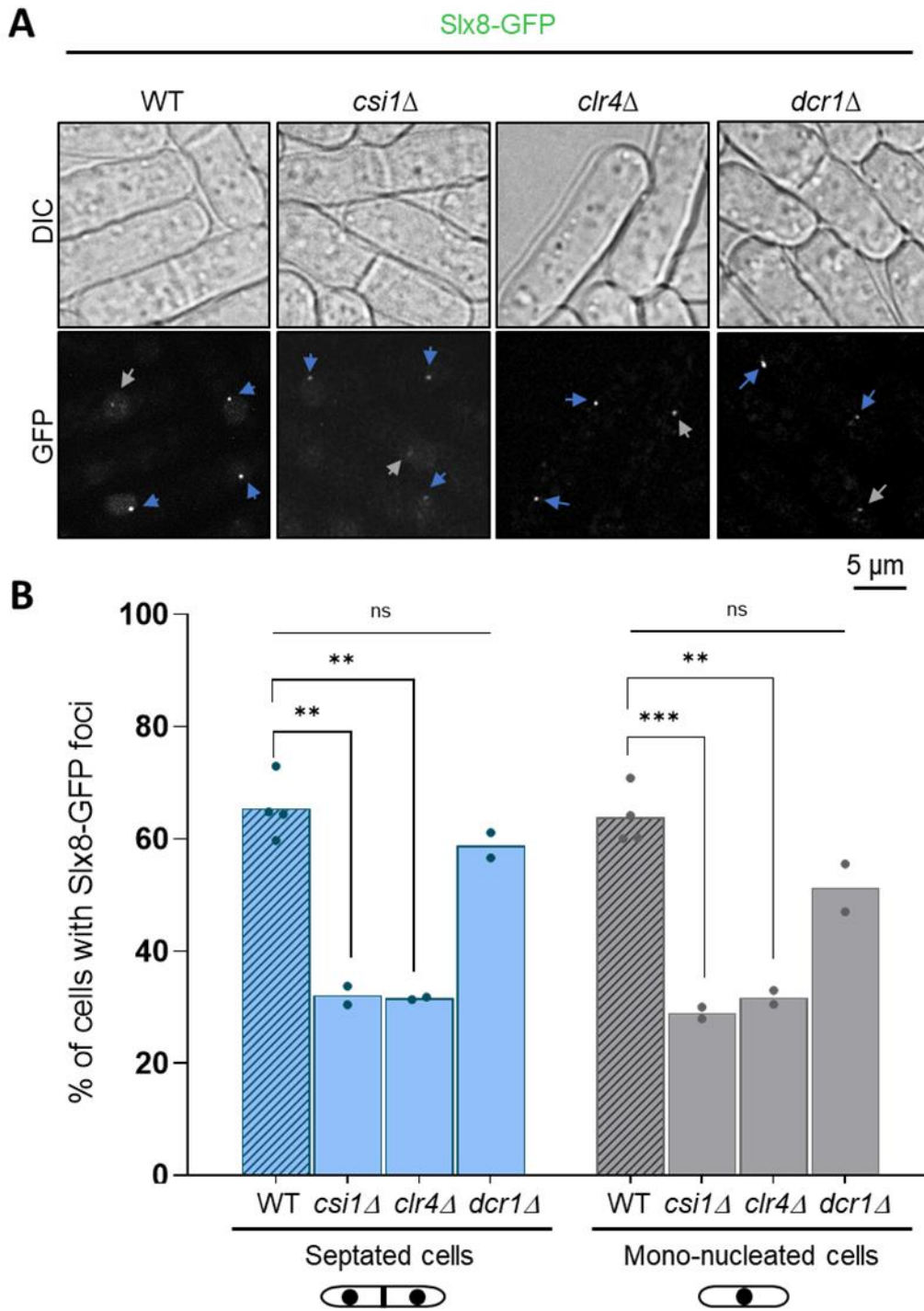
**Figure 5: Slx8-GFP focus marks the SPB, centromere and the mating type locus.**

**A.** Representative cell images of strains expressing Slx8-GFP and either Sid4-RFP (a SPB marker) or Mis6-RFP (a kinetochore marker) or Taz1-RFP (a telomere marker), or harboring the endogenous *mat2* locus tagged with a LacO arrays bound by Lacl-Mcherry (*mat2:mCherry*). Red, green and yellow arrows indicate RFP, GFP and co-localization events, respectively. Scale bar is 5  $\mu$ m. **B & C.** Histogram plots showing the percentage of co-localization events between Slx8-GFP and the above described markers. *p* value was calculated by Brown-Forsythe and Welch ANOVA test (\*\*\*\*  $p \leq 0.0001$ ; \*\*\*  $p \leq 0.001$ ; ns: non-significant). Dots represent values obtained from two independent biological experiments. At least 200 nuclei were analyzed for each strain and cell type.

SUMOylation of centromeres being an important mediator of centromere identity, and indicate that Slx8 plays a critical role in regulating SUMO homeostasis in the nuclear space to safeguard centromere biology.

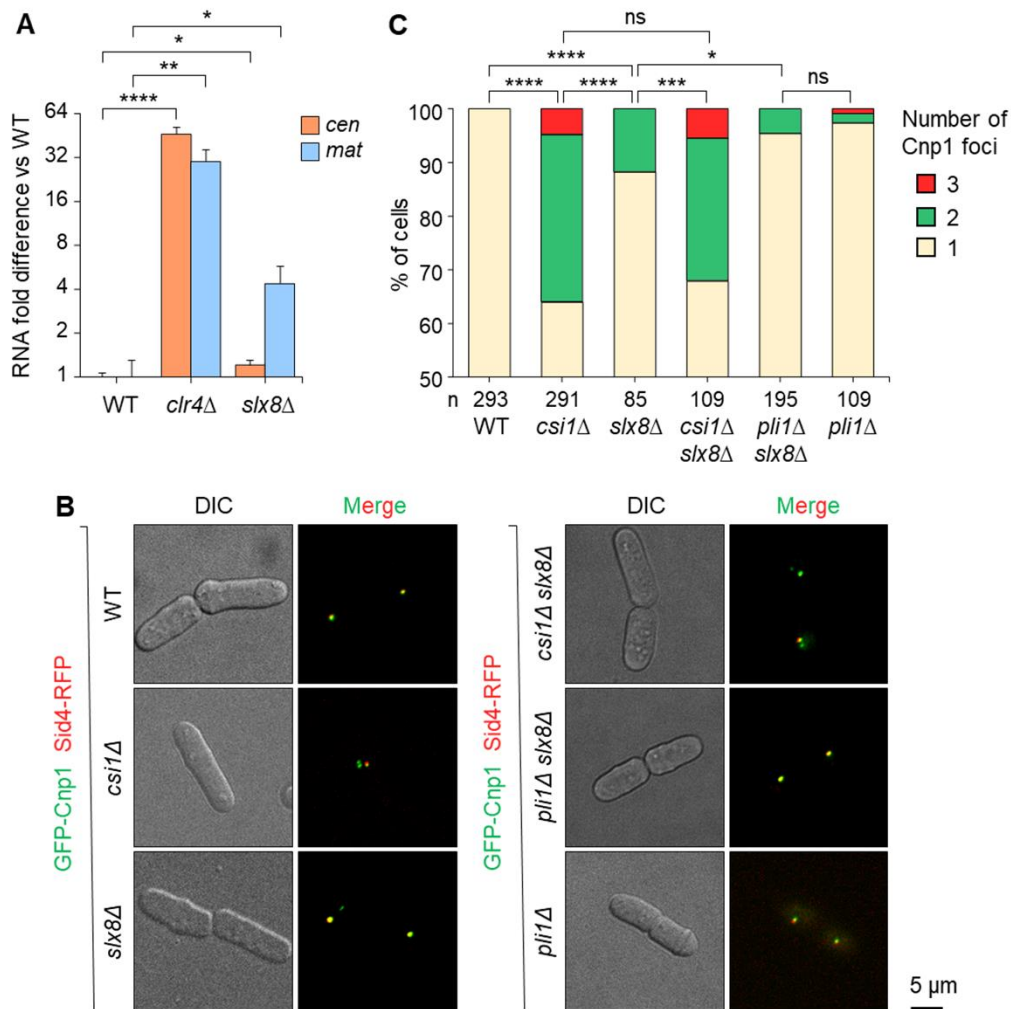
In several organisms, STUbL activities are linked to the maintenance of genome stability and resistance to DNA damage (reviewed in (Y. C. Chang et al., 2021)). In *S. pombe*, Slx8 operates with Ufd1, a component of the Cdc48-Ufd1-Npl4 that allows the extraction of ubiquitylated proteins from higher-order complexes (Køhler et al., 2013). Both physical and functional overlaps between Ufd1 and Slx8 have revealed that Slx8 helps in channeling SUMOylated proteins towards such extraction process. This mechanism is part of the DNA damage response as Ufd1 forms DNA damage-induced foci, co-localizing with SUMO at the nuclear periphery. Similarly, ScSlx5 forms damage-induced nuclear foci in a SUMO-dependent manner, and co-localizing with DNA repair factors (Cook et al., 2009a). Therefore, it was unanticipated that DNA damage does not lead to a redistribution of Slx8 to form specific DNA repair-associated foci. One possibility is that the amount of Slx8 recruited at site of DNA damage is below the level of detection offered in our cell microscopy condition. Alternatively, DNA-damage induced Slx8 foci are dynamic and rapidly moving to the SPB, leading to the increased foci intensity that we observed without increasing foci number.

Our observation of a Slx8 focus co-localizing with centromeres is consistent with several previous studies indicating that STUbLs reside and function at centromeres. In budding yeast, genome-wide binding analyses revealed centromeric enrichment of Slx5, but not of Slx8, and cells lacking Slx5 or Slx8 display chromosome segregation defects (van de Pasch et al., 2013). Indeed, Slx5-Slx8 STUbL activity has been shown to be required for degradation of several centromere-associated substrates including cohesin subunit Mcd1 (D'Ambrosio & Lavoie, 2014), chromosome passenger complex (CPC) components Bir1 and Sli15 (Thu et al., 2016), and centromere-specific histone H3 variant Cse4<sup>CENP-A</sup> (Cheng et al., 2017; Ohkuni et al., 2018), thereby promoting the proper specification and function of centromeres. Similarly, mammalian RNF4 has been implicated in regulating centromere and kinetochore assembly, functioning antagonistically with SUMO protease SENP6 to modulate levels of CENP-A assembly factor Mis18BP1 (Fu et al., 2019; Liebelt et al., 2019) and inner kinetochore protein CENP-I (Mukhopadhyay et al., 2010). In *S. pombe*, it has been reported previously that loss of Slx8 results in chromosome segregation defects, dependent on the SUMO ligase Pli1 (Steinacher et al., 2013b); our findings that absence of Slx8 is associated with defects in both heterochromatic silencing and centromere clustering point to multifaceted roles of STUbL activity in supporting normal centromere function.



**Figure 6: Heterochromatin and centromere clustering promote Slx8-GFP focus formation**

**A.** Example of bright-field (top panel, DIC) and GFP fluorescence (bottom panel) images of cells expressing Slx8-GFP in indicated strains. Blue and white arrows indicate Slx8-GFP foci in septated and mono-nucleated cells, respectively. Scale bar is 5  $\mu$ m. **B.** Histogram plots showing the percentage of septated and mono-nucleated cells with nuclear Slx8-GFP foci.  $p$  value was calculated by two-tailed t test (\*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ; ns: non-significant). Dots represent values obtained from two independent biological experiments. At least 200 nuclei were analyzed for each strain and cell type.



### Figure 7: *Slx8* promotes heterochromatic silencing and centromere clustering

**A.** RT-qPCR analysis of pericentromere (*cen[dg]*) and mating type locus (*mat*) transcript levels, normalized to control transcript *act1*<sup>+</sup>. Data are plotted as fold difference relative to wild-type, on log<sub>2</sub> scale. *p* value was calculated by two-tailed t test (\*\*\*\* *p* ≤ 0.0001; \*\* *p* ≤ 0.01; ns: non-significant). Values are mean ± s.d. from three independent experiments. **B.** Representative images of cells expressing GFP-Cnp1 (centromere marker) and Sid4-RFP (SPB marker). Scale bar is 5 μm. **C.** Stacked bar charts showing the percentage of cells displaying one, two or three Cnp1 foci, based on analysis of *n* nuclei. *p* value was calculated by chi-squared test (\*\*\*) *p* ≤ 0.001; \*\*\*\* *p* ≤ 0.0001; ns: non-significant).

Heterochromatin is a key structural and regulatory component of centromeres in most eukaryotes, functioning to promote accurate chromosome segregation and silence repetitive DNA elements. Perturbation of SUMOylation has been linked to defects in heterochromatic silencing in several systems, including flies (Ninova et al., 2020), mammals (Marshall et al., 2010), and *S. pombe* (Shin, Eun, et al., 2005). Moreover, large-scale studies in various organisms have identified heterochromatic regions including centromeres as SUMOylation hotspots (Cubeñas-Potts & Matunis, 2013; Ninova et al., 2023). Indeed, in *S. pombe*, proteomic analyses revealed that more than a third of

SUMOylated proteins regulated by Slx8 and Ufd1 are proteins associated with centromeres or telomeres, including key heterochromatin regulators, the H3K9 methyltransferase Clr4 and anti-silencing factor Epe1 (Køhler et al., 2015b). How SUMOylation impacts the function of these specific proteins is yet to be established. However, it has previously been shown that Epe1 is subject to ubiquitin-dependent cleavage and degradation that regulates its activity within heterochromatin domains (Braun et al., 2011) and in response to stress (Yaseen et al., 2022); it is tempting to speculate that Slx8 STUbL activity might contribute to Epe1 ubiquitination, and therefore that alleviation of heterochromatic silencing in *slx8Δ* cells could potentially be attributable, at least in part, to increased Epe1 activity. Since the localization of potential substrates such as Epe1 and Clr4 at centromeres and the *mat* locus is heterochromatin-dependent (Isaac et al., 2007; Zofall & Grewal, 2006), this could help explain why Slx8 association with these regions is both dependent on, and required for, proper heterochromatin maintenance.

The phenomenon of centromere clustering has been observed in many eukaryotes and also appears to be important for normal centromere function, although the underlying mechanisms are not yet fully understood. In fission yeast, Csi1 plays an important role in tethering kinetochores to the SPB, and loss of this protein results in centromere declustering (Hou et al., 2012b). We previously uncovered a role for SUMOylation in enhancing centromere clustering in conditions where Csi1 is absent, since removal of nucleoporin Nup132, which tethers SUMO protease Ulp1 to the NP, causes a SUMO-dependent rescue of clustering in *csi1Δ* cells (Strachan et al., 2023b). This effect was found to be dependent on SUMOylation of the inner nuclear membrane protein Lem2, which acts in parallel with Csi1 to promote clustering, but independent of Slx8 activity. In contrast, here we show that Slx8 is required to maintain proper centromere clustering in otherwise wild-type cells. The relevant substrate(s) in this case are yet to be determined; however, our genetic data suggests that substrate(s) likely lie in the same pathway as Csi1, and therefore could potentially include, for example, Csi1 itself, or the interacting NE protein Sad1, both of which have been shown to be subject to SUMOylation (Køhler et al., 2015b). In principle, Slx8 activity may be required either to temper the accumulation of SUMOylated proteins, or to actively promote protein extraction/turnover in a SUMO-dependent manner. However, we have shown previously that loss of the SUMO ligase Pli1 has only minimal effect on centromere clustering in this background (~2.5% of *pli1Δ* cells displaying declustering, as compared to ~12% of *slx8Δ* cells), whereas we confirm here that the clustering defects associated with absence of Slx8 are largely suppressed upon removal of Pli1. Thus, it is likely that Slx8 is primarily required to prevent the detrimental excess accumulation of



SUMOylated substrates, and therefore to help maintain an optimal balance of SUMOylation needed to support normal centromere clustering.

SUMOylation has been found to influence the dynamics of telomere maintenance in *S. pombe* by controlling the activity of positive or negative regulators of telomerase (Xhemalce et al., 2004a, 2007). In our microscopy analysis, we could not detect association between Slx8 and Taz1-marked telomeres. It is worth noting that, in budding yeast, telomeric factors are enriched for SUMO modifications upon telomere erosion (in the absence of telomerase), resulting in Slx5/Slx8-mediated relocation to the NPC to promote telomere length maintenance (Churikov et al., 2016a). Therefore, it is possible that, without stress-inducing SUMOylation at telomeres, the association between Slx8 and telomere is below limit of detection. Nonetheless, our results highlight that, in unchallenged conditions, Slx8 mainly acts at heterochromatic domains and the centromere to orchestrate the nuclear organization and functions of these specific domains.

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## **Author contributions**

S.C., J.S., K.S., E.V., K.F, H.Z. and N.Z. performed the experiments.

S.C., J.S., K.S., E.H.B. and S.A.E.L contributed to experimental design and data analysis.

L.B. provided expertise to perform and analyze cell imaging.

E.H.B. and S.A.E.L wrote the manuscript.

## **Declaration of interests**

The authors declare no competing interests.

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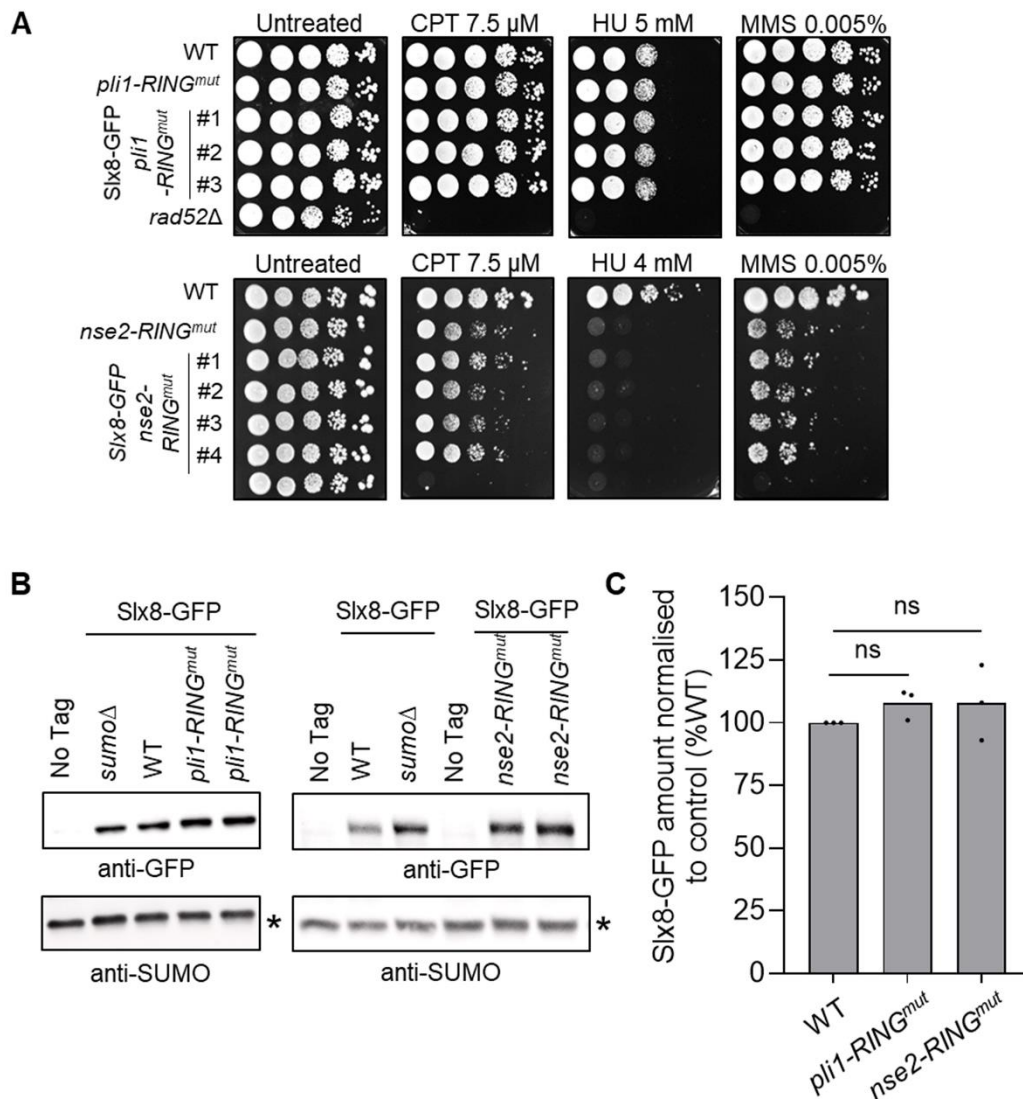
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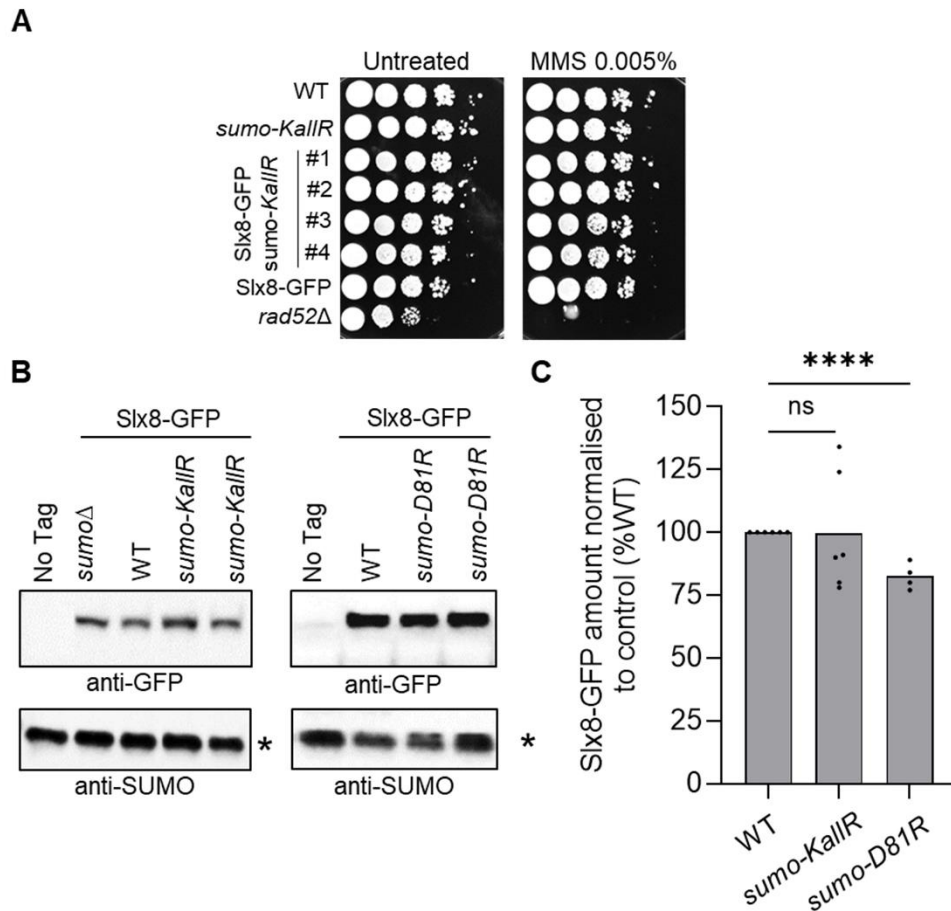


## Supplemental Information



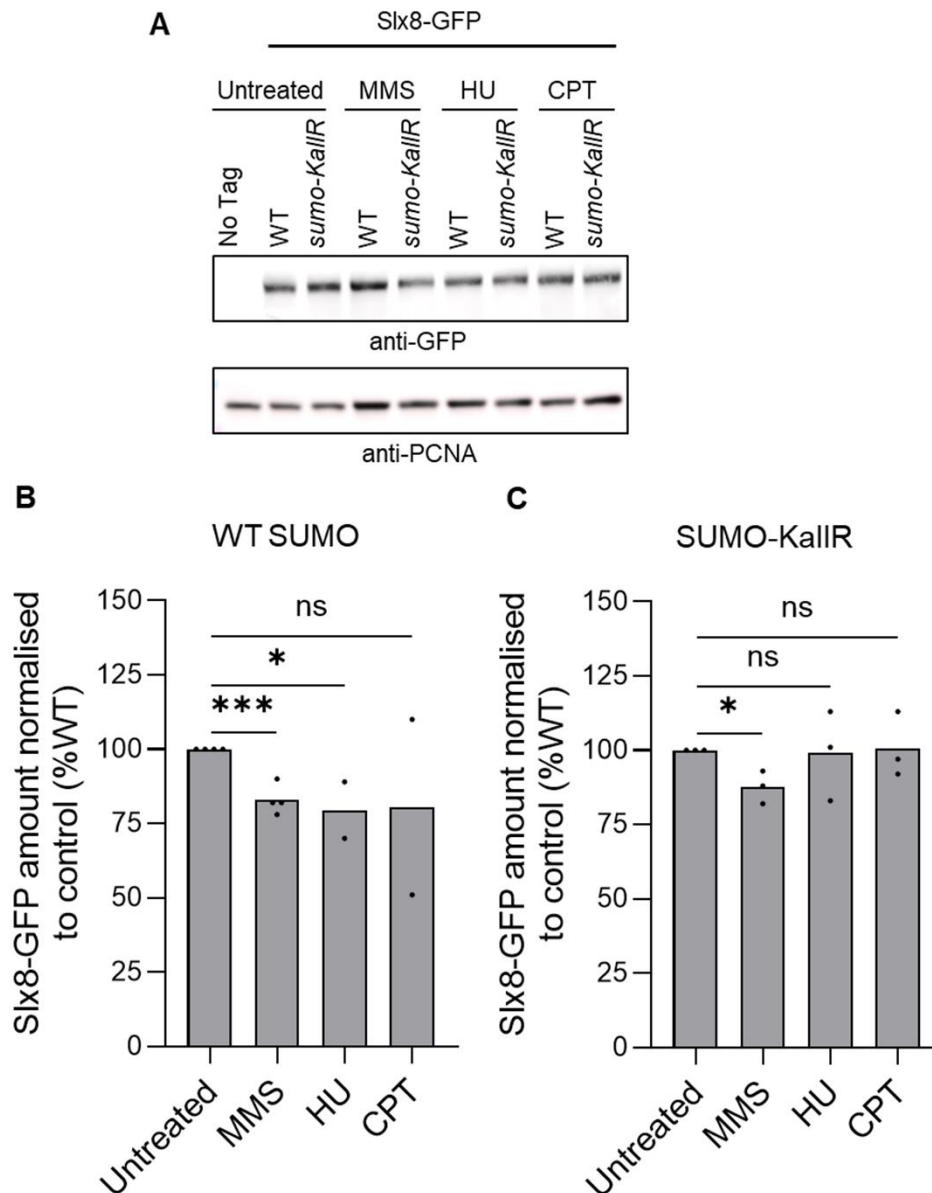
**Figure S1: Expression of Slx8-GFP is not affected by the absence of the E3 SUMO ligase activity of either Nse2 or Pli1.**

**A.** Sensitivity of indicated strains to genotoxic drugs. Ten-fold serial dilution of exponential cultures were dropped onto indicated plates. HU: hydroxyurea; CPT: camptothecin and MMS: methyl methane sulfonate. **B.** Expression of Slx8-GFP in indicated strains. An untagged WT strain (No Tag) was included as control for antibody specificity. An unspecific band (\*) from SUMO-blot was used as a loading control. **C.** Quantification of Slx8-GFP expression in indicated strains. Dots represent values obtained from independent biological experiments. The normalized amount of Slx8 was calculated by dividing the GFP signal by unspecific SUMO signal. The normalized amount of Slx8-GFP in mutants was indicated as a percentage of WT. *p* value was calculated by two-sided Fisher's exact test (ns: non-significant).



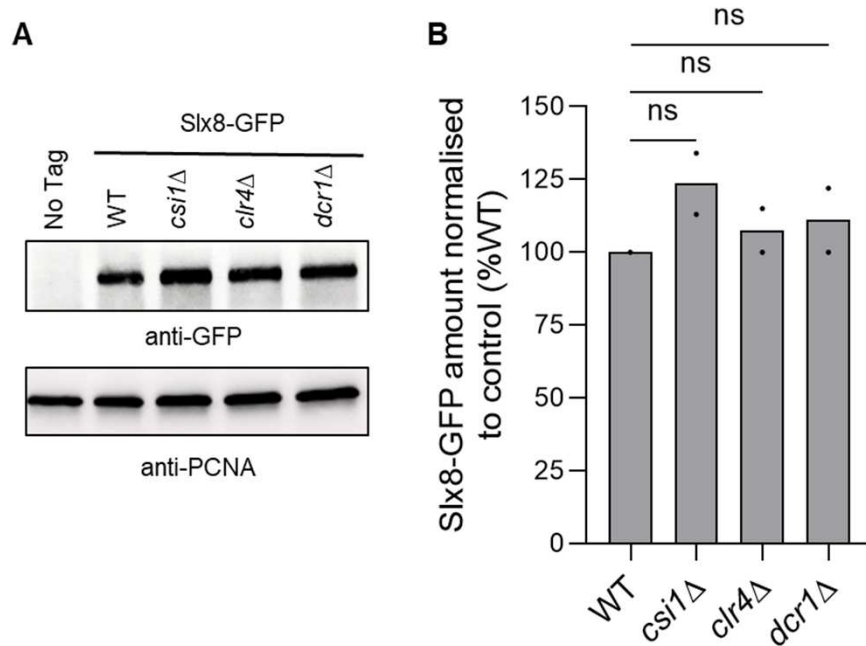
**Figure S2: Expression of Slx8-GFP is not affected in strains expressing SUMO-D81R or SUMO-KallR.**

**A.** Sensitivity of indicated strains to genotoxic drugs. Ten-fold serial dilution of exponential cultures were dropped onto indicated plates. MMS: methyl methane sulfonate. **B.** Expression of Slx8-GFP in indicated strains. An untagged WT strain (No Tag) was included as control for antibody specificity. An unspecific band (\*) from SUMO-blots was used as a loading control. **C.** Quantification of Slx8-GFP expression in indicated strains. Dots represent values obtained from independent biological experiments. The normalized amount of Slx8 was calculated by dividing the GFP signal by unspecific SUMO signal. The normalized amount of Slx8-GFP in mutants was indicated as a percentage of WT. *p* value was calculated by two-sided Fisher's exact test (\*\*\*\*  $p < 0.0001$ ; ns: non-significant).



**Figure S3: Genotoxic treatments have a variable effect upon Slx8-GFP protein expression profile.**

**A.** Expression of Slx8-GFP in indicated strains and conditions. An untagged WT strain (No Tag) was included as control for antibody specificity. PCNA was used as a loading control. HU: hydroxyurea; CPT: camptothecin and MMS: methyl methane sulfonate. **B & C.** Quantification of Slx8-GFP expression in indicated strains (WT SUMO: left panel, SUMO-KallR: right panel) and conditions. Dots represent values obtained from independent biological experiments. The normalized amount of Slx8 was calculated by dividing the GFP signal by PCNA signal. The normalized amount of Slx8-GFP in treated conditions was indicated as a percentage of the untreated conditions. *p* value was calculated by two-sided Fisher's exact test (\*\*\*)  $p \leq 0.001$ ; \*  $p \leq 0.05$ ; ns: non-significant).



**Figure S4: Expression of Slx8-GFP is not affected by the absence of Clr4, Dcr1 or Csi1.**

**A.** Expression of Slx8-GFP in indicated strains. An untagged WT strain (No Tag) was included as control for antibody specificity. PCNA was used as a loading control. **B.** Quantification of Slx8-GFP expression. Dots represent values obtained from independent biological experiments. The normalized amount of Slx8 was calculated by dividing the GFP signal by PCNA signal. The normalized amount of Slx8-GFP in mutants was indicated as a percentage of the WT. *p* value was calculated by two-sided Fisher's exact test (ns: non-significant).

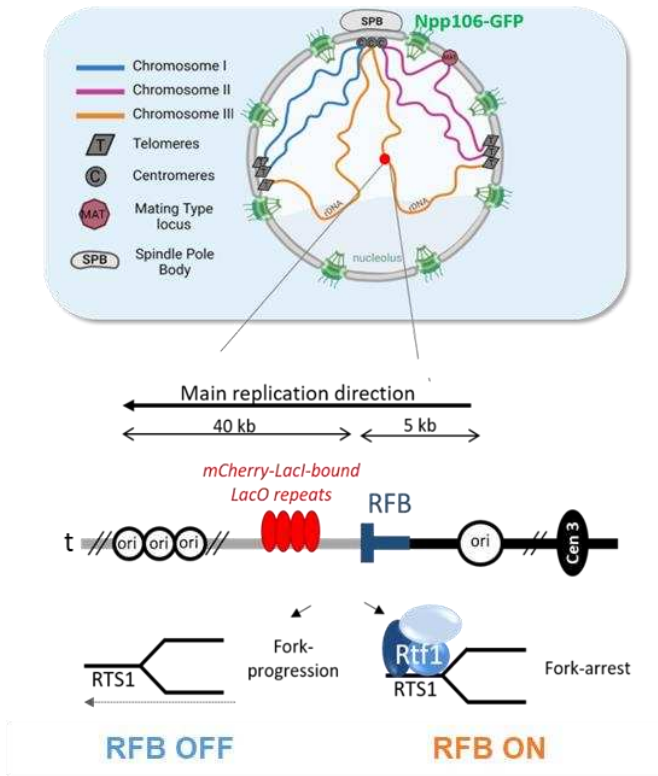
**Supplementary Table 1: strain list**

<b>Strain number</b>	<b>Mating type</b>	<b>Genotype</b>	<b>Reference</b>
<b>KK1492</b>	<i>h</i> -	<i>slx8-GFP:natMX6 nmt41:rtf1:sup35 ade6-704 leu1-32 t-ura4-SD20&lt;ori (uraR)</i>	this study
<b>KK2021</b>	<i>h</i> +	<i>slx8-GFP:natMX6 ade6-704 leu1-32 ura4-D18</i>	this study
<b>KK1377</b>	<i>h</i> +	<i>ade6-704 leu1-32 ura4-D18</i>	this study
<b>KK772</b>	<i>h</i> +	<i>rad52::kanMX6 nmt41:rtf1:sup35 ade6-704 t-ura4<sup>+</sup> &lt;ori (uraR) leu1-32</i>	this study
<b>KK1562</b>	<i>h</i> -	<i>pmt3::kanMX6-ura4<sup>+</sup> nmt41:rtf1:sup35 ade6-704 leu1-32 t-ura4-SD20&lt;ori (uraR)</i>	this study
<b>KK1025</b>	<i>h</i> -	<i>slx8-29:hphMX6 nmt41:rtf1:sup35 ade6-704 leu1-32 t-ura4-SD20&lt;ori (uraR)</i>	this study
<b>KK2096</b>	<i>h</i> -	<i>pli1-C321S-H323A-C326S (pli-RING<sup>mut</sup>) slx8-GFP:natMX6 ade6-704 leu1-32 ura4-D18</i>	this study
<b>KK2112</b>	<i>h</i> +	<i>nse2-C195S-H197A (nse2-RING<sup>mut</sup>) slx8-GFP:natMX6 ade6-704 leu1-32 ura4-D18</i>	this study
<b>KK2023</b>	<i>h</i> +	<i>pmt3-KallR (sumo-KallR) slx8-GFP:natMX6 ade6-704 leu1-32 ura4-D18</i>	this study
<b>KK2074</b>	<i>h</i> -	<i>pmt3-D81R (sumo-D81R) slx8-GFP:natMX6 ade6-704 leu1-32 ura4-D18</i>	this study
<b>KK2176</b>	<i>h</i> +	<i>slx8-GFP:natMX6 cut11-mCherry:hphMX6 ade6-704 leu1-32 ura4-D18</i>	this study
<b>KK2173</b>	<i>h</i> +	<i>pmt3-KallR (sumo-KallR) slx8-GFP:natMX6 cut11-mCherry:hphMX6 ade6-704 leu1-32 ura4-D18</i>	this study
<b>KK2294</b>	<i>h</i> -	<i>slx8-GFP:natMX6 sid4-mRFP:kanMX6 ade6-704 leu1-32 ura4-D18</i>	this study
<b>KK2201</b>	<i>h</i> +	<i>slx8-GFP:natMX6 mis6-mRFP:hphMX6 ade6-704 leu1-32 ura4-D18</i>	this study
<b>KK2217</b>	<i>h</i> +	<i>slx8-GFP:natMX6 taz1-mRFP:hphMX6 ade6-704 leu1-32 ura4-D18</i>	this study
<b>KK2602</b>	<i>h90</i>	<i>slx8-GFP:natMX6 arg3::mCherry-LacI his2::kanR-ura4<sup>+</sup>-lacOp ade6-704 leu1-32 ura4-D18</i>	this study
<b>KK2471</b>	<i>h</i> -	<i>csi1::hphMX6 slx8-GFP:natMX6 ade6-704 leu1-32 ura4-D18</i>	this study
<b>KK2432</b>	<i>h</i> +	<i>clr4::natMX6 slx8-GFP:natMX6 ade6-704 leu1-32 ura4-D18</i>	this study
<b>KK2436</b>	<i>h</i> -	<i>dcr1::hphMX6 slx8-GFP:natMX6 ade6-704 leu1-32 ura4-D18</i>	this study
<b>673</b>	<i>h90</i>	<i>mat3-M:ade6<sup>+</sup> ade6-DN/N leu1-32 ura4-D18</i>	this study

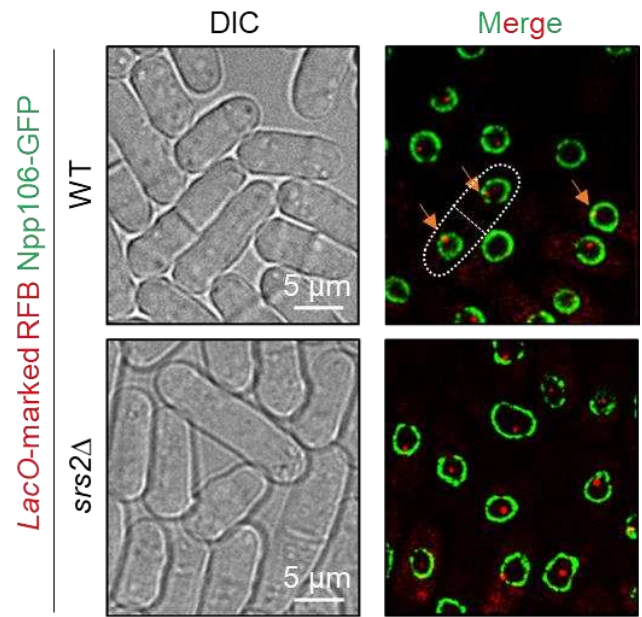
<b>674</b>	<i>h90</i>	<i>clr4Δ::leu2 mat3-M:ade6<sup>+</sup> ade6-DN/N leu1-32 ura4-D18</i>	this study
<b>6711</b>	<i>h90</i>	<i>slx8Δ::ura4<sup>+</sup> mat3-M:ade6<sup>+</sup> ade6-DN/N leu1-32 ura4-D18</i>	this study
<b>5513</b>	<i>h90</i>	<i>Sid4<sup>+</sup>-mRFP:Kan<sup>R</sup> GFP-Cnp1<sup>+</sup>:Nat<sup>R</sup> leu1-32 ura4-D18</i>	this study
<b>6363</b>	<i>h+</i>	<i>csi1Δ::Hyg<sup>R</sup> Sid4<sup>+</sup>-mRFP:Kan<sup>R</sup> GFP-Cnp1<sup>+</sup>:Nat<sup>R</sup> leu1-32 ura4-D18</i>	this study
<b>7681</b>		<i>slx8Δ::Kan<sup>R</sup> Sid4<sup>+</sup>-mRFP:Kan<sup>R</sup> GFP-Cnp1<sup>+</sup>:Nat<sup>R</sup> leu1-32 ura4-D18</i>	this study
<b>8000</b>		<i>csi1Δ::Hyg<sup>R</sup> slx8Δ::Kan<sup>R</sup> Sid4<sup>+</sup>-mRFP:Kan<sup>R</sup> GFP-Cnp1<sup>+</sup>:Nat<sup>R</sup> leu1-32 ura4-D18</i>	this study
<b>8028</b>		<i>slx8Δ::Kan<sup>R</sup> pli1Δ::ura4<sup>+</sup> Sid4<sup>+</sup>-mRFP:Kan<sup>R</sup> GFP-Cnp1<sup>+</sup>:Nat<sup>R</sup> leu1-32 ura4-D18</i>	this study
<b>7036</b>		<i>pli1Δ::ura4<sup>+</sup> Sid4<sup>+</sup>-mRFP:Kan<sup>R</sup> GFP-Cnp1<sup>+</sup>:Nat<sup>R</sup> leu1-32 ura4-D18</i>	this study

## Section IV: Additional data

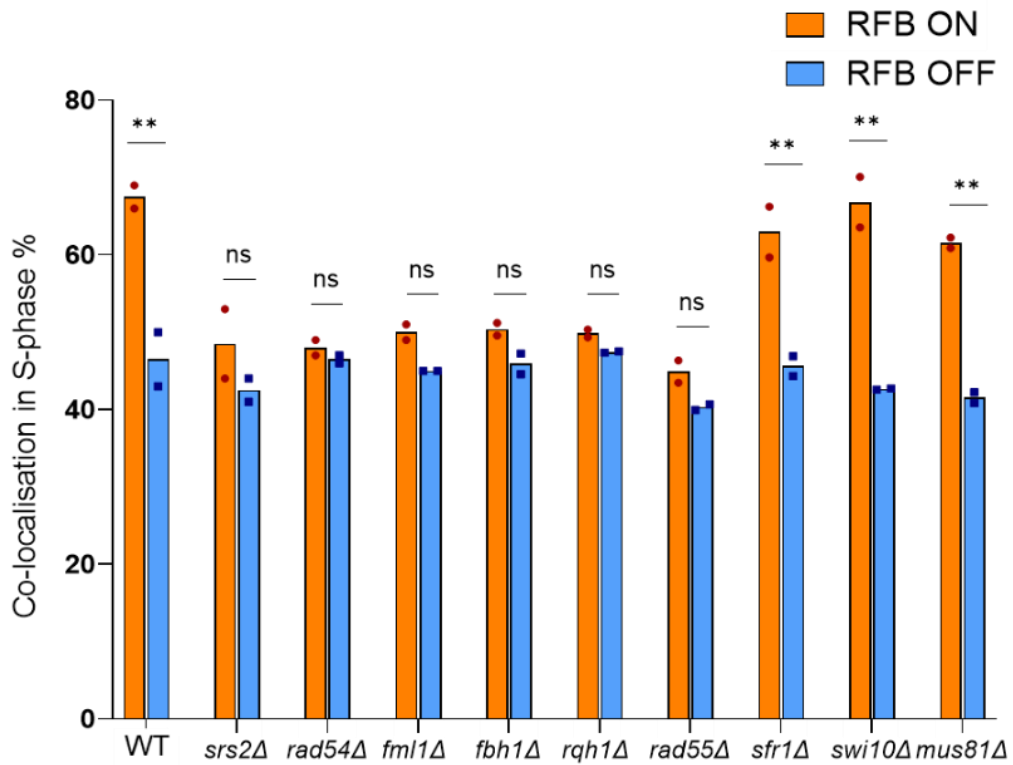
**A**



**B**



**C**





**Figure 1: Factors involved in D-loop processing are required to enrich the active RFB at the nuclear periphery. A:** Representative diagram showing the three chromosomes within the nucleus of a fission yeast cell. Chromosome III harbours a single *LacO*-marked *RTS1*-RFB that leads to the arrest of replisomes traveling from the centromere (Cen3) towards the telomere (t). "Ori" (black circles) indicate the main replication origins upstream and downstream of the RFB. Polar fork arrest is mediated by the binding of Rtf1 to the *RTS1* sequence. Rtf1 expression is under the thiamine-repressible *nmt41* promoter: with thiamine, Rtf1 is repressed and the RFB is poorly active (RFB OFF, light blue); without thiamine, Rtf1 is expressed and the RFB is strongly active (RFB ON, orange). *LacO* arrays bound by mCherry-LacI (red ellipses) are integrated ~7.9 kb away from the *RTS1*-RFB (blue bar). The nuclear periphery is marked in green by the nucleoporin, Npp106-GFP. **B:** Example of bright-field (left panel, DIC) and fluorescence (right panel) images of cells expressing the endogenous Npp106-GFP fusion protein and harboring the *LacO*-marked RFB in indicated strains. Mono-nucleated cells and septated bi-nucleated cells correspond to G2 and S-phase cells, respectively. Yellow arrows indicate co-localization events in S-phase cells. Scale bare: 5µm. **C:** Quantification of co-localization events, shown in B, in S-phase cells in indicated conditions and strains. Dots represent values obtained from two independent biological experiments. At least 200 nuclei were analyzed for each strain and condition. Fisher's exact test was used for group comparison to determine the *p* value (\*\*  $p \leq 0.01$ ; ns: non-significant).

#### **IV. Evidences for an NPC-independent recombination-dependent replication pathway.**

In fission yeast, arrested replication forks are reported to relocate to the nuclear periphery and anchor to Nuclear Pore Complexes (NPCs) for initiating fork restart mediated by the homologous recombination pathway. This phenomenon of relocation is triggered by the post-translation modification called SUMOylation. SUMOylation plays a key role in regulating the nuclear positioning of DNA lesions as well as the mechanisms that are further engaged to repair DNA (Kramarz et al., 2020a; Nagai et al., 2008a; Schirmeisen et al., 2023; X. A. Su et al., 2015a; Whalen et al., 2020a). Previous publications from the team have highlighted the significance of the location of arrested replication forks within the compartmentalised nucleus. Relocation of arrested forks to the NPC requires the formation of SUMO chains along with the strand exchange activity of Rad51 (Kramarz et al., 2020a). This suggested that arrested forks require to be remodelled to be prone to anchorage. Using a proxy fork restart genetic assay, that exploit the mutagenic DNA synthesis associated with restarted forks, it was noticed that the frequency of replication slippage induced by the *RTS1*-RFB, was increased when SUMO chains are unable to form (Kramarz et al., 2020a). This was interpreted as a role for SUMO chains in limiting recombination-mediated fork restart, creating a need to anchor arrested forks at the NPC to clear off SUMO conjugates by the SUMO protease Ulp1 and the proteasome, two activities enriched at the NPC. Interestingly, cells proficient for mono-SUMOylation only, maintains the arrested forks within the nucleoplasm, resulting in a higher frequency of replication slippage during replication restart. These data suggested the existence of a replication fork restart pathway in the nucleoplasm, without a routing to the nuclear periphery. This gave rise to the idea of the existence of at least two spatially segregated RDR pathways, whose choice is under SUMO control: SUMO chains formation vs mono-SUMOylation. In this context, by employing the *RTS1*-RFB system in fission yeast, I aimed at gathering genetics and molecular evidences of the NPC-independent and mono-SUMOylation-dependent RDR pathway for a better understanding of how the nuclear compartments have distinct competence towards fostering DNA repair pathways.

##### **1. Factors involved in D-loop processing are required to enrich the active RFB at the nuclear periphery.**

The team has previously showed that relocation of the RFB to the NPC is dependent on Rad51's strand exchange activity. Both the deletion of Rad51 or the use of a Rad51 mutant that is defective in its strand exchange activity, *rad51-II3A* (Cloud et al., 2012)

is sufficient to abrogate the shift to the nuclear periphery (Kramarz et al., 2020a). These data suggested that the formation of joint-molecules is a critical determinant for the relocation of the RFB to the nuclear periphery. I therefore tested the role of several factors, involved in the formation/stability of early joint molecules (D-loop) or the resolution of late joint-molecules (Holliday junctions) for RFB relocation.

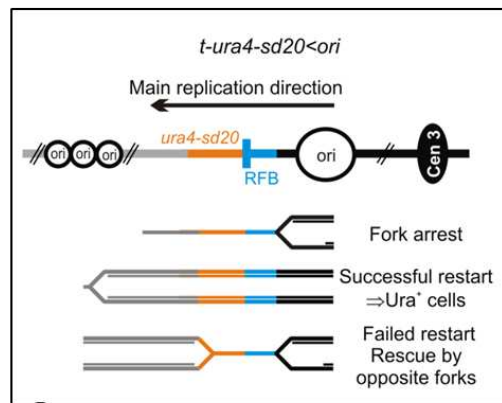
Subsequently, I tested different HR factors to check their respective contribution towards the relocation of RFB to the nuclear periphery. In order to do so, I employed a fluorescence microscopy based co-localization approach to ask how frequently the *LacO*-marked RFB co-localizes with the nuclear periphery labelled by the expression of Npp106-GFP, in RFB OFF vs RFB ON condition ([Figure 40A and 40B](#)).

Deletion mutants including *srs2Δ*, *rad54Δ*, *fml1Δ*, *fbh1Δ*, *rqh1Δ*, *rad55Δ*, *sfr1Δ*, *swi10Δ* and *mus81Δ* were tested respectively. Interestingly, I first looked at the factors involved in the early joint molecule formation or processing of D-loops. Here, I observed that a significant reduction in the RFB enrichment at the nuclear periphery, in ON condition for *srs2Δ*, *rad54Δ*, *fml1Δ*, *fbh1Δ*, *rqh1Δ* and *rad55Δ* as compared to the wildtype condition ([Figure 40C](#)). However, upon testing factors that participate in the resolution of joint molecules, I did not detect any significant loss in RFB enrichment as was observed in the *swi10Δ* and *mus81Δ* strains. One important observation worth noticing was the fact that all the factors involved in the formation/stability of early joint molecules or D-loop were critical for RFB relocation rather than the ones required for the resolution of the late joint-molecules or Holliday junctions.

Amongst the factors tested, the Srs2 DNA helicase that displayed a defect in RFB relocation is involved in maintaining the stability of the Rad51 filament. Srs2 harbours both, pro- and anti-recombinogenic functions towards Rad51 filament dynamics, thereby balancing out beneficial recombination and preventing harmful events for maintenance of genomic integrity (Doe & Whitby, 2004, p. 200; S.-W. Wang et al., 2001). Similarly, Rad55 and Sfr1 are different paralogue pathways that are associated with Rad51 filament formation and stability. Rad55 works along with Rad57 to protect Rad51 foci and filaments from disassembly rather than assisting its initial loading onto ssDNA (J. Liu et al., 2011). While, Sfr1 functions with Swi5 to enhance the assembly of Rad51 filament by stabilizing it (Kokabu et al., 2011; C.-H. Lu et al., 2018). Since, both Rad55 and Sfr1 are involved in maintenance of Rad51 filaments, it was expected that deletion of both these factors would phenocopy the *rad51Δ* mutant. Interestingly, I identified that *rad55Δ* displayed a defect in RFB relocation. However, I did not observe any effect on RFB relocation upon the deletion of Sfr1. In fact, there could be a possibility that the relocation of RFB to the NP requires the Rad55 paralogue pathway

**Figure 41: Restarted forks in HR factor mutants show great variability in the rate of replication fork slippage.** A: Diagram of the *t-ura4sd20<ori* construct containing a single *RTS1*-RFB as described above. The non-functional *ura4-sd20* allele contains a 20 nucleotide duplication flanked by micro-homology. HR-mediated restart is associated to a non-canonical DNA synthesis prone to frequent replication slippage (RS) leading to the deletion of the duplication, thus restoring a functional *ura4+* marker. B: Frequency of RFB-induced *Ura*<sup>+</sup> reversion by replication slippage in indicated strains and conditions. Each dot represents one sample from independent biological replicate. Bars indicate mean values  $\pm$  standard deviation (SD). *p* value was calculated using two-tailed t-test, compare to WT.

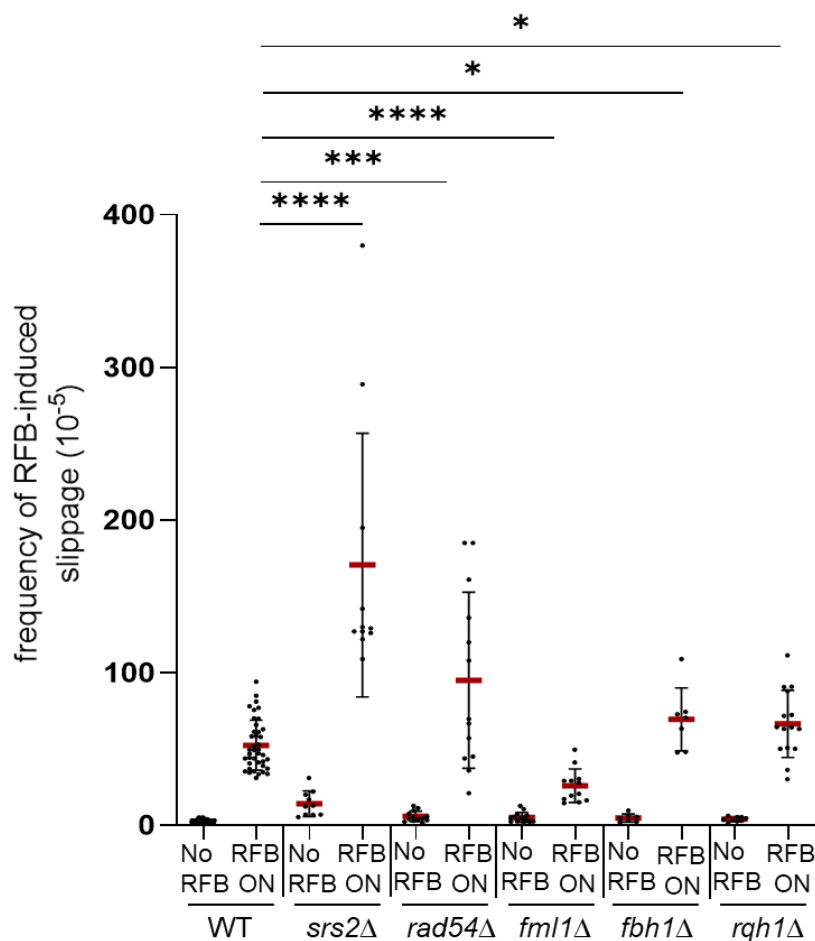
**A**



Recombination-dependent Replication is associated with a DNA synthesis prone to frequent replication slippage : a proxy restart assay

(Iraqui et al. PLoS Genetics 2012)

**B**



specifically and not the Sfr1 pathway. On the other hand, Rad54 is a DNA translocase that might mediate Rad51 filament formation through its ATPase and chromatin remodelling activities, promoting strand invasion and D-loop formation as in budding yeast (Muris et al., 1996; Petukhova et al., 1998). Rad54 could also be involved in the removal and recycling of Rad51 from DNA once repair is complete (Solinger et al., 2002). This function is important to prevent excessive recombination and ensure that Rad51 is available for subsequent repair processes. Additionally, Fbh1 possesses helicase activity that enables it to translocate along DNA and actively dismantle Rad51 filaments (Tsutsui et al., 2014). This helps to regulate the length and stability of Rad51 nucleoprotein filaments on single-stranded DNA (ssDNA). Similar to the other helicases like Srs2 and Fbh1, Rqh1 functions as an anti-recombinase by regulating Rad51 filament formation (Ahmad et al., 2002; Doe et al., 2000). It can unwind DNA structures that arise during homologous recombination and prevent excessive Rad51 filament accumulation (Stewart et al., 1997, p. 1). Altogether, except for Sfr1, all of the factors necessary for promoting and maintaining the stability of Rad51 filaments for D-loop formation are crucial for RFB relocation.

Conversely, factors such as Swi10 and Mus81 did not showcase a defect in RFB relocation ([Figure 40C](#)). Both factors are structure-specific endonucleases that are involved in the processing of DNA ends or Holliday junctions, which are structures formed following D-loop formation (A. M. Carr et al., 1994; Doe et al., 2004; Osman & Whitby, 2007). The processing of Holliday junctions help to resolve recombination intermediates into non-crossover or crossover products, depending on the context of the repair. Importantly, resolution of the D-loop was not essential for RFB relocation. This majorly reflects the idea that the phenomenon of RFB relocation to the nuclear periphery is reliant upon the helicases and translocases that promote the formation of the early joint molecules, rather than the downstream activity of resolution of the Holliday junction.

## 2. The efficiency of RFB-induced replication slippage does not correlate with nuclear positioning of the RFB.

Having observed that the formation/stability of D-loop is a critical determinant for RFB relocation, the next imminent question was to investigate the efficiency of RDR in the aforementioned mutants, for addressing the consequences of the lack of relocation. To accomplish that, a proxy restart genetic assay was used, which exploits the principle of HR-mediated fork restart being associated with a non-processive DNA synthesis being liable to replication slippage (RS). The rate of restarted forks are calculated by the

frequency of RFB-induced RS, based on the restoration of a functional *ura4<sup>+</sup>* gene to select for Ura<sup>+</sup> cells (Figure 41A). The frequency of Ura<sup>+</sup> reversion is used as a readout of the frequency at which the *ura4-sd20* allele is replicated by a restarted fork in the cell population (Iraqi et al., 2012a).

Surprisingly, RFB-induced RS in all the various HR factor mutants that showed defective relocation, displayed a great spectrum of variability amongst each other. Cell deleted for Srs2 (*srs2Δ*) exhibited a significantly higher increase in the rate of RFB-induced RS compared to the wild type (WT), while *rad54Δ* cells displayed an increase to a much lesser extent than *srs2Δ* (Figure 41B). The *fbh1Δ* and *rqh1Δ* mutants had a comparable frequency of RFB-induced RS as that of the wild type (WT), whereas the *fml1Δ* mutant was the only one that showed a significant decrease in the rate of fork restart compared to WT (Figure 41B).

Hence, the rate of RFB-induced RS tested in the HR factor mutants displayed no correlation amongst each other, despite all of them being defective for relocation. This hints towards the idea that arrested forks unable to shift to the nuclear periphery might be restarted via different pathways within the nucleoplasm. On the other hand, it cannot be ruled out that the RFB-induced RS assay reflects not only the efficiency of HR-mediated fork restart, but also the degree of mutagenicity of the restarted DNA synthesis. In other words, arrested forks may restart less frequently but DNA synthesis is more likely to be mutagenic and, conversely, arrested forks may restart more frequently but DNA synthesis is less prone to mutagenesis.

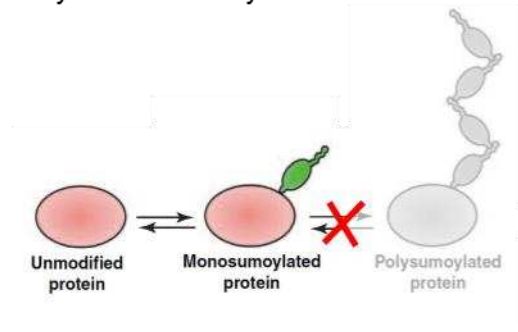
### 3. SUMO chains control the fidelity of restarted forks.

As mentioned earlier, SUMO chains are the “trigger” signal for the relocation of the RFB to the NPC, while mono-SUMOylation is not enough for triggering this response. The team has studied the effects of relocation in two mutants in which the level and type of SUMO conjugates has been manipulated by distinct means. In the *sumo-KallR* mutant, all internal lysines of the SUMO particle are mutated to prevent the formation of SUMO chains, while monoSUMOylation still occurs (Figure 42A). In the *sumo-D81R* mutant, the interaction between the E2 SUMO conjugating enzyme Ubc9 and SUMO is impaired and only mono- and di-SUMOylation can occur (Prudden et al., 2011b, p. 201). Hence, both mutants are incapable of generating SUMO chains. Previous publication from the team has demonstrated that both of these SUMO mutants are unable to relocate the RFB to the nuclear periphery indicating that SUMO chains are instrumental for RFB relocation (Kramarz et al., 2020a).

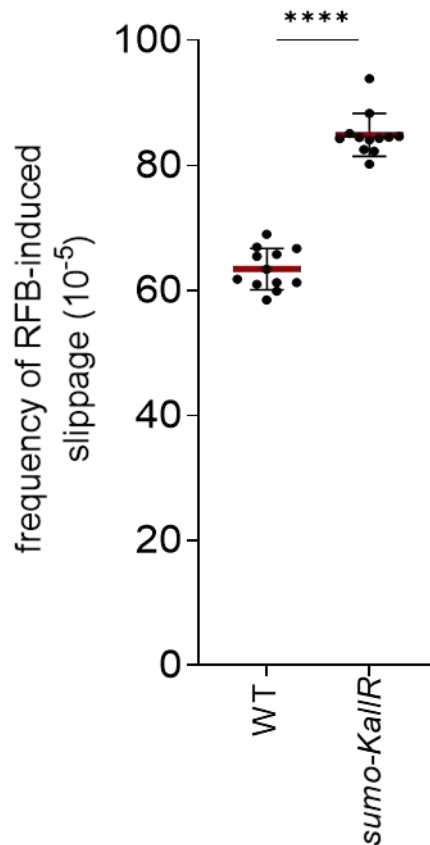
**Figure 42: Absence of SUMO chains enhance the efficiency of RFB-induced RS.** A: Diagrammatic representation of the *sumo-KallR* mutant. B: Frequency of RFB-induced Ura<sup>+</sup> reversion by replication slippage in indicated strains. Each dot represents one sample from independent biological replicate. Bars indicate mean values  $\pm$  standard deviation (SD). *p* value was calculated using two-tailed t-test, compare to WT.

**A**

***sumo-KallR* (all internal Lysines converted to Arginine):**  
allows only mono-SUMOylation



**B**



As mentioned above, the frequency of RFB-induced RS is significantly higher in the *sumo-KallR* mutant as compared to WT ([Figure 42B](#)). This was interpreted as a role for SUMO chains in limiting recombination-mediated fork restart, creating a need to anchor arrested forks at the NPC to clear off SUMO conjugates by the SUMO protease Ulp1 and the proteasome, two activities enriched at the NPC.

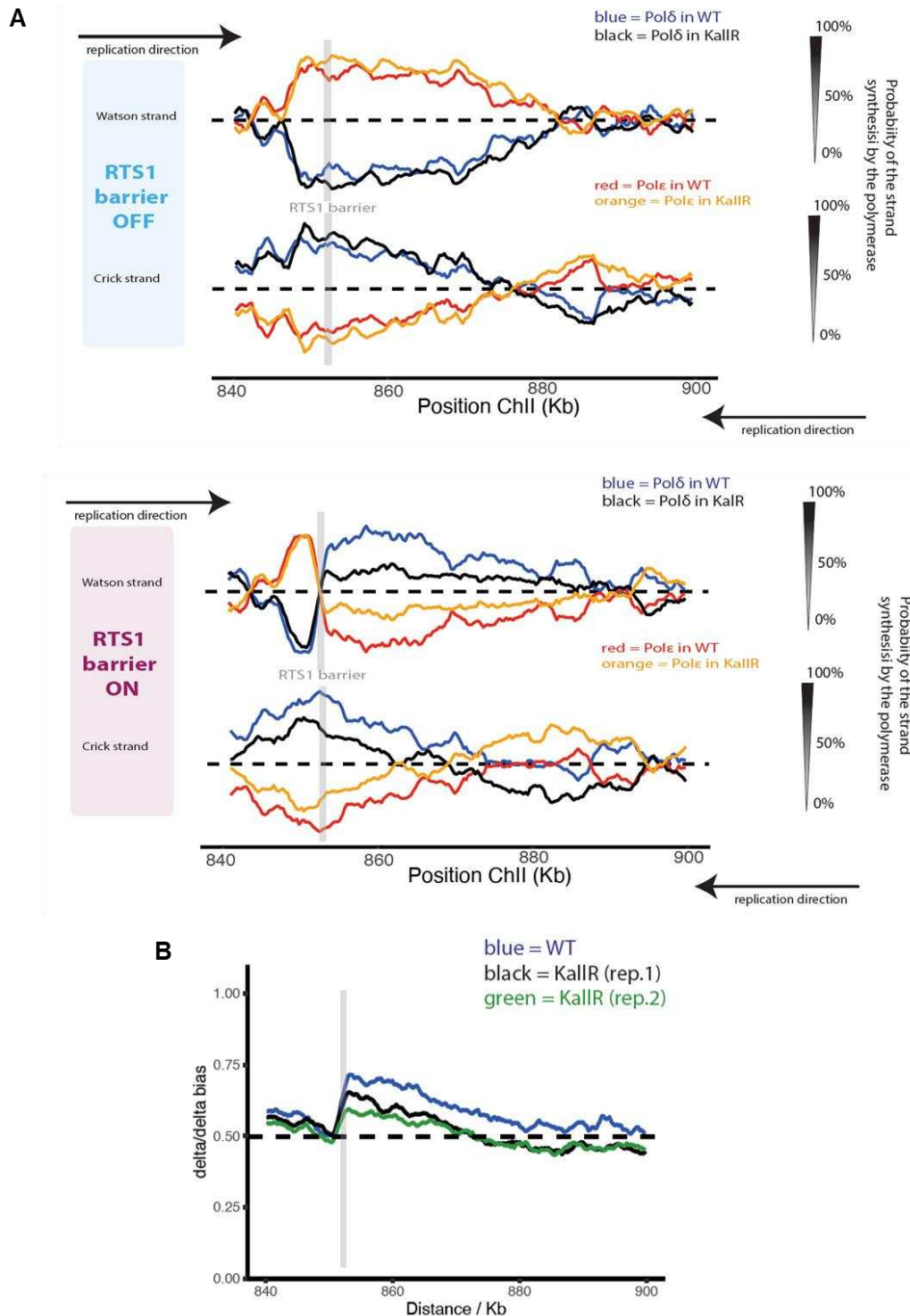
However, given the fact that I observed a lack of correlation between relocation of the RFB to nuclear periphery and the frequency of RFB-induced RS, I decided to analyse the dynamic of fork restart using the polymerase usage sequencing (Pu-Seq) technique. This technique was performed in collaboration with Tony Carr (University of Sussex) and Karel Naiman (Centre de Recherche en Cancérologie de Marseille). The technique allows a genome-wide mapping of the usage of polymerases, Pol  $\delta$  and Pol  $\epsilon$  during DNA replication (Daigaku et al., 2015). Pu-Seq utilizes a pair of yeast strains mutated in either Pol  $\delta$  or Pol  $\epsilon$ , leading to higher levels of ribonucleotide incorporation during DNA synthesis. By mapping ribonucleotides in a strand-specific manner in these mutated strains, it allows for genome-wide tracking of polymerase usage. When combined with the *RTS1*-RFB, the Pu-Seq method enables monitoring the usage frequency of each polymerase separately on both the Watson and Crick strands. This is done when the RFB is either inactive (RFB OFF, in an *rtf1* $\Delta$  genetic background) or constitutively active (RFB ON, with Rtf1 expressed from the *adh1* promoter to maximize fork arrest efficiency) (Naiman et al., 2021a).

In the RFB OFF condition, replication follows the canonical pattern: Pol  $\epsilon$  synthesizes the leading strand, and Pol  $\delta$  synthesizes the lagging strand. In most cells, replication proceeds from left to right, initiating from an early replication origin. ([Figure 43A](#)). In a RFB ON strain, the division of labor between Pol  $\epsilon$  and Pol  $\delta$  changes significantly: at the barrier site, Pol  $\delta$  takes over from Pol  $\epsilon$  in synthesizing the leading strand during the restart of the blocked fork. ([Figure 43A](#)). This precise transition characterizes the efficiency of the restart itself, creating a bias towards Pol  $\delta$  on both the Watson and Crick strands downstream of the *RTS1*-RFB site. The Pol  $\delta/\delta$  bias indicates the time required for the restart and the progression of the restarted fork with respect to the canonical convergent fork originating from a late replication origin. (Naiman et al., 2021a).

Based on the Pol  $\delta/\delta$  bias, around 60 to 70% of the arrested forks were restarted in the *sumo-KallR* mutant as compared to the WT, indicating a defect in fork restart ([Figure 43B](#)). Thus, surprisingly and contrary to previous interpretations, the absence of SUMO chains result in defective fork restart. The increase in Pol  $\epsilon$  usage on the Crick strand (in orange) after 10 Kb downstream of the *RTS1* barrier is indicative of the converging fork



**Figure 43: SUMO chains promotes Pol $\delta$ /Pol $\delta$  restarted forks at the RTS1-RFB.** A: Pu-Seq traces of the ChrII locus in *RTS1*-RFB OFF (top panel) and ON (bottom panel) conditions in *WT* and *sumo-KallR* strains. The usage of Pol delta (in blue and black for *WT* and *sumo-KallR* cells, respectively) are shown on the Watson and Crick strands. The usage of Pol epsilon (in red and orange for *WT* and *sumo-KallR* cells, respectively) are shown on the Watson and Crick strands. Note the switch from Pol epsilon to Pol delta on the Watson strand at the RFB site (gray bar), which is indicative of a change in polymerase usage on the leading strand in RFB ON condition. B: Graph of Pol delta/delta bias over both strands (Watson and Crick) around the RFB site in *WT* and two independent replicates of *sumo-KallR* strains. The gray bar indicates the position of the *RTS1*-RFB.



(Figure 43A bottom panel). Notably, I did not see the same response for WT (in red), which was possibly due to a technical issue as it is not an expected phenotype for WT condition.

Moreover, the relative slope of the Pol  $\delta/\delta$  curves between the WT and the *sumo-KallR* were the same (Figure 43B). This proposed that the progression of the Pol  $\delta/\delta$  mediated restarted forks proceeded at the same rate in both, *sumo-KallR* and WT strains. Hence, despite having a defective fork restart, the progression of restarted forks are unaffected when cells are unable to form SUMO chains, but are capable of mono-SUMOylation only.

Thus, the data challenges the previously suggested interpretation regarding SUMO chains and demonstrates that SUMO chains promote the HR-mediated restart of arrested forks. The data suggest that, in the absence of SUMO chains, fewer arrested forks are restarted, but those that have successfully restarted are associated with DNA synthesis more likely to commit replication slippage. It is therefore possible that SUMO chains regulate the fidelity of restarted DNA synthesis, at least when it occurs in the nucleoplasm.

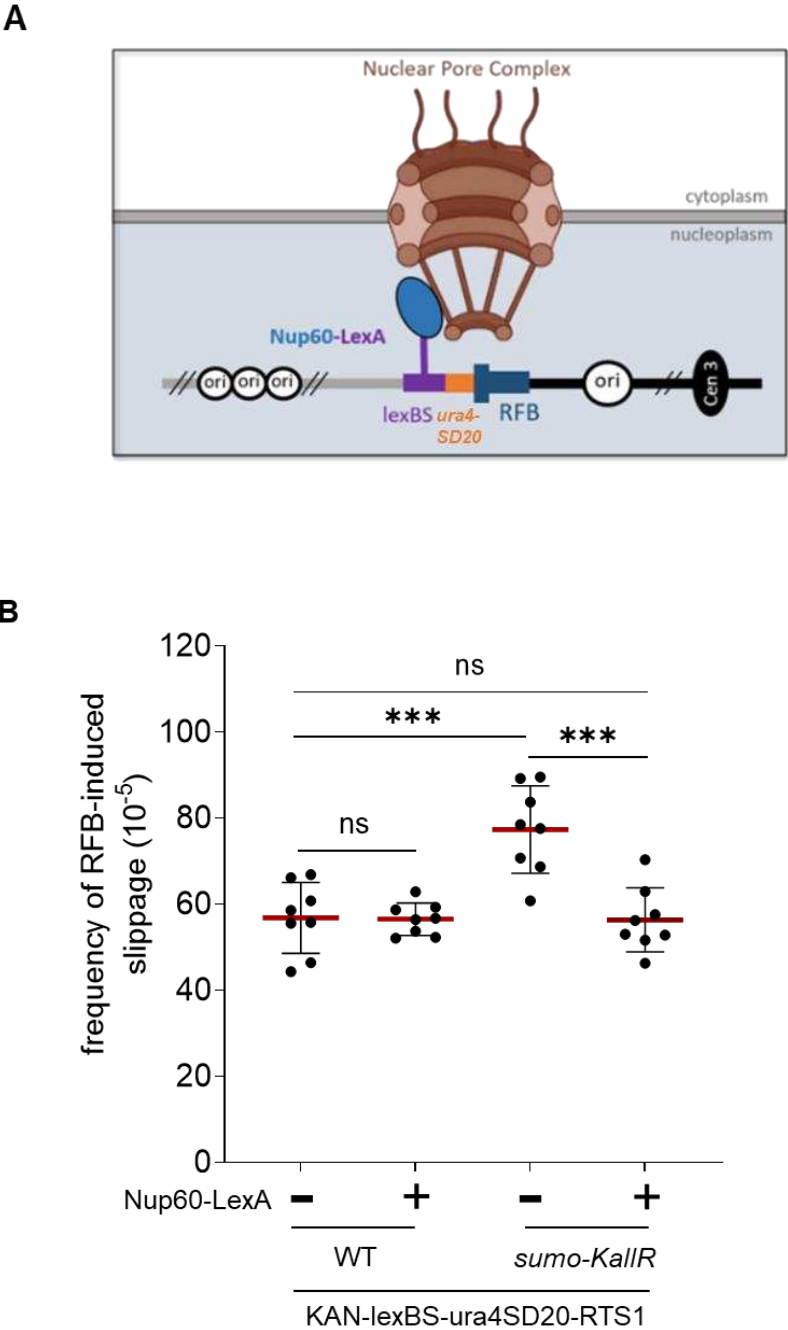
#### 4. The role of SUMO chains in limiting RFB-induced replication slippage is dispensable in the NPC environment.

Consequently, I investigated the dependence of SUMO chains in promoting RFB-induced RS when the RFB is constitutively enriched in the NPC environment. In order to do so, I exploited a LexA-based strategy to tether the RFB to the NPC developed in the lab by a former PhD student (Kamila Schirmeisen). In this construct, the RFB harbours eight LexA binding sites that are bound by LexA protein fused to Nup60 (a nuclear basket component) (Figure 44A). This way, the RFB was permanently enriched in the NPC environment whatever its activity (Schirmeisen et al., 2023).

Notably, I observed that the RFB-induced RS in the *sumo-KallR* mutant was reduced to WT levels upon tethering the RFB to the NPC (Figure 44B). Moreover, the level of RFB-induced RS in WT strains (*pmt3<sup>+</sup>*) were equal irrespective of whether they were tethered at the RFB or not. This was an important control to ensure that the rescue of the *sumo-KallR* mutant was unrelated to the LexA-based tethering.

Thereby, the data indicates that SUMO chains are no longer required to limit the frequency of RFB-induced RS in the NPC environment. These data suggest that the NPC environment bypasses the need of SUMO chains to limit the mutagenicity of restarted DNA synthesis.

**Figure 44: Tethering the *RTS1*-RFB to the nuclear pore complex results in the rescue of the *sumo-KallR* mutant phenotype.** A: Schematic representation of the LexA-based strategy to tether the RFB to the NPC. Nup60 fused to LexA tether *lexA*-binding sites (*lexBS*, purple) inserted in a close proximity to *RTS1*-RFB site at the chromosome III. B: Frequency of RFB-induced *Ura*<sup>+</sup> reversion by replication slippage in indicated strains. Each dot represents one sample from independent biological replicate. Bars indicate mean values  $\pm$  standard deviation (SD). *p* value was calculated using two-tailed t-test, compare to WT.



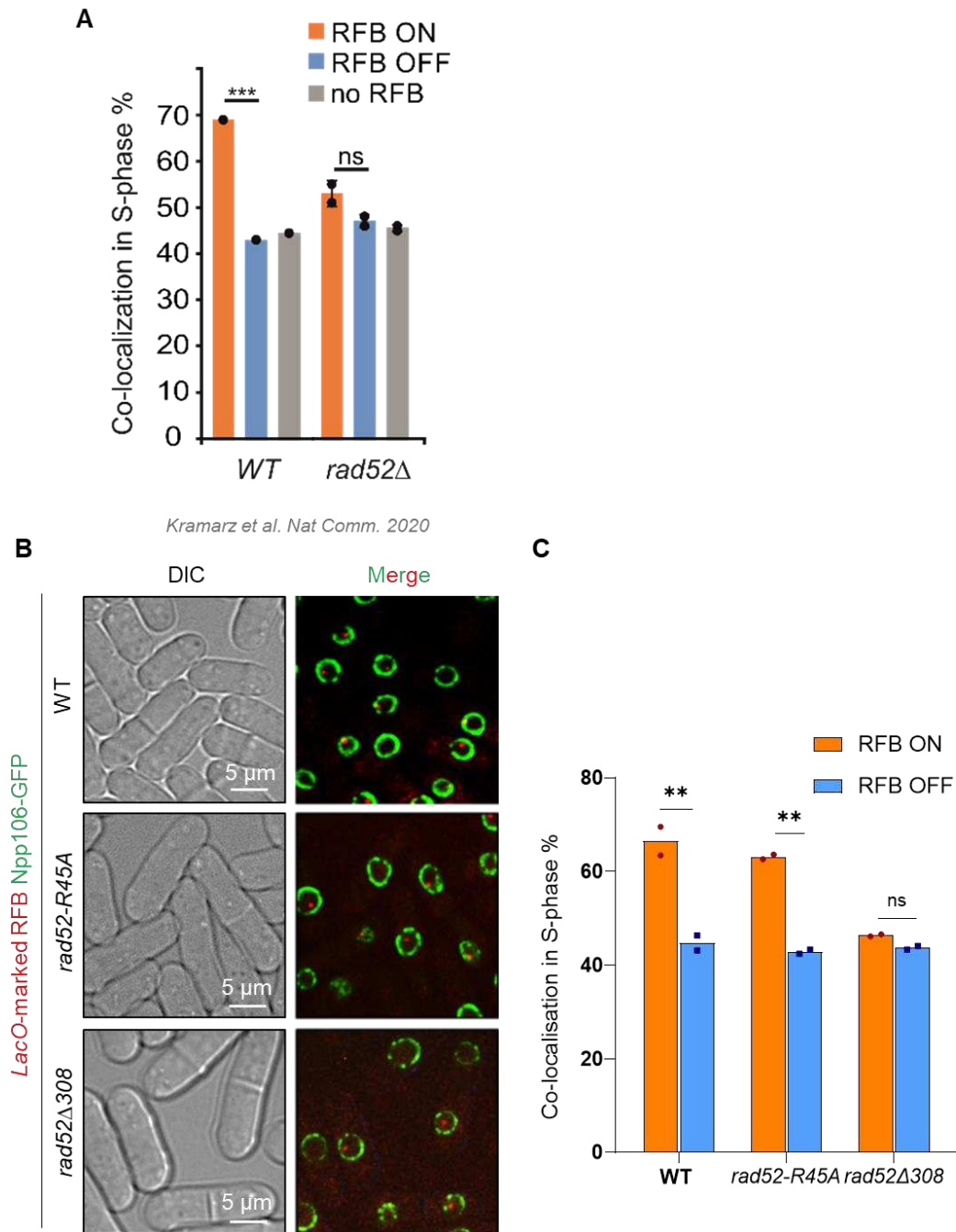
## 5. The Rad52 single strand annealing activity is dispensable for relocation of the RFB to the nuclear periphery.

Taking into consideration that there is no correlation between RFB relocation to the NPC and the frequency of RFB-induced RS which monitor both restart efficiency and the fidelity of restarted DNA synthesis, it might be possible that there are at least two RDR pathways with distinct control of DNA synthesis fidelity, depending on nuclear positioning. One of which is the previously established Rad51-dependent pathway that shifts towards the nuclear periphery for subsequent SUMO chains processing and fork restart (Kramarz et al., 2020a). While the other would be independent of Rad51 and occurs in the nucleoplasm without the need of NPC routing. Therefore, my next aim was to better understand the nucleoplasmic fork restart pathway, if any. However, the data I have obtained point to the limitation that the RFB-induced RS assay monitors two features of RDR: restart efficiency and the fidelity of the restarted DNA synthesis. Interestingly, a recent article from the lab of M. Whitby reported that the initiation of RDR is driven mainly by the Rad51 recombinase, whereas template switching mediated homology, during the elongation phase of RDR, relies more on DNA annealing by Rad52 (Kishkevich et al., 2022). Moreover, this report also suggested that the DNA annealing activity of Rad52 would promote replication restart at the RTS1-RFB, independently of Rad51.

It has been already documented that Rad52 is required for the relocation of the *RTS1*-RFB to the nuclear periphery ([Figure 45A](#)) (Kramarz et al., 2020a). However, Rad52 has two distinct functions: it promotes single strand annealing (SSA) and the loading of the recombinase Rad51. This last function requires the C-terminal domain, which interacts with Rad51. Whereas, the SSA activity requires the N-terminal domain. A SSA mutant for Rad52 was constructed in fission yeast by a single amino acid substitution at the conserved R45 position, named *rad52-R45A* that was severely impaired for Rad52's SSA activity (Yan et al., 2019).

Therefore, it was important to distinguish between the two functions towards the contribution of Rad52 to RFB relocation to the nuclear periphery. In order to do so, I took advantage of the SSA mutant of Rad52, *rad52-R45A* and tested its competence in RFB relocation (Yan et al., 2019). I discovered that the SSA activity is not required for the relocation of the *RTS1*-RFB to the nuclear periphery ([Figure 45B and 45C](#)). Whereas, another Rad52 mutant, *rad52-Δ308* was impaired in relocation of the RFB to the nuclear

**Figure 45: Relocation of the *RTS1*-RFB to the nuclear periphery requires *Rad52*-*Rad51* interaction but not the single-strand annealing activity of *Rad52*.** A & C: Quantification of co-localization events, shown in B, in S-phase cells in indicated conditions and strains. Dots represent values obtained from two independent biological experiments. At least 200 nuclei were analyzed for each strain and condition. Fisher's exact test was used for group comparison to determine the *p* value (\*\*  $p \leq 0.01$ ; ns: non-significant). B: Example of bright-field (left panel, DIC) and fluorescence (right panel) images of cells expressing the endogenous *Npp106*-GFP fusion protein and harboring the *LacO*-marked RFB in indicated strains. Mono-nucleated cells and septated bi-nucleated cells correspond to G2 and S-phase cells, respectively. Yellow arrows indicate co-localization events in S-phase cells. Scale bar: 5  $\mu$ m.



periphery ([Figure 45B and 45C](#)). The *rad52-Δ308* has been described to be lacking of the C-terminal disordered domain and most of the Rad51 binding domain (Kishkevich et al., 2022). Hence, the data reveal that the SSA activity of Rad52 is dispensable for relocation of arrested forks to the nuclear periphery. However, it also re-confirms our previous conclusion that RFB relocation is dependent upon Rad51 since, the *rad52Δ308* mutant, which abolishes Rad52's interaction with Rad51 is no longer capable of enriching the RFB at the nuclear periphery.

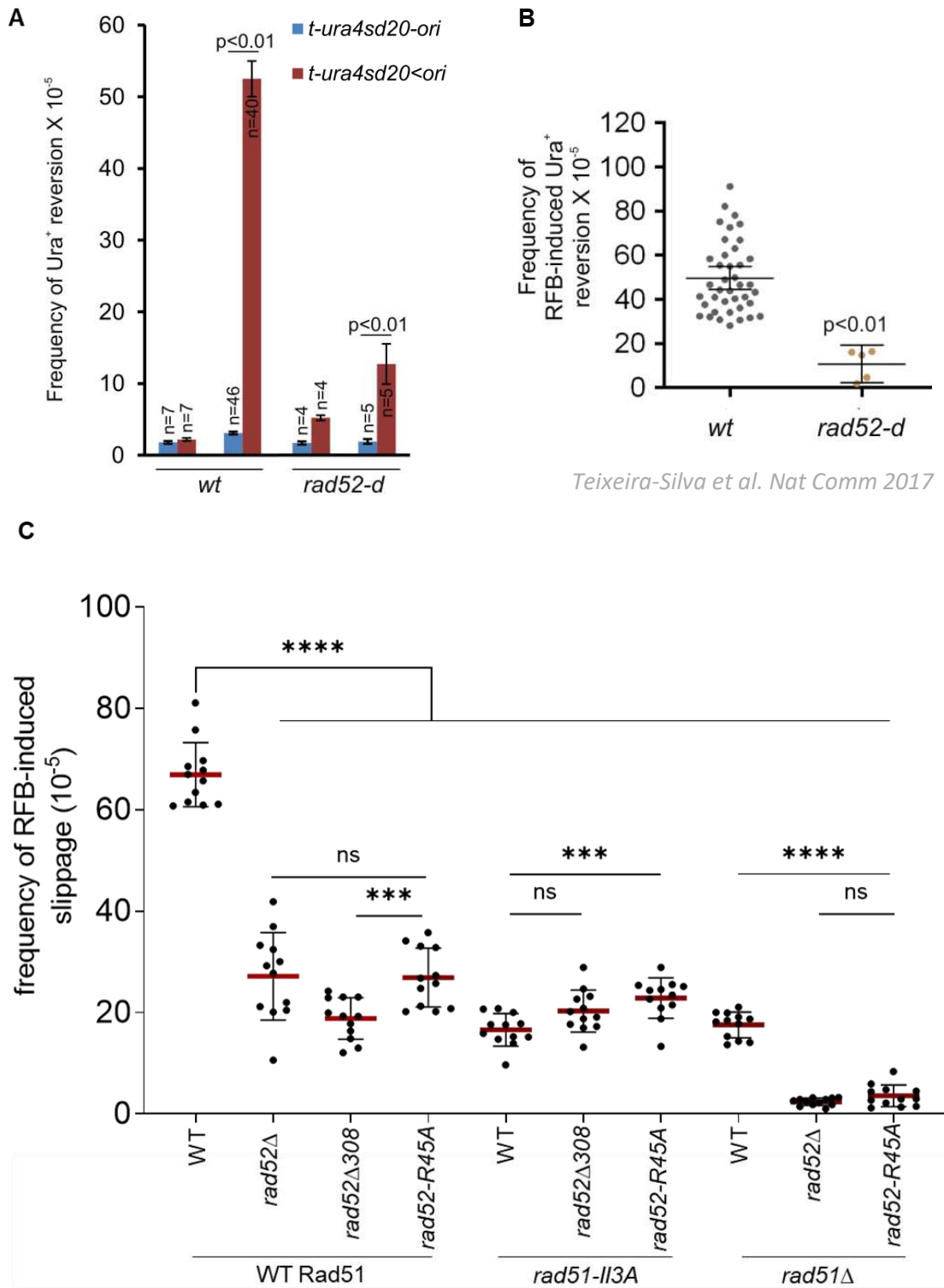
## 6. The single-strand annealing activity of Rad52 acts in Rad51-dependent and independent pathways of RDR.

As mentioned above, the *rad51-113A* mutant (see section 1) is capable of binding to DNA, but defective in strand exchange activity (Cloud et al., 2012). It was previously demonstrated by the team that the *rad51-113A* mutant, was also defective in HR-mediated fork restart and the frequency of restarted forks decreased by 70% (Ait Saada et al., 2017b). It was unclear as to which factor/pathway was responsible for the residual frequency of RFB-induced RS in this mutant. Therefore, I tested if the SSA activity of Rad52 was responsible for this ([Figure 46C](#)).

It has been previously published by the team that the *rad52Δ* mutant has a 5-fold reduction in the RFB-induced RS as compared to WT ([Figure 46A and 46B](#)) (Teixeira-Silva et al., 2017). However, I was not able to reproduce this result, as I observed only a 2-fold reduction in the RFB-induced RS in the *rad52Δ* mutant ([Figure 46C](#)). This discrepancy could be explained by the fact that *rad52Δ* is able to accumulate spontaneous, suppressor mutations, which can substitute for the loss of Rad52 activity to produce the increase in RFB-induced RS in the *rad52Δ* background. For example, one study has previously demonstrated that deletion of the Fbh1 helicase is able to suppress the severe defects in DNA repair and recombination observed in the *rad52Δ* mutant in *S. pombe* (Osman et al., 2005). This indicates that suppressor mutants could be responsible for the increased RFB-induced RS observed in the *rad52Δ* mutant ([Figure 46C](#)) and requires to be re-validated with further experiments.

Additionally, I observed a comparable reduction in the frequency of RFB-induced RS in *rad52Δ*, *rad52-Δ308* and *rad52ΔR45A* cells, indicating that both Rad51-Rad52 interaction and Rad52's SSA activity are required to promote RFB-induced RS. Interestingly, the level of RFB-induced RS in these mutants was similar to that of the *rad51-113A* or *rad51Δ* mutants.

**Figure 46: The single-strand annealing activity of Rad52 acts in Rad51-dependent and independent pathways of RDR.** A & B: Frequency of RFB-induced Ura<sup>+</sup> reversion by replication slippage in indicated strains. Values are means of n samples from independent biological replicates ± standard error of the mean (SEM). Statistics were calculated using the Mann and Whitney U test. C: Frequency of RFB-induced Ura<sup>+</sup> reversion by replication slippage in indicated strains. Each dot represents one sample from independent biological replicate. Bars indicate mean values ± standard deviation (SD). p value was calculated using two-tailed t-test, compare to WT.



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Furthermore, the *rad51Δ* and *rad51-113A* mutant displayed around 70% reduction in the frequency of RFB-induced RS, as previously published (Ait Saada et al., 2017b) (Figure 46). The remaining RFB-induced RS observed in the absence of Rad51 (i.e. in *rad51Δ* cells) was dependent on Rad52 and its SSA activity. Importantly, the frequency of RFB-induced RS in the double mutant *rad51Δ rad52-45A* was similar to that of the *rad51Δ rad52Δ* double mutant, suggesting that the Rad52 SSA activity triggers a Rad51-independent RDR pathway. An important information missing here is the level of RFB-induced RS in the *rad51Δ rad52-Δ308* to confirm that Rad51-Rad52 interaction is required or not to promote the Rad51-dependent RDR pathway.

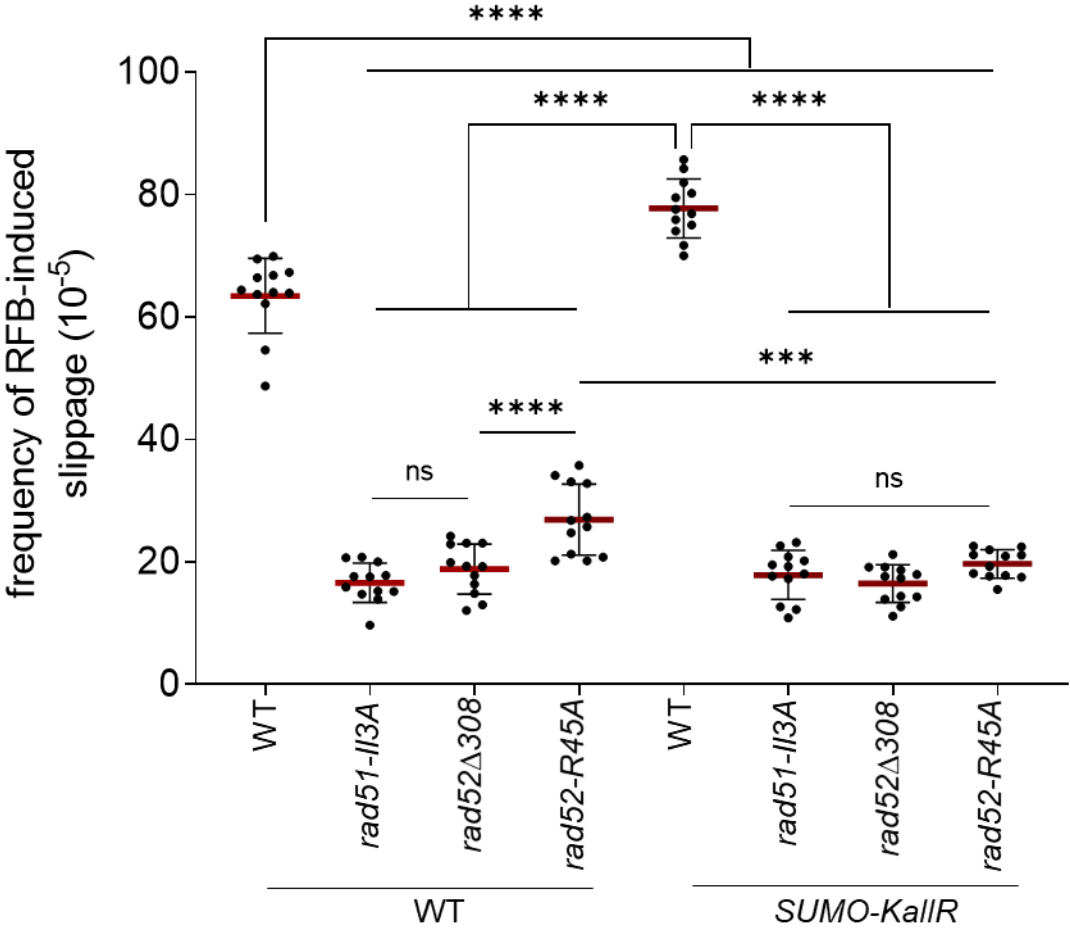
Surprisingly, opposite results were obtained in a *rad51-113A* genetic background. The *rad51-113A rad52Δ308* double mutant behaved like each single mutant, indicating that Rad52-Rad51 interaction and the strand exchange activity of Rad51 act in the same pathway to promote RDR. Moreover, the *rad51-113A rad52-R45A* double mutant behaved similarly to each single mutant, although I noticed a slight dominant effect of the *rad52-R45A* mutation over *rad51-113A*. These data support the hypothesis that the Rad52 SSA activity also acts with the strand exchange activity of Rad51 to promote RDR. One possible explanation is that RFB-induced RS occurring during the progression of fork restarted by the strand exchange activity of Rad51 are promoted by the SSA activity of Rad52. It's worth noting that the RFB-induced RS are mediated by micro-homology, making these events distinct from the homology-mediated template switches reported by the lab of M. Whitby. Altogether, these data point for a critical role of Rad52 SSA activity in triggering replication errors, homology-dependent and independent, during the progression of restarted forks.

In addition, the data suggest that the SSA activity of Rad52 promotes an alternative RDR pathway, but only in the absence of Rad51, and that this SSA-dependent RDR pathway is repressed by the binding of Rad51 to DNA. Previous report have suggested that the binding of Rad51 to DNA is sufficient to repress Rad52 SSA activity (Kishkevich et al., 2022; So et al., 2022). Moreover, my data indicate that this SSA-dependent RDR pathway would occur in the nucleoplasm. However, an important information missing here is the level of RFB-induced RS in the *rad52Δ rad51-113A*.

Nevertheless, to completely understand the dynamics of recombination-dependent DNA synthesis and follow the progression of restarted forks, Pu-seq analysis needs to be applied to the *rad51-113A*, *rad52-R45A* and their respective double mutants. This will provide us a comprehensive idea about the fate of the progressing restarted forks.



**Figure 47: SUMO chains repress replication slippage triggered by Rad52 SSA activity during the progression of Rad51-dependent restarted forks.** Frequency of RFB-induced Ura<sup>+</sup> reversion by replication slippage in indicated strains. Each dot represents one sample from independent biological replicate. Bars indicate mean values ± standard deviation (SD). *p* value was calculated using two-tailed t-test, compare to WT.



## 7. SUMO chains repress replication slippage triggered by Rad52 SSA activity during the progression of Rad51-dependent restarted forks.

The previous experiments support the idea that Rad52 SSA activity act both in a Rad51-dependent and in a Rad51-independent pathway to promote RFB-induced RS. Since SUMO chains rather control the liability of restarted forks to commit replication slippage, I questioned which pathway/activity is under the control of SUMO chains.

In the WT context, I observed a 70% reduction in the frequency of RFB-induced in the *rad51-113A* or in the *rad52-Δ308* mutant and a 60% reduction in the *rad52-R45A* mutant (Figure 47). However, in the context of the *sumo-KallR* mutant, in which fewer forks are able to restart, I observed a similar suppression of the frequency of RFB-induced RS by the *rad51-113A* mutation and *rad52-R45A* mutation. These data indicate that the Rad51-dependent RDR pathway remains functional in the absence of SUMO chains, but the forks that succeeded in restarting are more liable to replication slippage triggered by the Rad52 SSA activity.

### Contributions

Data presented in the co-localisation experiment of Figure 40C was carried out in part by Karol Kramarz, a former postdoc in the team. The RFB-induced RS experiments presented in Figure 41B were carried out by Ismail Iraqui, a former postdoc in the team. Data in Figure 43A and 43B were obtained in collaboration with Tony Carr (University of Sussex) and Karel Naiman (Centre de Recherche en Cancérologie de Marseille). Karel Naiman performed the Pu-Seq experiment. Data in Figure 44B was carried out in part by Kamila Schirmeisen, a former PhD in the team.

# DISCUSSION

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## **I. Slx8 STUbL maintains SUMO homeostasis to protect centromere function.**

SUMO-targeted Ubiquitin Ligases (STUbL) are a unique class of E3-ubiquitin ligases responsible for genome maintenance (refer to chapter 3). The Slx8 STUbL in *S. pombe* is required to target DNA lesions to the nuclear periphery in order to promote replication stress tolerance by counteracting the toxicity of SUMO conjugates (Kramarz et al., 2020a). The precise mechanism via which Slx8 functions in response to replication stress has not been elucidated. Upon further investigation, I uncovered that Slx8 does not form specific DNA-damage induced foci as expected. However, I revealed that Slx8 is responsible for heterochromatin silencing and centromere clustering to support normal centromere function in fission yeast.

### **1. Slx8-GFP does not form supernumerary foci in response to replication stress.**

Replication stress is widely associated with elevated levels of substrate SUMOylation, which is crucial for maintaining genome stability under perturbed replication conditions. This includes SUMOylation-dependent protection of damaged forks via Ubc9 and Mms21 in budding yeast (Branzei et al., 2006). There is also evidence of RPA polySUMOylation during replicative senescence (Churikov et al., 2016b). Additionally, several factors involved in replication restart, including Mre11, Ku, Sgs1, and Rad52, become SUMOylated at stalled replisomes (Psakhye & Jentsch, 2012; Sarangi & Zhao, 2015). Finally, the fate of the SUMOylated substrates are to be bound by STUbLs that are capable of ubiquitinating and degrading the targets to regulate their function (Seeler & Dejean, 2017). Besides promoting protein degradation, evidence shows that SUMO chains regulate chromatin dynamics. An important report in budding yeast demonstrated that difficult-to-repair DSBs and collapsed forks anchor to the NPC in a process requiring the ScSlx5-Slx8 STUbL that physically associates with the Nup84 complex, a component of the NPC (Nagai et al., 2008b). Moreover, newly published work from the team suggests that the Slx8 STUbL activity is essential for the relocation of arrested replication forks to the nuclear periphery (Kramarz et al., 2020a). Thereby, highlighting the idea of Slx8 mediated SUMO metabolism being critical towards mobility of DNA lesions within the nucleus.

Furthermore, previous reports indicate that Slx5, part of the Slx5-Slx8 heterodimeric STUbL complex, forms distinct nuclear foci in budding yeast (Cook et al., 2009b). These Slx5-GFP foci are SUMO dependent and associate with DSBs. Hence, to bridge the gap between SUMO-targeted ubiquitination by Slx8 and the relocation process of stressed

forks, the initial hypothesis for my PhD project was developed. This hypothesis aimed to investigate the temporal and spatial dynamics of Slx8 through live cell imaging in response to global replication stress in *S. pombe*.

Using the live-cell microscopy approach, first I observed that Slx8-GFP forms a single bright focus in 60-70% of S and G2 phase cells (**Publication #3: Figure 1D & 1E**). The remaining 30-40% of cells that did not display a focus may be attributed to the recruitment of Slx8 being below the detectable level of the available microscopy conditions. Additionally, I demonstrated that the single Slx8-GFP focus was partly dependent on the E3-SUMO ligase, Pli1 and not on Nse2 (**Publication #3: Figure 2B & 2C**). This observation was supported by the fact that global SUMOylation is majorly sustained by Pli1, while Nse2 is only responsible for catalysing SUMOylation in response to DNA damage (Prudden et al., 2011b). Although, there is evidence of compensatory activity between Pli1 and Nse2 (Andrews et al., 2005a; X. Zhao & Blobel, 2005), I could not test the overlapping functions between the E3-SUMO ligases in Slx8-GFP foci formation due to the inviability of the *pli1-RING<sup>mut</sup>* and *nse2-RING<sup>mut</sup>* double mutants. I am inclined to believe that, in the absence of Pli1 activity, Nse2 might still be able to sustain the level of SUMOylation necessary to partially form Slx8-GFP foci.

Further investigation established that another determinant for supporting the formation of the Slx8-GFP foci was the formation of SUMO chains. I identified that strains which were incapable of forming SUMO chains (SUMO-*KallR* and SUMO-*D81R*) harboured a two third reduction in Slx8-GFP foci formation in both S and G2 cells (**Publication #3: Figure 2B & 2C**). Moreover, Slx8-GFP foci formed in the SUMO-*KallR* strain were 3 to 4 times less intense (**Publication #3: Figure 2D**). Slx8-GFP foci intensity in the SUMO-*D81R* strain were not analysed, however, they also had a similar appearance as that of SUMO-*KallR* in cell images (**Publication #3: Figure 2B**). This was a clear indication that SUMO chains are crucial parameter for the formation of Slx8-GFP foci.

Ultimately, I aimed to test my initial hypothesis of exploiting Slx8-GFP as a readout of SUMO chains induced by DNA damage. Unfortunately, I could not detect a surplus of Slx8-GFP foci upon treatment with genotoxic agents like MMS (methylmethane sulfonate), HU (Hydroxyurea) and CPT (Camptothecin) in living cells (**Publication #3: Figure 3B & 3C**). Notably, even though there were no additional Slx8-GFP foci, the intensity of the single Slx8-GFP focus increased markedly in response to genotoxic stresses, especially following MMS treatment, compared to the untreated condition. (**Publication #3: Figure 3D**). One potential explanation is that the amount of Slx8 recruited to the DNA damage sites is too low to be detected under our cell microscopy

conditions. On the other hand, the Slx8 foci induced by DNA damage may be highly dynamic and quickly migrate to a specific nuclear region, which could explain the increased intensity of foci we observed without a corresponding increase in their number. Conversely, Slx8-GFP foci formation is critically reduced in the SUMO-*KallR* strain upon treatment with genotoxic agents (**Publication #3: [Figure 3B & 3C](#)**). This suggests that SUMO chains are significantly crucial for maintaining Slx8-GFP foci upon induction of replication stress compared to untreated conditions.

Altogether, this work identifies that Slx8-GFP is capable of forming a single nuclear focus, which is dependent on the formation of SUMO chains and sustained by the E3-SUMO ligase Pli1. However, Slx8-GFP foci cannot be used as a marker of damage-induced SUMO chain formation but the behaviour of the single Slx8-GFP focus is modulated by replication stress in a SUMO-chain dependent manner.

## 2. Localization of Slx8 to the spindle pole body (SPB) sustains centromere structure and function.

Interestingly, I observed that the single Slx8-GFP focus within the nucleus was frequently located at the periphery of the nucleus. First, I validated that more than half (~ 65 %) of the visible Slx8-GFP foci were located at the NP in S-phase cells in WT conditions (**Publication #3: [Figure 4A & 4B](#)**). This frequency dropped to ~ 35 % in WT G2 cells. Moreover, the peripheral Slx8-GFP foci also reduced to ~ 35 % in S-phase cells expressing SUMO-*KallR*. This led to the understanding that most Slx8-GFP foci are enriched at the NP. Notably, the formation as well as the positioning of Slx8-GFP foci is controlled by SUMO chains only during the S-phase.

Importantly, centromeres cluster together at the NP in many organisms and this spatial organisation has been shown to be important for promoting the loading of centromeric proteins (W. Wu et al., 2022b), silencing of repetitive elements (Padeken et al., 2013), and the prevention of micronuclei formation (Jagannathan et al., 2018). In fission yeast, three centromeres are clustered adjacent to the SPB embedded in the nuclear envelope (NE) (Mizuguchi et al., 2015a). Having confirmed that Slx8 is enriched at the NP, I subsequently established that Slx8 displayed a significant co-localization with the clustered centromeres in fission yeast (**Publication #3: [Figure 5A & 5B](#)**). Around 60% of cells harbouring Slx8-GFP foci successfully co-localised with the centromere (marked by Mis6-RFP) and the SPB (marked by Sid4-RFP). Two other chromosomal regions that are enriched at the nuclear periphery include the telomeres and the sexual mating loci in *S. pombe*. A significant amount of co-localization (~ 20 %

of the cells) was observed between Slx8-GFP and the mating region (marked by *mat2:mCherry*). However, no significant co-localization was detected between Slx8-GFP and the telomere (marked by Taz1-RFP) (**Publication #3: Figure 5A & 5B**). Therefore, Slx8-GFP foci enriched at the nuclear periphery specifically marks the clustered centromeres, the SPB and the *mat* region, but not the telomeres.

Additionally, since centromeres are enriched in heterochromatin that maintains gene silencing. I investigated the relationship between the formation of Slx8-GFP foci, which I had previously observed to be enriched at the centromere and the SPB, with heterochromatin formation at the clustered centromeres. I detected a 2 fold reduction in the formation of Slx8-GFP foci in the absence of the histone methyl transferase, Clr4 that promotes H3-K9 methylation for ensuring heterochromatin maintenance and gene silencing (**Publication #3: Figure 6A & 6B**). Contrary to the reduction in Slx8-GFP foci formation observed in *clr4Δ* cells, no effect was observed in cells lacking Dicer (Dcr1) (**Publication #3: Figure 6A & 6B**), which is a core component of the RNA interference (RNAi) pathway that processes double-stranded RNA (dsRNA) to small interfering RNAs (siRNAs). The RNAi pathway is rather a pre-requisite for targeting the nucleation of heterochromatin assembly at the centromere (Emmerth et al., 2010). Further maintenance of heterochromatin is majorly dependent upon the Clr4-complex rather than the RNAi pathway (Shanker et al., 2010). This could be a possible explanation for the dependence of Slx8-GFP foci formation in the *clr4Δ* mutant rather than the *dcr1Δ* mutant. Besides, I monitored the loss of Slx8-GFP foci in the Csi1 deletion mutant (*csi1Δ*) and observed a similar phenotype as the *clr4Δ* strain. The lack of Csi1 led to severe defect in centromere clustering in *S. pombe* (Hou et al., 2012a) and displayed a 2 fold reduction in the frequency of Slx8-GFP foci in the *csi1Δ* cells as compared to WT (**Publication #3: Figure 6A & 6B**). Therefore, I concluded that both heterochromatin formation and centromere clustering contribute to Slx8-GFP focus formation.

The next critical question was whether Slx8 plays a role in promoting heterochromatin silencing and centromere clustering. This question was addressed in collaboration with Joe Strachan and Prof. Elizabeth Bayne from the University of Edinburgh, UK. Our collaborators performed RT-qPCR analysis of transcripts from the heterochromatic pericentromere and the silent mating-type (*mat*) regions, in order to assess the rate of heterochromatin loss in the absence of factors like Clr4 and Slx8. Interestingly, compared to the WT, they observed an increased accumulation of transcripts in the *clr4Δ* mutant and a small but significant increase in the *slx8Δ* mutant (**Publication #3: Figure 7A**). Remarkably, loss of heterochromatin in the absence of Slx8 seemed to have a more pronounced effect on the *mat* region as compared to the pericentromeric

region (2.5-fold increase in transcript expression). This indicates that Slx8 is involved in gene silencing at the centromere and the mating type locus, with variable efficiency.

In addition, centromere clustering was assessed by the co-localisation between GFP-Cnp1 (*S. pombe* CENP-A, the centromere-specific histone variant) and Sid4-RFP that marks the SPB. Centromere clustering was recognised to be significantly disrupted in the *slx8Δ* mutant (**Publication #3: Figure 7B & 7C**). Around 12% of *slx8Δ* cells displayed more than one GFP-Cnp1 focus revealing the loss in centromere clustering. Moreover, an epistatic phenotype was observed in the *slx8Δ csi1Δ* double mutant cells, which exhibited clustering defects similar to those of the *csi1Δ* single mutant, suggesting that Slx8 might operate in the same pathway as Csi1 (**Publication #3: Figure 7B & 7C**). Likewise, deletion of the SUMO ligase Pli1 significantly mitigated the defect linked to the absence of Slx8. This indicates that the absence of Slx8 may be resulting in excessive SUMOylation and hence, deletion of Pli1 rescues the loss of centromere clustering observed in the *slx8Δ* mutant to a certain extent.

Therefore, we characterize that the localization of Slx8 near the SPB is both reliant on and crucial for maintaining heterochromatin integrity and centromere clustering.

So what could be the function of Slx8 at the centromeres in *S. pombe*? One straightforward explanation would be that the ubiquitin ligase activity of Slx8 promotes the ubiquitination and removal of SUMOylated factors from the centromere to sustain centromere function. This is in accordance with the rescue of centromere clustering observed in the *slx8Δ pli1Δ* double mutants as compared to the *slx8Δ* mutant (**Publication #3: Figure 7B & 7C**). Evidence in *S. pombe* showing chromosome segregation defects in cells lacking Slx8, which are dependent on the Pli1 SUMO ligase, further supports the explanation mentioned earlier (Steinacher et al., 2013a). Additionally, proteomic analyses revealed that over a third of SUMOylated proteins regulated by Slx8 and Ufd1 are associated with centromeres or telomeres, including key heterochromatin regulators such as the H3K9 methyltransferase Clr4 and the anti-silencing factor Epe1 (Køhler et al., 2015a). Alternatively, Slx8 might indirectly promote the maintenance of protein factors that are required for centromere clustering and supports the downstream centromere structure and function. However, I lean towards the former hypothesis due to the clear indication of *pli1Δ* rescuing the *slx8Δ* phenotype. Thereby, suggesting the importance of Slx8 in removing deleterious SUMOylation and maintaining an optimal balance for preserving centromere function.



## II. Existence of a spatially segregated, NPC-independent recombination-dependent replication pathway.

The relocation of perturbed replication forks to the nuclear periphery and their anchorage to NPCs facilitate the resumption of DNA synthesis at terminally dysfunctional forks by promoting a recombination-dependent replication restart mechanism.

However, it was noticed that in some genetic backgrounds recombination-dependent replication restart can also occur without a routing towards the nuclear periphery and NPC anchorage. This applied to cells expressing the mutated allele of SUMO, which is unable to form SUMO chains (*SUMO-KallR*) ([Figure 42B](#)). In both cases SUMOylation is hampered, which likely bypasses the need for anchorage to Ulp1-associated NPC to overcome the inhibitory effects of SUMO conjugates on the initiation of DNA synthesis. Therefore, one pathway is triggered by SUMO chains formation and followed by the relocation to the nuclear periphery, while a second pathway could exist, which is signalled by mono-SUMOylation and occurs within the nucleoplasm. Previous work from our team demonstrated that in *rad51Δ* and *rad51-ll3A* (incapable of strand-exchange activity) cells the effectiveness of recombination-dependent fork restart was decreased by 60-70% (Ait Saada et al., 2017a). Such partial reduction indicated that the remaining fork restart occurs through a Rad51-independent pathway. Consistent with this observation, a recent study in fission yeast from the lab of Prof. Whitby has reported that replication forks arrested at the RFB can be restarted by a Rad51-independent pathway, which relies on the single strand annealing (SSA) activity of Rad52 (Kishkevich et al., 2022). This study reported that the initiation of restarted forks are driven by Rad51.

In the quest to decipher the existence of a second pathway of fork restart, first, I inspected the processing of D-loops downstream of arrested forks at the *RTS1*-RFB to identify factors that contribute to RFB relocation to the NP. I observed that the most important determinant of RFB relocation to the nuclear periphery is the formation of D-loops ([Figure 40B & 40C](#)). I revealed that none of the mutants involved in maintaining the stability of D-loops go to the NP. However, I cannot rule out the possibility of a transient association with the NPC, which is insufficient to visualise the RFB enrichment at the NP via snapshot microscopy. Surprisingly, I further noticed that all the mutants (apart from *rad55Δ*) showing a defect in RFB relocation to the NP, had absolutely no correlation in the rate of fork restart assayed by the RFB-induced RS assay ([Figure 41A & 41B](#)). Mutants like *srs2Δ* and *rad54Δ* displayed a variable increase, *fbh1Δ* and *rqh1Δ* behaved more like WT, while *fml1Δ* showed a reduction. There could be several

hypotheses: either different restart pathways occur within the nucleoplasm and lead to the variability observed in the RFB-induced RS of different mutants, or the RFB-induced RS assay may not be the readout of restart efficiency only.

The second hypothesis was supported by the Pu-seq data on the *sumo-KallR* mutant that we received from our collaborators, Tony Carr and Karel Naiman ([Figure 43A & 43B](#)). It has been previously published and re-confirmed by myself that RFB-induced RS is increased in the *sumo-KallR* mutant (Kramarz et al., 2020a) ([Figure 42A & 42B](#)). Our previous hypothesis was inclined towards the idea that SUMO chains have an inhibitory effect on restarted DNA synthesis. However, the Pu-seq data revealed a contradictory result, revealing a defect in fork restart without any effect on the speed of fork progression in the *sumo-KallR* mutant. This was a clear indication that the results of RFB-induced RS assay might have been over-simplified and encompasses other parameters of restarted DNA synthesis. One possible way of interpretation is that the RFB-induced RS is not just a readout of fork restart, but also monitors the fidelity of the restarted forks. Hence, the inability to form SUMO chains led to a 30-40% defect in fork restart as observed from the Pu-seq data and SUMO chains are indeed required to promote fork restart. However, the increased RFB-induced RS in the *sumo-KallR* mutant might reflect an increased rate of replication slippage occurring in the forks (60-70%) that were capable of doing the fork restart. Thus, I conclude that SUMO chains might be important to regulate the fidelity of restarted DNA synthesis at least in the nucleoplasm. Other factor(s) may be involved in modulating the fidelity of restarted replication, potentially requiring poly-SUMOylation to minimize the mutagenicity of restarted forks.

Consequently, I established that SUMO chains are no longer required to limit the frequency of RFB-induced RS when forks are constitutively enriched in the NPC environment ([Figure 44A & 44B](#)). This data suggests that SUMO chains ensure the fidelity of fork restart and limits mutagenicity of restarted DNA synthesis predominantly in the nucleoplasm. This result along with the previously identified lack of correlation between RFB relocation with the frequency of RFB-induced RS, (that monitors both restart efficiency and the fidelity of restarted DNA synthesis) led to the possibility of having at least two RDR pathways, each with distinct control on DNA synthesis fidelity, depending on nuclear positioning.

So what is this pathway? As proposed by Prof. Whitby where they describe that template switching during the elongation phase of restarted forks are more reliant on the SSA activity of Rad52 (Kishkevich et al., 2022), I hypothesized that Rad52's SSA activity might contribute to the RDR pathway occurring in the nucleoplasm. In order to test that, first I ruled out the possibility of Rad52's SSA activity in RFB relocation to

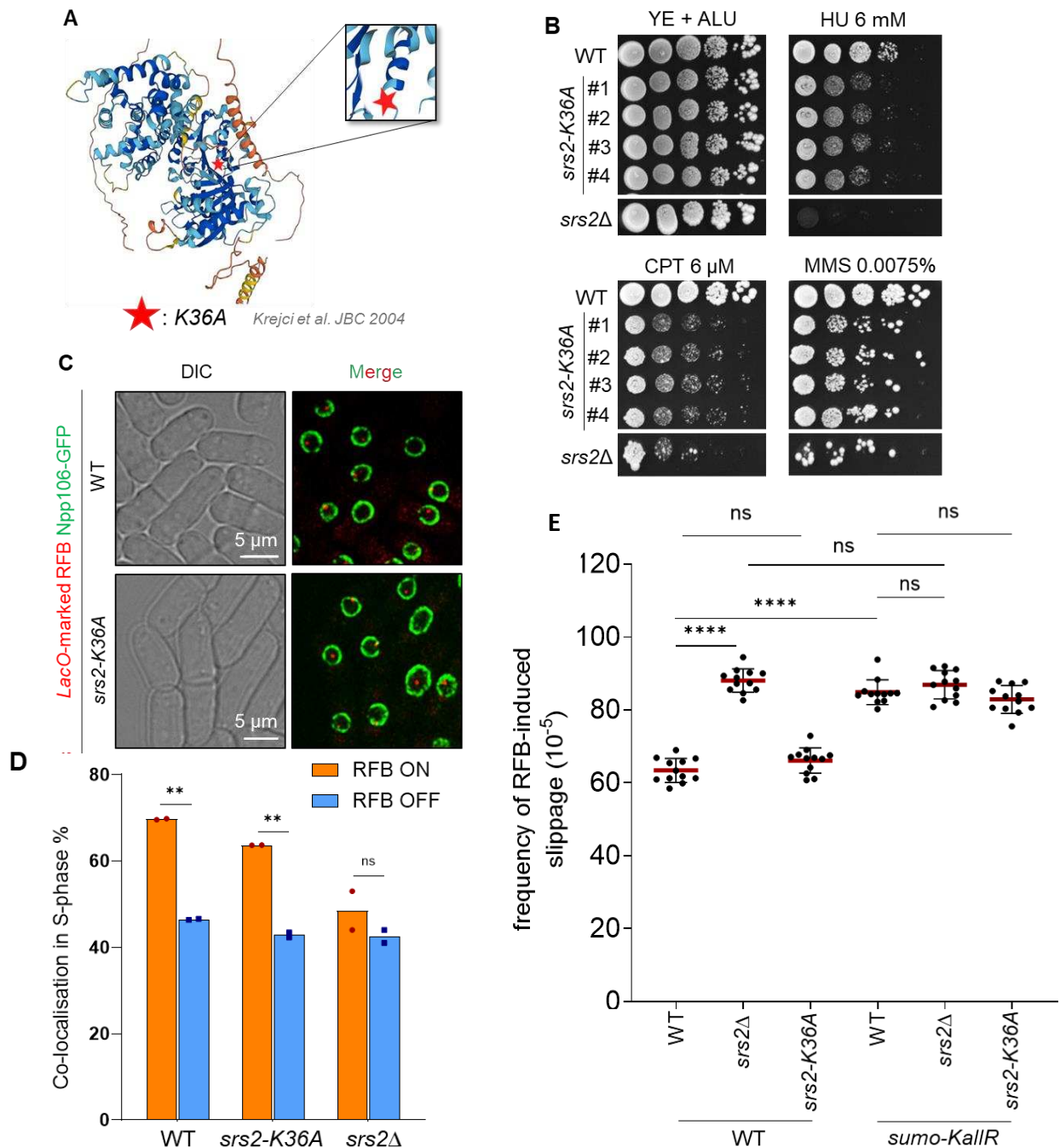
ensure that Rad52 SSA is dispensable for favouring NPC routing. Further, I addressed how the SSA activity of Rad52 affects RFB-induced RS. I observed that a large fraction of the slippage are dependent on the SSA activity of Rad52 in the WT condition ([Figure 46](#)). Moreover, RFB-induced RS in the *rad51-113A* mutant were epistatic to the *rad52-R45A*, with a slight dominance of Rad52's SSA activity over the strand-exchange activity of Rad51. This difference in the RFB-induced RS could be a genetic effect of replication slippage or fidelity, or just a growth defect. However, the most eye-opening data was that the RFB-induced RS in the *rad51-113A* single mutant (showing a 70% reduction in RFB-induced RS) was not abolished in the *rad51-113A rad52-R45A* double mutant. This indicates that the role of the SSA activity of Rad52 in promoting slippage is in the same pathway as the strand-exchange activity of Rad51. Nevertheless, the frequency of RFB-induced RS in the *rad51Δ rad52-R45A* double mutant was almost completely abolished, similar to that of the *rad51Δ rad52Δ* double mutant. This is the only data that suggests there may be an alternative fork restart pathway in the nucleoplasm that operates exclusively in the absence of Rad51. This pathway of RFB-induced RS is entirely dependent on the SSA activity of Rad52. Additionally, this data goes in view with the results obtained by other researchers, suggesting that the binding of Rad51 can repress the SSA activity of Rad52 (Kishkevich et al., 2022; So et al., 2022). Hence, Rad52 SSA activity act both in a Rad51-dependent and in a Rad51-independent pathway to promote RFB-induced RS.

The next question was, what could be affected in the *sumo-KallR* mutant?

Most of the RFB-induced RS is known to rely on Rad51's strand exchange activity when SUMO chains can form (WT condition). Nonetheless, I observed that even in the absence of SUMO chains, RFB-induced RS remains largely dependent on the strand-exchange activity of Rad51 and the Rad51-Rad52 interaction (*sumo-KallR* mutant). ([Figure 47](#)). This indicates that the increase in the rate of RFB-induced RS in *sumo-KallR* requires the classical Rad51-mediated pathway. Additionally, it is worth noting that RFB-induced RS was not completely dependent on the strand-exchange activity of Rad51 in the WT condition. However, in the *sumo-KallR* mutant, RFB-induced RS is completely dependent on the strand exchange activity of Rad51, even when forks reside within the nucleoplasm. This emphasizes that a lesser fraction of the Rad51-dependent RFB-induced RS also occurs within the nucleoplasm. Although what SUMO chains do seem to control is the SSA activity of Rad52, which is in part responsible for the RFB-induced RS occurring in the Rad51-mediated pathway. This is due to the observed reduction in the frequency of RFB-induced RS in the *rad52-R45A sumo-KallR* double mutant as compared to the *rad52-R45A* single mutant ([Figure 47](#)). So there could be two possibilities of fork restart and subsequent progression: either fork restart

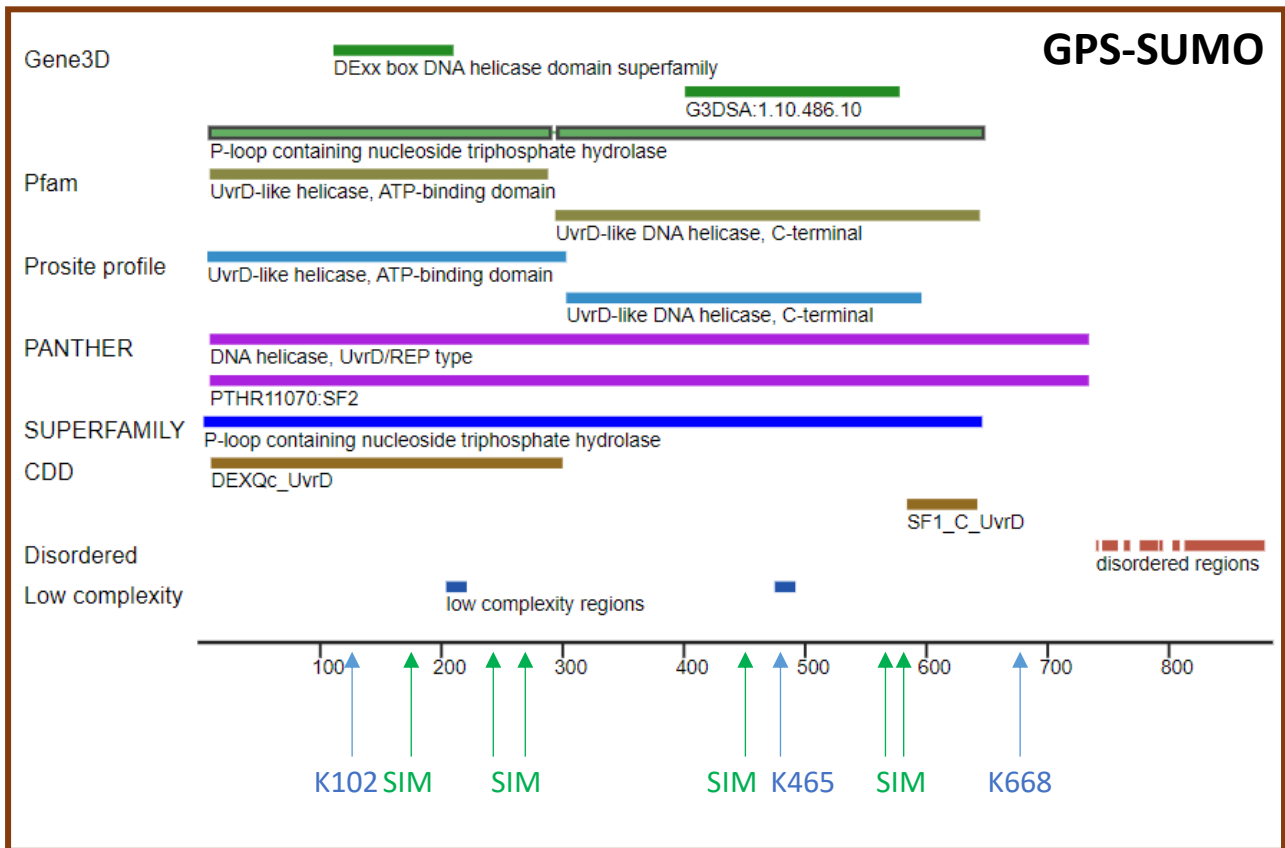
**Figure 48: Helicase activity of Srs2 is dispensable for relocation of arrested forks and limiting RFB-induced RS.**

**A:** Diagrammatic representation of the helicase-dead mutant of *srs2* in fission yeast. **B:** Sensitivity of indicated strains to indicated genotoxic drugs. Ten-fold serial dilution of exponential cultures were dropped on appropriate plates. HU: hydroxyurea; CPT: camptothecin and MMS: methyl methane sulfonate. **C:** Example of bright-field (left panel, DIC) and fluorescence (right panel) images of cells expressing the endogenous Npp106-GFP fusion protein and harboring the *LacO*-marked RFB in indicated strains. Mono-nucleated cells and septated bi-nucleated cells correspond to G2 and S-phase cells, respectively. Scale bare: 5µm. **D:** Quantification of co-localization events, shown in C, in S-phase cells in indicated conditions and strains. Dots represent values obtained from two independent biological experiments. At least 200 nuclei were analyzed for each strain and condition. Fisher's exact test was used for group comparison to determine the *p* value (\*\*  $p \leq 0.01$ ; ns: non-significant). **E:** Frequency of RFB-induced *Ura*<sup>+</sup> reversion by replication slippage in indicated strains. Each dot represents one sample from independent biological replicate. Bars indicate mean values  $\pm$  standard deviation (SD). *p* value was calculated using two-tailed t-test, compare to WT.



is brought about by the combined action of Rad51's strand exchange activity and SSA activity of Rad52. However, since Rad51 binding represses the SSA activity of Rad52 (Kishkevich et al., 2022; So et al., 2022), and fork restart depends on the SSA activity of Rad52 entirely, only when Rad51 is absent. I favour the second hypothesis, which proposes that fork restart is initiated by Rad51's strand exchange activity, while during fork progression, the restarted DNA synthesis which is error prone might be mediated by the SSA activity of Rad52. This error-prone DNA synthesis could actually be limited by the formation of SUMO chains. Hence, SUMO chains might repress the SSA activity of Rad52 during the progression of Rad51-dependent restarted forks. This also hints towards the idea that Rad52 could be a target of SUMOylation. Indeed, Rad52 is reported to be SUMOylated in budding yeast. SUMOylation of Rad52 is known to attenuate the DNA binding and single-strand annealing activities of Rad52, which affects the regulation of recombination *in vivo* (Altmannova et al., 2010). Another possible target that could be controlled by SUMO chains is Srs2. Preliminary data suggests that Srs2 is a promising target of SUMOylation due to various reasons. Firstly, the absence of Srs2 displays a defect in RFB relocation to the NP and indicates that Srs2 is required for RFB relocation (Figure 40C). Interestingly, the relocation of RFB to the NP is not dependent upon the helicase activity of Srs2. (Figure 40C). Secondly, *srs2Δ* exhibits an increased rate of RFB-induced RS, similar to what has been observed in the *sumo-KallR* mutant (Figure 48E). However, the level of RFB-induced RS in the double mutant *srs2Δ sumo-KallR* was similar to the ones observed in each single mutant, indicating that Srs2 and SUMO chains act in a same pathway to prevent RFB-induced RS (Figure 48E). Additionally, I also revealed that the role of Srs2 in preventing RFB-induced RS was not dependent on its ATPase activity, since the frequency observed in *srs2-K36A* (ATPase-dead mutant of Srs2) was comparable to that of *srs2Δ* (Figure 48E). Hence, if the ATPase activity is dispensable for RFB relocation and the increased RFB-induced RS, there remains a fair chance of Srs2 being regulated by SUMOylation to mediate fork positioning and restart. Furthermore, it is essential to highlight that Srs2 is reported to interact with SUMOylated PCNA in budding yeast (Armstrong et al., 2012). Budding yeast Srs2 contains a C-terminal PIP (PCNA-interacting protein) box motif, which allows Srs2 to interact with PCNA and affect downstream repair process (Burkovics et al., 2013). However, it is critical to mention here that the PIP box motif is not conserved in *S. pombe*'s Srs2, but putative SIM (SUMO-interaction Motif) domains and SUMOylation sites have been identified using the GPS SUMO software (Figure 49). Overall, this suggests that Srs2 could be a good candidate for SUMO-based regulation of fork positioning and restart within the nucleus.

**Figure 49: Putative SUMOylation and SIM domains identified in Srs2 using the GPS SUMO software in budding yeast.** Three residues marked with blue arrows depict the putative lysine residues capable of SUMOylation. Six regions marked with green arrows represent the putative SIM domains.



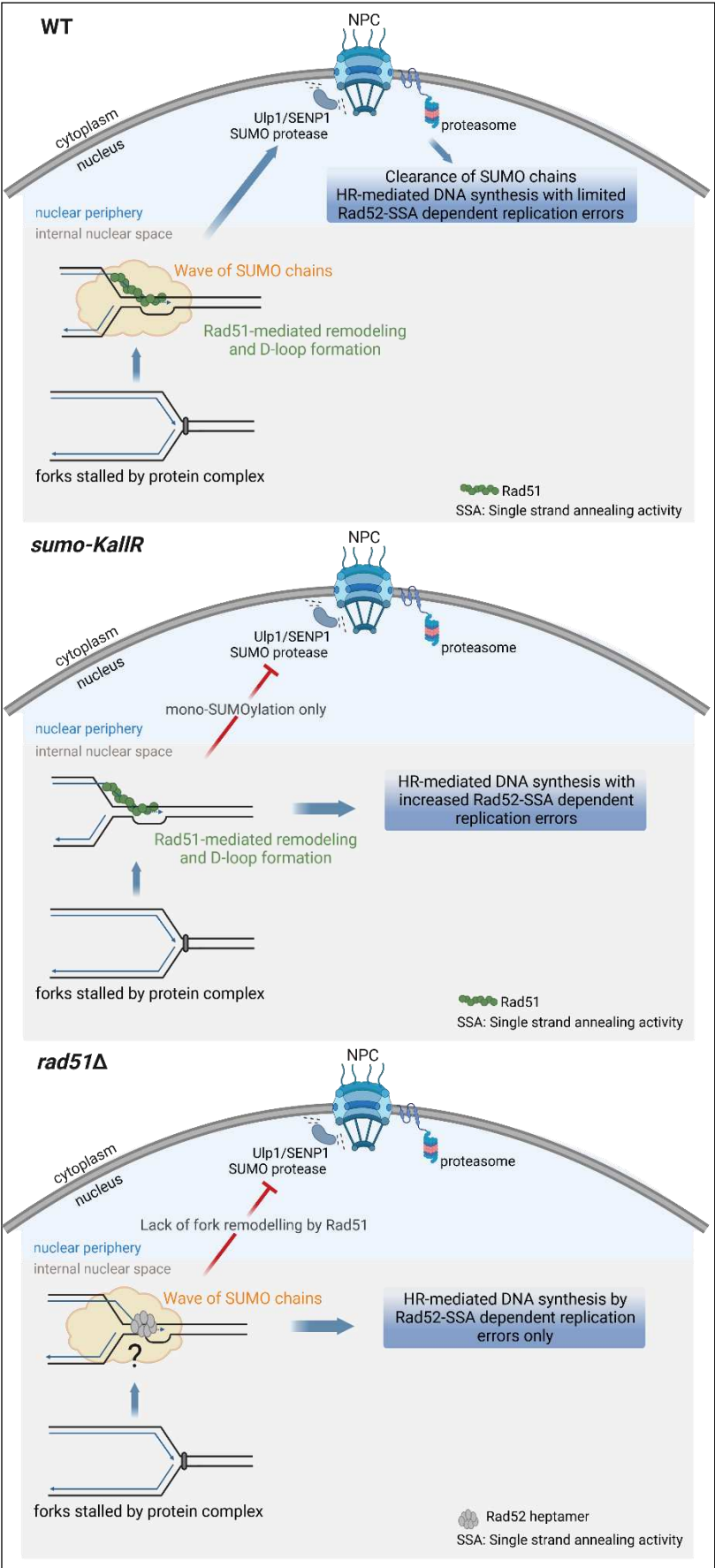
To sum up the data obtained so far, I have introduced a schematic representation of the Rad51-dependent and -independent pathways active within the nucleus ([Figure 50](#)). This visual depiction illustrates the step-wise flow of events starting from fork arrest to the restart of DNA synthesis in WT, the *sumo-KallR* mutant and the *rad51Δ* mutant. In the WT condition, RFBs relocate to the NPC to get rid of the SUMO chains by the Ulp1 SUMO protease enriched at the NP. This pathway of RDR is largely dependent on the strand-exchange activity of Rad51. Additionally, in the WT condition, the rate of replication errors decrease because of the reduced reliance on Rad52's SSA activity for RFB-induced RS due to the formation of SUMO chains. However, in the *sumo-KallR* mutant, where RFBs are no more enriched at the NPC, a fraction of the Rad51-dependent pathway is still active within the nucleoplasm. In this condition, where cells are capable of performing mono-SUMOylation only, increased RFB-induced RS in the *sumo-KallR* mutant ([Figure 42B](#)) could be explained by the increased replication errors carried out by the SSA activity of Rad52 working in concert with the nucleoplasmic Rad51-dependent pathway. Finally, in the *rad51Δ*, the sole RDR pathway that remains active is completely independent of Rad51. The RDR pathway in this mutant is dependent on the SSA activity of Rad52 entirely and therefore, the RFB-induced RS in the *rad51Δ rad52-R45A* is totally abolished ([Figure 46](#)). Due to the dependence of RDR upon the SSA activity of Rad52, this mutant is much more prone to replication errors. However, in this scenario, it cannot be excluded that the replication errors are caused due to unprotected forks that results from the absence of Rad51. Additionally, it needs to be mentioned that evidence suggests that the N-terminal domain of Rad52, which harbours the SSA activity is capable of promoting the annealing between complementary ssDNA and forms D-loops between ssDNA and supercoiled plasmid DNA (Doe et al., 2004; Kagawa et al., 2001, 2008; Mortensen et al., 1996). However, it is not clear if D-loops are still formed in the absence of Rad51 or exactly how Rad52's SSA activity operates between the resected fork and the region of homologous or homeologous ssDNA to bring about fork restart when Rad51 is not present.

Overall, my data elucidates an alternative pathway of fork restart within the nucleoplasm, and highlights the novel role of the SSA activity of Rad52 in orchestrating fork restart within the nucleoplasm.

### III. Conclusions

The research of our team is focused onto better understand how replication stress is resolved within the nuclear architecture. Previous studies have shown how protein-mediated fork stalled at unique sequences are routed to the NPC for further fork restart by homologous recombination dependent pathways. The NPC routing of stalled forks

**Figure 50: Graphical abstract representing the spatially-segregated pathways of fork restart as elucidated in fission yeast.**





has been demonstrated to be dependent upon the formation of SUMO chains and requires the Slx8 STUbL at the same time. Therefore, my PhD project was initially aimed at deciphering if the Slx8 STUbL could be utilised as a readout of SUMO chains induced by DNA damage, based on the crosstalk between the STUbL family and maintenance of genome stability. Although I was not able to fulfil the primary hypothesis, I characterised how Slx8 promotes centromere clustering and gene silencing at heterochromatin domains. This work has given rise to an article that established a functional and physical interaction between the STUbL and heterochromatin to maintain the nuclear organization of specific domains in fission yeast. Additionally, I was interested in a second project that aimed to better understand the hypothesis of the existence of a second, spatially segregated RDR pathway prevailing in the nucleoplasm as compared to the one at the NP. The NPC routing pathway of fork restart requires the formation of SUMO chains, HR factors that promote D-loop stability followed by processing of SUMO chains by the Ulp1 protease at the NP to initiate fork restart. Progression of restarted forks is maintained by the proteasome whose activity is enriched at the nuclear periphery. However, I present for the first time, evidence for the existence of another pathway that occurs by mono-SUMOylation, in the nucleoplasm when the NPC pathway is disrupted. It is crucial to mention here, that the second RDR pathway identified is actually not independent of Rad51's strand exchange activity. Since, the team has previously demonstrated that the RDR pathway taking place by NPC routing is dependent on the strand exchange activity of Rad51 (Kramarz et al., 2020a). My initial hypothesis was inclined towards the idea that RDR pathway within the nucleoplasm would be independent of Rad51's strand exchange activity. However, the pathway taking place within the nucleoplasm is also partly dependent upon Rad51's strand exchange activity. Interestingly, what I rather identify here is the essential role of SUMOylation in limiting the mutagenicity of restarted forks. SUMO chains are required to limit mutagenicity when forks are maintained in the nucleoplasm and are no longer required when forks are enriched at the NP, which provides a better "fork protective" environment by mechanisms that remain to be discovered.

Taken together, my results suggest that the key determinant of replication fork integrity and restart depends on SUMO metabolism. SUMOylation controls the spatially-segregated pathways of fork restart in the context of nuclear positioning of replication stress sites. This highlights how the disruption of SUMO balance, commonly observed in various human diseases, including cancer, affects the preservation of genome integrity at sites of replication stress. Such essential research, which deeply uncovers the mechanisms by which cells can naturally combat genetic instability, is crucial for developing effective anti-cancer therapies.

# MATERIALS AND METHODS

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## **Standard yeast genetics and biological resources**

Gene mutation and tagging were performed by classical genetic techniques.

Plate viability assay: To assess the sensitivity of the strains (in this study) to genotoxic agents, mid log-phase cells were serially diluted and spotted onto yeast extract agar plates containing hydroxyurea (HU), methyl methanesulfonate (MMS) and camptothecin (CPT).

## **Live cell imaging**

For snapshot microscopy, cells were grown in filtered EMMg with or without 60  $\mu$ M thiamine for 24 hours to exponential phase (RFB OFF and RFB ON), then centrifuged and resuspended in 500  $\mu$ L of fresh EMMg. 1  $\mu$ L from the resulting solution was dropped onto Thermo Scientific slide (ER-201B-CE24) covered with a thin layer of 1.4 % agarose in filtered EMMg (Kramarz et al., 2020c). 21 z-stack pictures (each z step of 200 nm) were captured using a Nipkow Spinning Disk confocal system (Yokogawa CSU-X1-A1) mounted on a Nikon Eclipse Ti E inverted microscope, equipped with a 100x Apochromat TIRF oil-immersion objective (NA: 1.49) and captured on sCMOS Prime 95B camera (Photometrics) operated through MetaMorph<sup>®</sup> software (Molecular Devices). GFP and m-Cherry proteins were excited with a 488 nm (Stradus<sup>®</sup> - Vortran Laser Technology, 150mW) and a 561 nm (Jive<sup>™</sup> - Cobolt, 100mW) lasers, respectively. A quad band dichroic mirror (405/488/568/647 nm, Semrock) was used in combination with single band-pass filters of 525/50 or 630/75 for the detection of GFP and m-Cherry, respectively. Fluorescence and bright-field 3D images were taken at every 0.3 $\mu$ m over a total of 4.5 $\mu$ m thickness. Exposure time for GFP channel was 500 ms, for mCherry 1000 ms. During the imaging, the microscope was set up at 25°C.

## **Image analysis**

To study the co-localization time between lacO/LacI RFB foci and Npp106-GFP cells grown in the above conditions and visualized with a Nikon inverted microscope described above, using two fluorescent channels with 491 and 561 nm diode lasers, 100 mX (Cobolt). Images were captured every 10 s with 14 optical slices (each z step of 300 nm) for 30 min with 100 ms exposure time both for GFP and mCherry channels at 15% of laser power using METAMORPH software. Images were mounted and analyzed with ImageJ software (description below). The 3D Z series of both fluorophore channels were merged together in order to analyze the co-localization between Npp106-GFP marking the nuclear periphery in green along with the red dot of the LacO-marked RFB within the nucleus, at each stack. The co-localization of the red dot with the green periphery was done carefully in order to calculate the percentage of cells having the RFB enriched at the nuclear periphery in ON vs OFF condition.

### **Whole protein extract analysis**

Aliquots of  $1 \times 10^8$  cells were collected and disrupted by bead beating in 1 mL of 20 % TCA (Sigma, T9159). Pellets of denatured proteins were washed with 1M Tris pH 8 and resuspended in 2x Laemmli buffer (62.5 mM Tris pH 6.8, 20 % glycerol, 2 % SDS, 5 %  $\beta$ -mercaptoethanol with bromophenol blue). Samples were boiled before being subjected to SDS-PAGE on Mini-PROTEAN TGX Precast Gel 4-15 % (Biorad, 4561086). Western blot using anti-FLAG (Sigma, F1804), anti-tubulin (Abcam, Ab6160) and anti-SUMO (non-commercial, produced in rabbit by Agro-Bio) antibodies were performed.

### **Pulldown of Srs2-FLAG**

Aliquots of  $1.10^7$  cells (50 mL culture for each strain) of WT cells and Srs2-FLAG tagged cells were grown to. Cell pellets were collected, washed once with 1 ml of 20% TCA. For immunoprecipitation (IP) in denaturing conditions, cells were resuspended in 1 ml of 20% TCA and disrupted by bead beating (3 x 6500 rpm). Precipitates were recovered by centrifugation (10 min, 13,000 rpm, 4°). Pellets were washed with 1 mL of 1M Tris pH8. Denatured proteins in pellets were resuspended in 500  $\mu$ l of 2x Laemmli buffer without bromophenol blue and boiled for 5 min. Then, 10  $\mu$ l of the resulting protein extracts was used as a loading control for the IP reaction. For each IP, 100  $\mu$ l of protein extract was diluted with 400  $\mu$ l of IP buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40) and 5  $\mu$ l of anti-FLAG (F-1804; Sigma) antibody was added. The IP reaction was done overnight at 4° on a rotator wheel. The negative control for each IP was the diluted protein extract with IP buffer without anti-Srs2 antibody. Prewashed (washed like in ChIP) Protein G Magnetic Beads (Dynabeads; Invitrogen, Carlsbad, CA) were added for additional 2 hr at 4°. Beads were washed three times with IP buffer and bound proteins were eluted with 20  $\mu$ l of 2x Laemmli buffer (with bromophenol blue) for 10 min at 65°. The eluted proteins are subjected to be resolved on 2 different Mini-PROTEAN TGX Precast Gel 4–15% (Bio-Rad, Hercules, CA). Western blot using anti-FLAG (F-1804; Sigma) and anti-SUMO antibody was performed.

### **Replication slippage assay**

The frequency of *ura4+* revertants using the *ura4-sd20* allele was determined as follows. 5-FOA (EUROMEDEX, 1555) resistant colonies were grown on plates containing uracil with or without thiamine for 2 days at 30 °C and subsequently inoculated into EMMg supplemented with uracil for 24 h. Then cultures were diluted and plated on EMMg complete (for cell survival) and on EMMg without uracil, both supplemented with 60  $\mu$ M thiamine. After 5-7 days of incubation at 30°C colonies were counted to determine the frequency of *ura4+* reversion. To obtain the occurrence of replication slippage by the *RTS1*-RFB, independently of the genetic background, we subtracted

the replication slippage frequency of the strain having RFB in OFF condition (considered as spontaneous frequency) from the frequency of the strain containing the *t-ura4sd20<ori* construct, upon expression of Rtf1.

### **Pu-Seq**

The published protocol (Keszthelyi et al., 2015) was used with minor modifications: size selection was performed using a Blue Pippin (Sage Science). We used *rnh201-RED* instead of *rnh201::kan* (Naiman et al., 2021b). Sequence files were aligned with Bowtie2 and alignment data converted to counts with custom Perl script (Keszthelyi et al., 2015). Analysis of polymerase usage was performed with custom R script (Keszthelyi et al., 2015). Sequence data is available under GEO dataset GSE247371.

### **STATISTICAL ANALYSIS**

Cell imaging was performed using METAMORPH software and processed and analyzed using ImageJ software (Schindelin et al., 2012). The explanation and definitions of values and error bars are mentioned within the figure legends. In most experiments, the number of samples is > 2 and obtained from independent experiments to ensure biological reproducibility. For all experiments based on the analysis of cell imaging, the number of nuclei analyzed is mentioned in the figure legends. Statistical analysis was carried out using Fisher's exact test and Student's *t*-test. N/S,  $P \geq 0.05$ , \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ .

Supplementary Table 1: strain list

Strain number	Mating type	Genotype	Reference
KK1311	<i>h</i> -	<i>npp106-GFP:natMX6 arg3::mCherry-LacI nmt41:rtf1:sup35 ade6-704 leu1-32 t-LacO 7,9Kb:Kan:ura4<sup>+</sup>&lt;ori (uraR)</i>	this study
KK600		<i>srs2::natMX6 npp106-GFP:natMX6 arg3::mCherry-LacI nmt41:rtf1:sup35 ade6-704 leu1-32 t-LacO 7,9Kb:Kan:ura4<sup>+</sup>&lt;ori (uraR)</i>	this study
KK601	<i>h</i> +	<i>rad54::hphMX6 npp106-GFP:natMX6 arg3::mCherry-LacI nmt41:rtf1:sup35 ade6-704 leu1-32 t-LacO 7,9Kb:Kan:ura4<sup>+</sup>&lt;ori (uraR)</i>	this study
KK648		<i>fml1::hphMX6 npp106-GFP:natMX6 arg3::mCherry-LacI nmt41:rtf1:sup35 ade6-704 leu1-32 t-LacO 7,9Kb:Kan:ura4<sup>+</sup>&lt;ori (uraR)</i>	this study
KK2039	<i>h</i> +	<i>fbh1::hphMX6 npp106-GFP:natMX6 arg3::mCherry-LacI nmt41:rtf1:sup35 ade6-704 leu1-32 t-LacO 7,9Kb:Kan:ura4<sup>+</sup>&lt;ori (uraR)</i>	this study
KK2025	<i>h</i> -	<i>rqh1::kanMX6 npp106-GFP:natMX6 arg3::mCherry-LacI nmt41:rtf1:sup35 ade6-704 leu1-32 t-LacO 7,9Kb:Kan:ura4<sup>+</sup>&lt;ori (uraR)</i>	this study
KK2042	<i>h</i> +	<i>rhp55::hphMX6 npp106-GFP:natMX6 arg3::mCherry-LacI nmt41:rtf1:sup35 ade6-704 leu1-32 t-LacO 7,9Kb:Kan:ura4<sup>+</sup>&lt;ori (uraR)</i>	this study
KK2047	<i>h</i> +	<i>sfr1::kanMX6 npp106-GFP:natMX6 arg3::mCherry-LacI nmt41:rtf1:sup35 ade6-704 leu1-32 t-LacO 7,9Kb:Kan:ura4<sup>+</sup>&lt;ori (uraR)</i>	this study
KK2049		<i>swi10::kanMX6 npp106-GFP:natMX6 arg3::mCherry-LacI nmt41:rtf1:sup35 ade6-704 leu1-32 t-LacO 7,9Kb:Kan:ura4<sup>+</sup>&lt;ori (uraR)</i>	this study
KK1707	<i>h</i> +	<i>nmt41:rtf1:sup35 ade6-704 leu1-32 t- ura4-SD20&lt;ori (uraR)</i>	this study
KK1222	<i>h</i> -	<i>srs2::natMX6 nmt41:rtf1:sup35 ade6-704 leu1-32 t- ura4-SD20&lt;ori (uraR)</i>	this study
KK1230	<i>h</i> -	<i>rad54::hphMX6 nmt41:rtf1:sup35 ade6-704 leu1-32 t- ura4-SD20&lt;ori (uraR)</i>	this study
KK2014	<i>h</i> +	<i>srs2-K36A ade6-704 leu1-32 ura4-D18</i>	this study
KK2069		<i>srs2-K36A npp106-GFP:natMX6 arg3::mCherry-LacI nmt41:rtf1:sup35 ade6-704 leu1-32 t-LacO 7,9Kb:Kan:ura4<sup>+</sup>&lt;ori (uraR)</i>	this study
KK2083	<i>h</i> -	<i>rqh1-K547A npp106-GFP:natMX6 arg3::mCherry-LacI nmt41:rtf1:sup35 ade6-704 leu1-32 t-LacO 7,9Kb:Kan:ura4<sup>+</sup>&lt;ori (uraR)</i>	this study
KK2088		<i>rqh1-SM-8Gly-13xMyc:kanMX6 npp106-GFP:natMX6 arg3::mCherry-LacI nmt41:rtf1:sup35 ade6-704 leu1-32 t-LacO 7,9Kb:Kan:ura4<sup>+</sup>&lt;ori (uraR)</i>	this study

KK1950	<i>h+</i>	<i>srs2-5xFLAG:hphMX6 ade6-704 leu1-32 ura4-D18</i>	this study
KK1342	<i>h+</i>	<i>pmt3-KallR (sumo-KallR) nmt41:rtf1:sup35 ade6-704 leu1-32 t- ura4-SD20&lt;ori (uraR)</i>	this study
KK1467	<i>h-</i>	<i>LoxP-cdc6-L591G-LoxM3 rtf1::Nat loxP-rnh201RED:KAN-loxM3 ChrII-8535:Rura RTS1::phleo ura4-D18 ade6-704 leu1-32</i>	this study
KK2089	<i>h-</i>	<i>pmt3-KallR (sumo-KallR) LoxP-cdc6-L591G-LoxM3 rtf1::Nat loxP-rnh201RED:KAN-loxM3 ChrII-8535:Rura RTS1::phleo ura4-D18 ade6-704 leu1-32</i>	this study
KK2531	<i>h+</i>	<i>srs2-K36A nmt41:rtf1:sup35 ade6-704 leu1-32 t- ura4-SD20&lt;ori (uraR)</i>	this study
KK2537	<i>h-</i>	<i>pmt3-KallR (sumo-KallR) srs2-K36A nmt41:rtf1:sup35 ade6-704 leu1-32 t- ura4-SD20&lt;ori (uraR)</i>	this study
KK2534	<i>h+</i>	<i>pmt3-KallR (sumo-KallR) srs2::matMX6 nmt41:rtf1:sup35 ade6-704 leu1-32 t- ura4-SD20&lt;ori (uraR)</i>	this study
KK2243	<i>h-</i>	<i>nmt41:rtf1:sup35 ade6-704 leu1-32 t-Kan-lexBS-ura4-SD20&lt;ori (uraR)</i>	this study
KK2247	<i>h+</i>	<i>nup60-lexA:hphMX6 nmt41:rtf1:sup35 ade6-704 leu1-32 t-ura4-SD20&lt;ori (uraR)</i>	this study
KK2251	<i>h+</i>	<i>nup60-lexA:hphMX6 nmt41:rtf1:sup35 ade6-704 leu1-32 t-Kan-lexBS-ura4-SD20&lt;ori (uraR)</i>	this study
KK2260	<i>h-</i>	<i>pmt3-KallR (sumo-KallR) nup60-lexA:hphMX6 nmt41:rtf1:sup35 ade6-704 leu1-32 t-ura4-SD20&lt;ori (uraR)</i>	this study
KK2263		<i>pmt3-KallR (sumo-KallR) nup60-lexA:hphMX6 nmt41:rtf1:sup35 ade6-704 leu1-32 t-Kan-lexBS-ura4-SD20&lt;ori (uraR)</i>	this study
KK1220	<i>h+</i>	<i>nup132::natMX6 nmt41:rtf1:sup35 ade6-704 leu1-32 t- ura4-SD20&lt;ori (uraR)</i>	this study
KK1226	<i>h+</i>	<i>nup132::natMX6 srs2::natMX6 nmt41:rtf1:sup35 ade6-704 leu1-32 t- ura4-SD20&lt;ori (uraR)</i>	this study
KK2300	<i>h+</i>	<i>rad52-R45A:kanMX6 npp106-GFP:natMX6 arg3::mCherry-LacI nmt41:rtf1:sup35 ade6-704 leu1-32 t-LacO 7,9Kb:Kan:ura4<sup>+</sup>&lt;ori (uraR)</i>	this study
KK2304	<i>h+</i>	<i>rad52Δ308:kanMX6 npp106-GFP:natMX6 arg3::mCherry-LacI nmt41:rtf1:sup35 ade6-704 leu1-32 t-LacO 7,9Kb:Kan:ura4<sup>+</sup>&lt;ori (uraR)</i>	this study
KK2408	<i>h-</i>	<i>rad52-R45A:kanMX6 nmt41:rtf1:sup35 ade6-704 leu1-32 t- ura4-SD20&lt;ori (uraR)</i>	this study
KK2412	<i>h+</i>	<i>rad52Δ308:kanMX6 nmt41:rtf1:sup35 ade6-704 leu1-32 t- ura4-SD20&lt;ori (uraR)</i>	this study
YC86	<i>h+</i>	<i>rad52::natMX6 nmt41:rtf1:sup35 ade6-704 leu1-32 t- ura4-SD20&lt;ori (uraR)</i>	this study
YC78	<i>h+</i>	<i>rad51::kanMX6 nmt41:rtf1:sup35 ade6-704 leu1-32 t- ura4-SD20&lt;ori (uraR)</i>	this study
YC109	<i>h+</i>	<i>rad51::kanMX6 rad52::natMX6 nmt41:rtf1:sup35 ade6-704 leu1-32 t- ura4-SD20&lt;ori (uraR)</i>	this study

KK2527	<i>h-</i>	<i>rad51::kanMX6 rad52-R45A:kanMX6 nmt41:rtf1:sup35 ade6-704 leu1-32 t- ura4-SD20&lt;ori (uraR)</i>	this study
KK2423	<i>h+</i>	<i>loxP:rad51-R152A-R324A-K334A:loxM3 nmt41:rtf1:sup35 ade6-704 leu1-32 t- ura4-SD20&lt;ori (uraR)</i>	this study
KK2463	<i>h-</i>	<i>loxP:rad51-R152A-R324A-K334A:loxM3 rad52Δ308:kanMX6 nmt41:rtf1:sup35 ade6-704 leu1-32 t- ura4-SD20&lt;ori (uraR)</i>	this study
KK2456	<i>h+</i>	<i>loxP:rad51-R152A-R324A-K334A:loxM3 rad52-R45A:kanMX6 nmt41:rtf1:sup35 ade6-704 leu1-32 t- ura4-SD20&lt;ori (uraR)</i>	this study
KK2452	<i>h+</i>	<i>pmt3-KallR (sumo-KallR) loxP:rad51-R152A-R324A-K334A:loxM3 nmt41:rtf1:sup35 ade6-704 leu1-32 t- ura4-SD20&lt;ori (uraR)</i>	this study
KK2448	<i>h+</i>	<i>pmt3-KallR (sumo-KallR) rad52Δ308:kanMX6 nmt41:rtf1:sup35 ade6-704 leu1-32 t- ura4-SD20&lt;ori (uraR)</i>	this study
KK2442	<i>h-</i>	<i>pmt3-KallR (sumo-KallR) rad52-R45A:kanMX6 nmt41:rtf1:sup35 ade6-704 leu1-32 t- ura4-SD20&lt;ori (uraR)</i>	this study



## Extended Summary in French

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## Résumé étendu en français

### INTRODUCTION

Maintenir la stabilité du génome est essentiel pour garantir la transmission fidèle de l'information génétique. Les défauts dans le processus de réplication de l'ADN, connus sous le nom de stress réplicatif, sont une source majeure d'instabilité génomique, contribuant au développement du cancer. Le stress réplicatif résulte principalement de l'altération de la progression des fourches de réplication, provoquée par divers obstacles physiques ou par un déséquilibre métabolique, qui entraîne des ressources insuffisantes pour la duplication de la chromatine. Une incapacité à répliquer correctement le génome peut conduire à des erreurs de ségrégation des chromosomes pendant la mitose. Pour éviter une duplication incomplète des chromosomes, les cellules mettent en œuvre plusieurs voies de réparation pour : i) restaurer la capacité de synthèse de l'ADN au niveau des fourches dysfonctionnelles ou cassées, ii) protéger l'intégrité des fourches de réplication bloquées, et iii) réparer les brèches d'ADN simple brin laissées derrière les fourches de réplication.

Parmi les mécanismes de réparation de l'ADN qui aident à tolérer le stress réplicatif, la voie de la recombinaison homologe (RH) est cruciale pour assurer une synthèse continue de l'ADN et une réplication complète du génome (Chakraborty et al., 2023). Des recherches sur la levure ont montré que la machinerie de la RH aide à une réplication complète de l'ADN en rétablissant la capacité des fourches dysfonctionnelles à reprendre la synthèse d'ADN grâce au mécanisme de « réplication dépendante de la recombinaison (RDR) ». Contrairement à de nombreux modèles établis, ce mécanisme se produit sans cassures double-brin de l'ADN. De manière inattendue, le RDR est associé à une synthèse d'ADN non canonique, avec un taux de mutation plus élevé que la réplication normale de l'ADN (Mizuno et al., 2009; Lambert et al., 2010). Restart and slippage ? Cela suggère qu'en cas d'échec de la réplication, l'achèvement de la duplication des chromosomes fait appel à des mécanismes mutagènes, augmentant ainsi l'instabilité du génome.

Le noyau des cellules eucaryotes est divisé en plusieurs compartiments en fonction de la nature de la chromatine, avec des capacités de réparation de l'ADN distinctes. De plus, les mécanismes de réparation de l'ADN sont spatialement organisés (Kalousi et Soutoglou, 2016; Lemaitre et al., 2014). Par exemple, les régions d'hétérochromatine ou le nucléole sont moins favorables à certains modes de réparation, tandis que la région des pores nucléaires est plus propice à des mécanismes de réparation alternatifs (Hauer et Gasser 2017; Wootton et Soutoglou 2021). Par conséquent, la chromatine

endommagée peut se déplacer d'un compartiment nucléaire à un autre pour une réparation plus efficace.

Des études chez différents eucaryotes ont montré que les cassures double-brin dans l'hétérochromatine quittent leur compartiment pour permettre une réparation par la RH. De plus, les cassures d'ADN difficiles à réparer et les télomères érodés migrent vers les complexes du pore nucléaire (NPC, pour nuclear pore complex). Ce phénomène de relocalisation vers la périphérie nucléaire a également été observé en cas de dommages induits par des stress réplicatifs. Par exemple, chez la levure, les fourches de réplication bloquées par des séquences formant des structures secondaires, des protéines liées à l'ADN ou des séquences télomériques se déplacent vers la périphérie nucléaire pour s'ancrer aux NPC (Nagai et al., 2008; Su et al., 2015; Whalen et al., 2020; Aguilera et al., 2020; Kramarz et al., 2020). Des résultats similaires ont été observés lorsque la réplication des télomères humains est perturbée (Pinzaru et al., 2020). Enfin, l'inhibition globale de la synthèse de l'ADN chez l'homme entraîne une relocalisation des fourches altérées vers la périphérie nucléaire (Lamm et al., 2020).

Des recherches pionnières ont révélé que la SUMOylation joue un rôle majeur dans la coordination de la position nucléaire des cassures double-brin et des fourches de réplication bloquées. La SUMOylation est une modification post-traductionnelle où une petite protéine SUMO (Small Ubiquitin-like Modifier) est fixée de manière covalente à une protéine cible (Celen et Sahin, 2020). Ce processus repose sur une cascade enzymatique impliquant l'enzyme activatrice E1, l'enzyme de conjugaison E2, et la ligase E3 SUMO qui catalyse l'attachement de SUMO à un substrat. SUMO peut être attachée en tant que monomère sur une lysine cible, entraînant une monoSUMOylation, ou sur plusieurs lysines, générant une multiSUMOylation. De plus, SUMO peut former des chaînes polymériques (polySUMOylation), où plusieurs molécules de SUMO sont liées entre elles par des lysines internes (Pichler et al., 2017). Comme d'autres modifications post-traductionnelles, la SUMOylation est dynamique et réversible, avec des protéases spécifiques, appartenant à la famille Ulp/SENp, qui clivent les particules SUMO des protéines cibles.

Au niveau moléculaire, la SUMOylation peut avoir diverses conséquences biologiques, influençant l'activité, la localisation et la stabilité de la protéine cible (Geiss-Friedlander et Melchior, 2007; Wilkinson et Henley, 2010). Elle peut également servir de signal pour recruter d'autres protéines, notamment une classe particulière de ligases E3 d'ubiquitine appelées ubiquitines ligases ciblées par SUMO (STUbL, pour SUMO-targeted ubiquitin ligase). Toutes les STUbL possèdent deux éléments structurels clés : des motifs d'interaction SUMO (SIMs), qui facilitent la liaison avec des substrats

multiSUMOylés ou polySUMOylés, et un domaine de type RING E3 requis pour leur activité enzymatique d'ubiquitination. Des études génétiques et biochimiques ont montré que les STUbL régulent l'homéostasie de la SUMOylation en ciblant les protéines SUMOylées pour une dégradation par le protéasome (Chang et al., 2021).

Le métabolisme lié à la SUMOylation est également distribué spatialement dans les cellules eucaryotes. La localisation nucléaire des enzymes impliquées dans ce processus détermine la spécificité de leurs substrats, ajoutant ainsi un niveau supplémentaire de régulation des niveaux de SUMOylation. Par exemple, la localisation des protéases SUMO près des pores nucléaires a été démontrée. Des études chez la levure de fission et la levure bourgeonnante ont révélé que la localisation d'Ulp1 à la périphérie nucléaire nécessite des nucléoporines du NPC, soit le complexe Y, soit le panier nucléaire. Chez ces deux organismes, un défaut d'ancrage de Ulp1 aux NPC entraîne sa mauvaise localisation dans le nucléoplasme et sa dégradation via le protéasome (Zhao et al., 2004 ; Palancade et al., 2007 ; Nie et al., 2015). De même, chez les mammifères, la protéase SUMO SENP2 est enrichie au niveau des NPC, et la perte de cet ancrage réduit globalement la SUMOylation (Zhang et al., 2002). Ces découvertes soulignent le rôle des NPC comme centres de signalisation médiée par la SUMOylation, un rôle conservé au cours de l'évolution. En outre, les STUbL fonctionnent dans des contextes spécifiques, tels que l'enveloppe nucléaire, le centromère, le kinétochore ou les corps nucléaires PML dans les cellules humaines (Chang et al., 2021). La SUMOylation des centromères est un facteur clé dans l'identité du centromère, soulignant l'importance des STUbL dans la régulation de l'homéostasie de la SUMOylation au sein du noyau, essentielle à la biologie du centromère. Ainsi, la SUMOylation se révèle être un processus complexe, jouant un rôle crucial dans le maintien de l'homéostasie globale des voies de signalisation cellulaire.

## **OBJECTIFS**

La SUMOylation est un processus réversible et dynamique qui affecte l'activité, la localisation et la stabilité d'une protéine cible. Une publication récente de l'équipe, chez *S. pombe*, a montré que les chaînes SUMO déclenchent le déplacement d'une seule fourche de réplication arrêtée vers la périphérie nucléaire pour s'ancrer au NPC (Kramarz et al., 2020). Cet ancrage nécessite des chaînes SUMO et la voie Slx8 STUbL, mais les cibles SUMOylées exactes restent inconnues. Cependant, dans le même temps, les chaînes SUMO limitent la fréquence du glissement de la réplication au cours de la progression des fourches redémarrées. L'ancrage du NPC favorise l'élimination des conjugués SUMO par la protéase SUMO Ulp1 et le protéasome, dont les activités sont enrichies à la périphérie nucléaire. Le déplacement des fourches de réplication vers la

périphérie nucléaire nécessite l'activité d'échange de brins de la recombinaise Rad51. Cela suggère que les fourches de réplication arrêtées doivent être remodelées pour pouvoir s'ancrer. Une possibilité est que la formation de molécules conjointes par la recombinaise Rad51 facilite la formation de chaînes SUMO essentielles à la relocalisation. Il est important de noter que l'absence de chaînes SUMO ou la capacité à effectuer une mono-SUMOylation seulement, abroge la relocalisation à la périphérie nucléaire, mais permet à une voie de redémarrage de la fourche de réplication de se produire dans le nucléoplasme sans acheminement vers le NPC. Cela suggère l'existence d'au moins deux voies RDR séparées dans l'espace, dont l'utilisation pourrait être contrôlée par SUMO. Une voie, déclenchée par les chaînes SUMO, nécessiterait un ancrage dans le NPC; une deuxième voie, efficace lorsque les chaînes SUMO ne sont pas formées, se produirait dans le nucléoplasme. Dans ce contexte, les objectifs de ma thèse ont été divisés en deux axes :

#### Développement d'approches in vivo pour suivre la formation des chaînes SUMO dans l'espace nucléaire.

- Peut-on marquer Slx8 STUbL avec la GFP?
- Si oui, pouvons-nous exploiter la visualisation des foyers Slx8-GFP comme marqueur pour suivre les modifications SUMO dans les cellules vivantes?
- Les foyers de Slx8-GFP sont-ils suffisants pour suivre la formation de chaînes SUMO spontanées et induites par le stress dans l'espace nucléaire? Si oui, comment?
- Si non, comment caractériser les foyers de Slx8-GFP en termes d'espace et d'architecture nucléaires? Marquent-ils un locus ou une région nucléaire spécifique?
- Quel est le rôle des chaînes SUMO dans le contrôle de la localisation susmentionnée de Slx8-GFP, le cas échéant?

#### Étudier la voie RDR résolue dans l'espace et se produisant dans le nucléoplasme.

- La modulation de la formation et de la stabilité des molécules jointes affecte-t-elle la relocalisation à la périphérie nucléaire?
- Existe-t-il des facteurs spécifiques qui sont des cibles de la SUMOylation?
- Pouvons-nous identifier une voie de réplication dépendante de la recombinaison dans le nucléoplasme?
- Si oui, quelle est la dépendance génétique de cette voie?

## SYSTÈME EXPÉRIMENTAL

Pour atteindre mes objectifs, j'ai utilisé une barrière de fourche de réplication (RFB) conditionnelle et spécifique à un site précis dans le génome de la levure de fission. Dans ce système, l'activité de la RFB est régulée par la protéine Rtf1, qui se lie à la séquence RTS1 pour bloquer la progression du réplisome de façon polaire. Pour rendre ce système inductible, le gène codant pour Rtf1 a été placé sous le contrôle du promoteur nmt41, répressible par la thiamine. En présence de thiamine, Rtf1 n'est pas exprimée, et la RTS1-RFB reste inactive (condition OFF). En l'absence de thiamine, nmt41 est activé, Rtf1 est exprimée, et la RTS1-RFB est active (condition ON). Les fourches bloquées par la RFB deviennent dysfonctionnelles et peuvent être sauvées soit par une fourche convergente, soit, si cela ne se produit pas à temps, par la réplication dépendante de la recombinaison (RDR), qui prend environ 20 minutes. Ce mécanisme de RDR implique une synthèse d'ADN non canonique, avec les deux brins répliqués par la polymérase delta (Lambert et al., 2010; Mizuno et al., 2013; Tsang et al., 2014; Miyabe et al., 2015; Nguyen et al., 2015). Au laboratoire, des essais génétiques, cellulaires et moléculaires ont été développés pour explorer les mécanismes moléculaires et les acteurs clés impliqués dans les événements se produisant au niveau du blocage de la fourche de réplication au locus RTS1-RFB. Ces techniques permettent (1) de mesurer l'efficacité du redémarrage des fourches via la recombinaison homologue (essai génétique), (2) de suivre le recrutement de protéines sur le locus en condition de blocage de fourche dans des cellules individuelles (microscopie avec système rapporteur fluorescent) ou dans une population cellulaire (immunoprécipitation de la chromatine, ChIP), et (3) de suivre *in vivo* le destin et la localisation nucléaire du locus (microscopie avec rapporteur fluorescent) (Ait Saada et al., 2017; Teixeira-Silva et al., 2017; Hardy et al., 2019; Kramarz et al., 2020).

## RÉSULTATS

### I. Slx8 STUbL maintient l'homéostasie SUMO pour protéger la fonction du centromère

Les ubiquitines ligases ciblées par SUMO (STUbL) constituent une classe unique d'ubiquitine ligases E3 essentielles au maintien du génome. Chez *S. pombe*, la STUbL Slx8 cible les lésions de l'ADN à la périphérie nucléaire, favorisant la tolérance au stress de réplication en contrecarrant la toxicité des conjugués SUMO (Kramarz et al., 2020). Bien que le mécanisme précis par lequel Slx8 répond au stress de réplication ne soit pas clair, mon étude a révélé que Slx8 ne forme pas de foyers spécifiques induits par les lésions de l'ADN comme attendu. Cependant, j'ai découvert que Slx8 joue un rôle

crucial dans le maintien de l'hétérochromatine et le regroupement des centromères, qui est essentiel pour la fonction normale des centromères chez la levure de fission.

### 1. Slx8-GFP ne forme pas de foyers surnuméraires en réponse à un stress de réplication

Le stress de réplication est étroitement associé à des niveaux élevés de SUMOylation des substrats, ce qui est essentiel pour la stabilité du génome dans des conditions de réplication perturbées. Cela inclut la protection des fourches endommagées de façon dépendante de la SUMOylation, comme observé avec Ubc9 et Mms21 chez la levure bourgeonnante (Branzei et al., 2006), et la SUMOylation des facteurs de redémarrage de la réplication comme Mre11, Ku, Sgs1, et Rad52 sur les replisomes bloqués (Psakhye & Jentsch, 2012 ; Sarangi & Zhao, 2015). Les STUbL, comme Slx8, ubiquitinent et dégradent les cibles SUMOylées, régulant leur fonction et impactant la dynamique de la chromatine. Chez la levure bourgeonnante, par exemple, les CDBs difficiles à réparer et les fourches effondrées s'ancrent au NPC via un processus nécessitant la STUbL ScSlx5-Slx8 (Nagai et al., 2008). Des données récentes suggèrent que l'activité STUbL de Slx8 est cruciale pour le déplacement des fourches de réplication arrêtées vers la périphérie nucléaire (Kramarz et al., 2020), soulignant l'importance du métabolisme SUMO médié par Slx8 dans la mobilité des lésions de l'ADN.

Alors que Slx5, qui fait partie du complexe STUbL Slx5-Slx8, forme des foyers nucléaires distincts chez la levure bourgeonnante (Cook et al., 2009), j'ai émis l'hypothèse que Slx8 pourrait présenter un comportement similaire en réponse à un stress de réplication chez *S. pombe*. En utilisant la microscopie sur cellules vivantes, j'ai observé que Slx8-GFP forme un seul foyer d'une fluorescence intense dans 60-70% des cellules en phase S et G2 (Publication #3 : Figure 1D & 1E). Il est intéressant de noter que ce foyer dépend en partie de la ligase E3-SUMO Pli1, mais pas de Nse2 (Publication #3 : Figure 2B & 2C), ce qui reflète le rôle majeur de Pli1 dans le maintien de la SUMOylation globale (Prudden et al., 2011). Bien qu'il existe une activité compensatoire entre Pli1 et Nse2 (Andrews et al., 2005 ; Zhao & Blobel, 2005), je n'ai pas pu l'explorer en raison de la non-viabilité des doubles mutants (*pli1-RING<sup>mut</sup>* et *nse2-RING<sup>mut</sup>*). Il est plausible que Nse2 puisse maintenir partiellement les niveaux de SUMOylation en l'absence de Pli1, permettant ainsi la formation de foci Slx8-GFP.

De plus, j'ai identifié les chaînes SUMO comme étant cruciales pour la formation du foyer Slx8-GFP. Les souches incapables de former des chaînes SUMO (*SUMO-KallR* et *SUMO-D81R*) présentaient une réduction de deux tiers des foyers de Slx8-GFP dans les cellules en phase S et G2 (Publication #3 : Figure 2B & 2C). De plus, l'intensité des foyers

de Slx8-GFP dans ces souches était significativement plus faible (Publication #3 : Figure 2D), indiquant le rôle essentiel des chaînes SUMO dans ce processus.

Contrairement à ce qui était attendu, Slx8-GFP ne forme pas de foyers supplémentaires lorsque les cellules sont traitées avec par des agents génotoxiques tels que le MMS, l'HU ou le CPT (Publication #3 : Figure 3B & 3C). Cependant, l'intensité du foyer unique de Slx8-GFP a augmenté, en particulier après traitement au MMS (Publication #3 : Figure 3D). Cela pourrait indiquer que la quantité de Slx8 recrutée sur les sites de dommages à l'ADN est trop faible pour être détectée ou que les foyers de Slx8 migrent rapidement vers des régions nucléaires spécifiques. Il est intéressant de noter que la formation des foyers de Slx8-GFP a été significativement réduite dans la souche *SUMO-KallR* en réponse à des stress génotoxiques (Publication #3 : Figure 3B & 3C), renforçant l'importance des chaînes SUMO dans le maintien des foyers de Slx8-GFP au cours du stress de réplication.

En résumé, ce travail montre que Slx8-GFP forme un foyer nucléaire unique dépendant de la formation de chaînes SUMO et de l'activité de Pli1. Cependant, les foyers de Slx8-GFP ne sont pas des marqueurs fiables de la formation de chaînes SUMO induite par les dommages, bien que leur comportement soit modulé par le stress de réplication d'une manière dépendante des chaînes SUMO.

## 2. La localisation de Slx8 au centre organisateur des microtubules ou SPB maintient la structure et la fonction des centromères

Il est intéressant de noter que le foyer unique Slx8-GFP se localise fréquemment à la périphérie nucléaire. J'ai confirmé que plus de 65% de ces foyers étaient situés à la périphérie nucléaire (PN) dans les cellules en phase S, tombant à ~35% dans les cellules en phase G2. Cette localisation périphérique était également réduite à ~35% dans les cellules en phase S exprimant *SUMO-KallR*, ce qui indique que les chaînes SUMO contrôlent la formation et le positionnement des foyers de Slx8-GFP pendant la phase S uniquement (Publication #3 : Figure 4A & 4B).

Chez de nombreux organismes, les centromères se regroupent au niveau de la PN, ce qui facilite le chargement des protéines centromériques, le silencing de gènes et la prévention de la formation de micronoyaux (Wu et al., 2022 ; Padeken et al., 2013 ; Jagannathan et al., 2018). Chez *S. pombe*, trois centromères sont regroupés à côté du centre organisateur des microtubules ou SPB (pour Spindle Pole Body), dans l'enveloppe nucléaire (NE) (Mizuguchi et al., 2015). J'ai observé une colocalisation significative de Slx8 avec les centromères regroupés (marqués par Mis6-RFP) et le SPB (marqué par Sid4-RFP) dans 60% des cellules avec des foyers de Slx8-GFP (Publication



#3 : Figure 5A & 5B). En outre, une certaine colocalisation a été observée avec la région *mat* (pour mating type) contenant le locus du changement de sexe (~20% des cellules), mais pas avec les télomères (Publication #3 : Figure 5A & 5B). Ainsi, les foyers de Slx8-GFP au niveau de la PN marquent spécifiquement les centromères regroupés, le SPB et la région *mat*, mais pas les télomères.

Étant donné que les centromères sont enrichis en hétérochromatine, j'ai étudié la relation entre les foyers Slx8-GFP et l'hétérochromatine au niveau des centromères regroupés. J'ai constaté que les foyers Slx8-GFP étaient deux fois moins nombreux dans les cellules dépourvues de l'histone méthyltransférase Clr4, qui favorise la méthylation H3-K9 et le *silencing* des gènes, mais pas dans les cellules dépourvues de Dicer (Dcr1), composant crucial du système d'interférence à ARN (publication n° 3 : figures 6A et 6B). Ceci suggère que la formation des foyers Slx8-GFP dépend plus du maintien de l'hétérochromatine médiée par Clr4 que de l'activité de la voie de l'ARNi. De plus, les cellules contenant des foyers de Slx8-GFP ont été réduits de façon similaire dans le mutant de délétion Csi1 (*csi1Δ*), qui perturbe le regroupement des centromères (Hou et al., 2012) (Publication #3 : Figure 6A & 6B). Ainsi, la formation de l'hétérochromatine et le regroupement des centromères sont tous deux cruciaux pour la formation du foyer Slx8-GFP.

Pour déterminer si Slx8 influence le maintien de l'hétérochromatine et le regroupement des centromères, j'ai collaboré avec Joe Strachan et le professeur Elizabeth Bayne de l'université d'Édimbourg, au Royaume-Uni. Leur analyse par RT-qPCR a révélé une accumulation accrue de transcrits dans les mutants *clr4Δ* et *slx8Δ*, avec un effet plus prononcé au niveau la région *mat* dans le mutant *slx8Δ* (publication n° 3 : figure 7A). Le regroupement des centromères, évalué par la colocalisation de la GFP-Cnp1 (*S. pombe* CENP-A) et de la Sid4-RFP, a été significativement perturbé dans les cellules *slx8Δ*, 12% d'entre elles présentant plus d'un foyer GFP-Cnp1 (Publication #3 : Figure 7B & 7C). Le phénotype épistatique observé dans le double mutant *slx8Δ csi1Δ* suggère que Slx8 pourrait opérer dans la même voie que Csi1 (Publication #3 : Figure 7B & 7C). En outre, la délétion de *pli1* a atténué le défaut de regroupement dans les mutants *slx8Δ*, ce qui indique que Slx8 aide à éliminer l'excès de SUMOylation pour préserver le regroupement des centromères.

En conclusion, la localisation de Slx8 près du SPB est essentielle pour maintenir l'intégrité de l'hétérochromatine et le regroupement des centromères, probablement grâce à son rôle dans l'élimination des facteurs SUMOylés du centromère pour maintenir la fonction du centromère.

## II. Existence d'une voie de réplication dépendante de la recombinaison, indépendante du NPC et résolue au niveau spatial

Le déplacement des fourches de réplication perturbées vers la périphérie nucléaire et leur ancrage aux complexes du pore nucléaire facilitent la reprise de la synthèse de l'ADN au niveau des fourches dysfonctionnelles en favorisant un mécanisme de redémarrage de la réplication dépendant de la recombinaison (RDR). Cependant, dans certains contextes génétiques, RDR peut se produire sans déplacement vers ou ancrage au NPC. Ce phénomène a été observé dans des cellules exprimant un allèle SUMO muté incapable de former des chaînes SUMO (*SUMO-KallR*). Dans ce cas, la SUMOylation est altérée, contournant probablement le besoin d'ancrage aux NPC associés à Ulp1 pour surmonter les effets inhibiteurs des conjugués SUMO sur l'initiation de la synthèse de l'ADN. Ainsi, deux voies peuvent exister : l'une déclenchée par la formation de chaînes SUMO et la relocalisation au NPC, et l'autre, éventuellement signalée par la mono-SUMOylation, se produisant dans le nucléoplasme.

Nos travaux précédents ont démontré que dans les cellules *rad51Δ* et *rad51-I13A* (dépourvu de l'activité d'échange de brins), l'efficacité du redémarrage par le RDR diminuait de 60 à 70 % (Ait Saada et al., 2017). Cette réduction partielle suggère que le redémarrage restant se produit par une voie indépendante de Rad51. Une étude récente menée dans la levure de fission par le laboratoire du professeur Whitby a rapporté que les fourches de réplication arrêtées au niveau de la RFB peuvent redémarrer par une voie indépendante de Rad51, en s'appuyant sur l'activité d'appariement d'ADN simple brin ou SSA (pour *Single-Strand Annealing*) de Rad52 (Kishkevich et al., 2022). Ce résultat confirme l'existence de mécanismes alternatifs de redémarrage.

Pour étudier cette voie alternative, j'ai analysé des facteurs impliqués dans la formation/stabilisation de la D-loop en aval des fourches arrêtées au niveau de *RTS1*-RFB afin d'identifier les facteurs contribuant au déplacement de la RFB vers la périphérie nucléaire. J'ai découvert que la formation des D-loop est un déterminant clé de la relocalisation de la RFB (Figure 1B & 1C). De façon remarquable, les mutants impliqués dans le maintien de la stabilité de la D-loop n'ont pas montré de relocalisation à la périphérie nucléaire. Cependant, je ne peux pas exclure une association transitoire au NPC qui est insuffisante pour visualiser l'enrichissement de la RFB par microscopie. De manière surprenante, le taux de redémarrage de la fourche n'est pas en corrélation avec le déplacement au NPC dans la plupart des mutants, ce qui suggère soit l'existence de voies de redémarrage nucléoplasmiques, soit que l'essai de glissements de la polymérase induits par la RFB, utilisé pour mesurer le redémarrage

des fourches, ne mesure pas uniquement une efficacité de redémarrage (Figure 2A & 2B).

A l'appui de cette dernière hypothèse, les données Pu-seq sur le mutant *SUMO-KallR* des collaborateurs Tony Carr et Karel Naiman (Figure 4A & 4B) ont révélé un défaut de redémarrage des fourches malgré une augmentation de la fréquence de glissements de la polymérase induite par la RFB (Kramarz et al., 2020) (Figure 3A & 3B). Ceci suggère que le test des glissements de la polymérase induits par la RFB pourrait également être indicatif de la fidélité des fourches redémarrées. Le mutant *SUMO-KallR* a montré un défaut de redémarrage de la fourche de 30-40% dans les données Pu-seq, indiquant que les chaînes SUMO sont nécessaires pour le redémarrage de la fourche. Cependant, l'augmentation des glissements de la polymérase induits par la RFB pourrait refléter un glissement accru de la réplication dans les fourches redémarrées, ce qui implique que les chaînes SUMO régulent la fidélité de la synthèse d'ADN redémarrée, en particulier dans le nucléoplasme.

Une analyse plus approfondie a révélé que les chaînes SUMO ne sont pas nécessaires pour limiter la fréquence des glissements de la polymérase induits par la RFB lorsque les fourches sont enrichies au niveau du NPC (Figure 5A & 5B), ce qui suggère des mécanismes distincts basés sur le positionnement nucléaire. Ces résultats, associés à l'absence de corrélation entre le déplacement de la RFB et la fréquence des glissements de la polymérase induite par la RFB, indiquent l'existence d'au moins deux voies de la RDR avec des contrôles différents sur la fidélité de la synthèse de l'ADN en fonction du positionnement nucléaire.

Qu'est-ce qui définit cette voie alternative? Les travaux du professeur Whitby suggèrent que template switch pendant la phase d'élongation des fourches redémarrées repose sur l'activité SSA de Rad52 (Kishkevich et al., 2022). J'ai émis l'hypothèse que l'activité SSA de Rad52 pourrait contribuer à la voie RDR nucléoplasmique. En testant cette hypothèse, j'ai exclu l'activité SSA de Rad52 dans la relocalisation de la RFB, confirmant qu'elle est dispensable pour la relocalisation au NPC. De plus, j'ai évalué l'impact de l'activité SSA de Rad52 sur les glissements de la polymérase induite par la RFB, révélant qu'une part importante du glissement dépend de l'activité SSA de Rad52 dans des conditions normales (Figure 7). En outre, les glissements de la polymérase induits par la RFB dans les mutants *rad51-I13A* étaient épistatiques avec *rad52-R45A*, l'activité SSA de Rad52 dominant légèrement l'activité d'échange de brins de Rad51. Il est important de noter que l'activité SSA de Rad52 est cruciale dans les voies dépendantes et indépendantes de Rad51, comme le montre la

quasi-absence de glissements de la polymérase induites par la RFB dans le double mutant *rad51Δ rad52-R45A*.

Dans le mutant *SUMO-KallR*, les glissements de la polymérase induits par la RFB repose toujours sur l'activité d'échange de brins de Rad51, même en l'absence de chaînes SUMO (Figure 8). Ceci suggère que l'activité SSA de Rad52, potentiellement régulée par les chaînes SUMO, joue un rôle dans le redémarrage médié par Rad51. En effet, il a été démontré que la SUMOylation de Rad52 atténue ses activités de liaison à l'ADN et de SSA, affectant la régulation de la recombinaison chez la levure (Altmannova et al., 2010). Srs2 apparaît également comme une cible potentielle régulée par SUMO en raison de son rôle dans la relocalisation de la RFB et dans la prévention des glissements de la polymérase induits par le RFB, indépendamment de son activité ATPase (Figure 9A-E).

En résumé, mes résultats suggèrent l'existence d'une voie RDR alternative, indépendante du NPC, dans le nucléoplasme, où l'activité SSA de Rad52 orchestre le redémarrage de la fourche. Cette voie dépendante du SSA opère à la fois dans le nucléoplasme et au niveau du NPC, soulignant son nouveau rôle dans le maintien de la fidélité de la réplication (Figure 10). L'existence de deux voies RDR, chacune ayant chacune des contrôles distincts sur la fidélité de la synthèse de l'ADN en fonction du positionnement nucléaire, donne un nouvel aperçu de la complexité des mécanismes de réparation de l'ADN et de redémarrage de la réplication.

## CONCLUSIONS

En résumé, mes résultats fournissent des informations clés sur la caractérisation de Slx8 dans la promotion du regroupement des centromères et du silencing des gènes dans les domaines de l'hétérochromatine. Mon travail de doctorat a établi une interaction fonctionnelle et physique entre STUbL et l'hétérochromatine pour maintenir l'organisation nucléaire de domaines spécifiques chez la levure de fission. En outre, je présente pour la première fois une preuve de l'existence d'une autre voie qui se produit avec la mono-SUMOylation dans le nucléoplasme lorsque la voie NPC est perturbée. Dans l'ensemble, mes résultats suggèrent que le déterminant clé de l'intégrité et du redémarrage des fourches de réplication dépend du métabolisme des SUMO. La SUMOylation contrôle les voies de redémarrage de la fourche qui sont résolues au niveau spatial dans le contexte du positionnement nucléaire des sites de stress de réplication. Ceci met en évidence la façon dont la perturbation de l'équilibre SUMO, communément observée dans diverses maladies humaines, y compris le cancer, affecte le maintien de l'intégrité du génome au niveau des sites de stress de réplication. Cette

recherche essentielle, qui permet de découvrir en profondeur les mécanismes par lesquels les cellules peuvent naturellement lutter contre l'instabilité génétique, est cruciale pour le développement de thérapies anticancéreuses efficaces.

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