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THÈSE DE DOCTORAT
DE L'UNIVERSITÉ PSL

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Role of 53BP1-RNA interaction in DNA Replication

Rôle de l'interaction entre 53BP1 et l'ARN dans la réplication de l'ADN

Soutenue par

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cellulaire**



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INTRODUCTION

Au cours de sa vie, l'ARN est associé avec des protéines de liaison à l'ARN (RBP) pour former des complexes ribonucléoprotéiques. Cette interaction dynamique permet de contrôler le métabolisme de l'ARN messager (ARNm) et est crucial pour de nombreux processus cellulaires impliqués dans la régulation post-transcriptionnelle de l'expression des gènes tels que la transcription, l'ajout d'une coiffe en 5', l'épissage alternatif, la polyadénylation de l'extrémité 3', la translocation du noyau au cytoplasme, la localisation subcellulaire de l'ARN, la traduction et enfin sa stabilité. Parmi les ARNm cibles des RBP, il y a des gènes de réponse aux dommages de l'ADN (DDR) ce qui positionne les RBP comme des acteurs dans la maintenance de l'intégrité du génome. En plus de ce rôle lié à leur fonction de liaison à l'ARN, les RBP sont localisées aux sites de lésions et aident ainsi dans les voies du DDR.

Les RBP participent à prévenir les dommages de l'ADN, en empêchant par exemple la formation des hybrides ARN:ADN (R-loop) et à résoudre les contraintes topologiques, en interagissant avec la topoisomérase TOP1. Elles sont également directement impliquées dans la signalisation, en s'associant à l'ADN endommagé ou à d'autres facteurs du DDR. Par ailleurs, même s'il y a de plus en plus d'évidences que l'ARN est produit au niveau des dommages et joue un rôle dans la signalisation de réparation, il n'est pas clairement défini si la fonction des RBP dans le DDR dépend de leur activité de liaison à l'ARN. En outre, plusieurs protéines du DDR ont été suggérées comme ayant des capacités de liaison à l'ARN, mais le rôle de cette éventuelle activité reste à être démontré. De part toutes ces interactions, les RBP sont donc des acteurs de la stabilité du génome et leur expression aberrante ou l'altération de leurs fonctions peuvent être associées à des pathologies, incluant les cancers.

Ces dernières années, environ 1400 RBP ont été identifiées dans les cellules humaines. Une RBP est communément définie comme étant une protéine liant directement l'ARN à travers un ou plusieurs domaines de liaison à l'ARN (RBD), induisant un changement dans le destin de l'ARN qu'elle lie. Ces domaines classiques de liaison à l'ARN sont le motif de reconnaissance de l'ARN (RRM), le domaine d'homologie K (KH), le doigt de

zinc (ZnF) et le domaine de liaison à l'ARN double-brin (dsRBD), pour ne citer que les mieux caractérisés. Plusieurs RBD peuvent être retrouvés dans une seule RBP, augmentant ainsi l'affinité et la spécificité des interactions protéine-ARN. Par ailleurs, de récentes études ont révélé l'existence de RBP dites non-conventionnelles qui ne présentent pas de domaine spécifique. Ces RBP sont souvent composées de régions désordonnées capables de lier l'ARN. L'absence de structure de ces régions leur permet de reconnaître des séquences et des structures d'ARN qui ne sont pas accessibles à des RBD plus structurés. Les régions désordonnées sont classées selon la répétition du motif : Serine/Arginine (SR), Arginine/Glycine (RGG ou GAR) et les patches basiques Arginine ou Lysine (R/K patches).

Ces régions désordonnées, et d'une manière globale les interactions ARN-protéine, ont pu être identifiées et caractérisées grâce aux approches à grande échelle développées au cours de la dernière décennie. Aujourd'hui, il existe un éventail de méthodes à adapter en fonction de la problématique à résoudre. Il existe des techniques centrées sur l'ARN, tel que le « complex capture » (2C) et des procédures centrées sur les protéines, tel que le « cross-linking immunoprecipitation » (CLIP). Dans le cas du CLIP et du 2C, comme pour de nombreuses techniques d'identification des RBP, la première étape est une étape de liaison covalente entre les protéines et les ARN pour figer les interactions dans les cellules vivantes. Pour induire ce crosslink, l'exposition aux UV-C est souvent utilisée. Les UV-C nécessitent un contact direct entre la protéine et les acides nucléiques et ne crosslinkent pas les interactions protéine-protéine. Couplés à ces méthodes expérimentales, de nombreux outils *in silico* permettent également la prédiction des sites d'interactions sur l'ARN ou sur les RBP. Ces techniques computationnelles ne cessent d'émerger et récemment, AlphaFold et RoseTTAFold ont révolutionné le domaine de la biologie structurale.

Comme décrit plus haut, les RBP jouent un rôle dans le métabolisme de l'ARNm et également dans le métabolisme de l'ADN, tels que la réplication ou la réparation des dommages où les RBP interagissent plutôt avec des facteurs de ces mécanismes. La réplication de l'ADN est un processus conservé et finement régulé, permettant à une cellule de dupliquer correctement son matériel génétique avant de le transmettre lors de la phase de division. Ce mécanisme bidirectionnel et semi-conservatif implique de nombreuses protéines, qui ne sont, par ailleurs, pas toujours bien déterminées.

La réplication de l'ADN commence au niveau des origines de réplication avec le chargement du complexe de pré-réplication en fin de phase G1. À la transition G1/S, ce complexe sera activé pour former alors le complexe de pré-initiation. Ce complexe protéique permet de dérouler l'ADN double-brin menant au recrutement de facteurs de réplication pour former le réplisome. Le réplisome commence alors à convertir le complexe de pré-initiation en deux fourches de réplication progressant dans des directions opposées. Deux brins sont alors déterminés : le brin précoce synthétisé de manière continue, et le brin retard synthétisé de manière discontinue et constitué de fragments d'Okazaki.

Pour initier la synthèse de l'ADN sur ces brins, et notamment sur le brin retard, des amorces ARN-ADN sont nécessaires. L'amorce ARN en 5', entre 7 et 12 ribonucléotides, est produite par les primases et l'amorce ADN, entre 10 et 25 désoxyribonucléotides, est générée par l'ADN polymérase α considérée comme sujette aux erreurs. À la suite de ces amorces, l'ADN polymérase δ synthétise, sur le brin retard et de manière fidèle, les fragments d'Okazaki jusqu'à 100-200 nucléotides. Lorsque l'ADN polymérase δ rencontre le fragment d'Okazaki en aval, elle déplace l'amorce ARN en une structure simple-brin appelée le « flap ». La maturation de ce flap est un processus critique pour le bon déroulé de la réplication de l'ADN et tous les acteurs impliqués dans la maturation ne sont pas encore bien décrits. Le flap obtenu est un substrat reconnu et clivé par l'endonucléase FEN1 laissant un espace entre deux fragments d'Okazaki que l'ADN ligase 1 (LIG1) peut ensuite utiliser pour joindre les fragments et ainsi obtenir un ADN double-brin fonctionnel. Dans certains cas, encore mal définis, il y a un retard de clivage de FEN1 induisant une voie non-canonique de maturation du brin retard : la voie du flap long. L'ADN polymérase δ continue alors le déplacement de l'amorce et les protéines de réplication A (RPA) se

lient à la partie ADN simple-brin pour recruter et stimuler l'activité de la nucléase DNA2. DNA2 raccourcit alors le long flap, permettant d'obtenir de nouveau un substrat clivable par FEN1. Le passage entre un flap court et long n'est pas complètement compris mais des hypothèses émergent. Il a cependant été suggéré que le long flap serait favorisé dans des régions transcriptionnellement actives, pour permettre à l'ADN polymérase δ de corriger les éventuelles erreurs de l'ADN polymérase α et ainsi diminuer le taux de mutation.

Enfin, à la suite de la maturation des fragments d'Okazaki, la réplication de l'ADN se termine, lorsque deux fourches convergentes se rencontrent. Les tensions topologiques sont alors résolues, les espaces restants sont remplis et ligaturés et les complexes de réplication sont dissociés de la chromatine. Chaque étape de la réplication doit donc être réalisée sans défaut et sans stress pour maintenir la stabilité du génome et la progression dans le cycle cellulaire.

En effet, des altérations dans la maturation des fragments d'Okazaki conduisent à une instabilité génétique et sont souvent associées à des pathologies. Ces défauts induisent un espacement (« gap ») d'ADN simple-brin, entre deux fragments d'Okazaki non maturés, qui est reconnu par la protéine PARP1 résultant alors en une accumulation des chaînes poly(ADP-ribose) (PAR) en phase S. Cibler PARP1 par des inhibiteurs (PARPi) est souvent utilisé en clinique, notamment pour cibler les cancers déficients pour les gènes *BRCA*. En effet, la perte de *BRCA* augmente ces gaps qui sont alors visés par les PARPi. La perte de 53BP1 dans ce contexte induit une résistance à ces drogues.

Par ailleurs, la cellule est constamment sujette à un stress, par des agents endogènes (dommage de l'ADN, gaps d'ADN simple-brin, hybrides ARN:ADN, structure G-quadruplex de l'ADN) ou exogènes (drogues ciblant le métabolisme des nucléotides, l'activité de polymérases, les topoisomérases), ce qui se traduit par le ralentissement ou l'arrêt de la fourche. La fourche doit alors être réparée avant de redémarrer pour éviter de s'effondrer, créant un état irréversible où la fourche ne peut pas reprendre la réplication. Plusieurs mécanismes de réparations existent : la tolérance aux dommages de l'ADN, la régression de la fourche et le redémarrage médié par la recombinaison homologue (HR). Par ailleurs, même si les facteurs de la HR sont les acteurs majeurs pour répondre au stress répliatif, certaines protéines de la voie de ligature des extrémités non homologues (NHEJ) peuvent aussi jouer un rôle.

Lorsqu'il y a une cassure double-brin, il y a deux voies de réparation possible. Si c'est en phase S alors la voie HR est favorisée, à l'inverse si c'est en phase G1 la voie NHEJ est choisie. Une protéine clé dans le choix de ces voies est la protéine 53BP1. 53BP1 est codée par le gène *TP53BP1* sur le chromosome 15 du génome humain et a été historiquement identifiée en 1994 comme partenaire de la protéine p53, un facteur de transcription considéré comme le « gardien du génome ». Le complexe 53BP1-p53 permet de réguler les fonctions de p53 pour répondre de manière adaptée à l'environnement cellulaire.

Par la suite, 53BP1 est devenue principalement connue pour son rôle dans la signalisation des voies de réparation de l'ADN et notamment dans le choix de ces voies. 53BP1 favorise la voie NHEJ en réponse à une cassure double-brin spontanée, mais aussi dans des contextes où la cassure est programmée : au niveau des télomères et pour la recombinaison V(D)J et isotypique nécessaires à la diversité des anticorps. L'activité anti-résection de 53BP1 est cruciale pour son rôle dans le choix des voies. En effet, la première étape de la voie HR est une étape de résection par les nucléases et 53BP1 protège donc l'ADN de cette dégradation, par ses interactions avec différentes protéines, tels que RIF1, DYNLL1 et Shieldin. Ces interactions protéiques sont médiées par la structure de la chromatine puisque 53BP1 reconnaît des marques d'histones : H4K20me2 via ses domaines Tudor et H2AK15ub via son domaine UDR.

En plus de son rôle dans la réparation de l'ADN, 53BP1 participe à la résolution du stress répliatif. Lorsque la fourche de réplication est bloquée, 53BP1 est recrutée à l'ADN naissant pour éviter sa résection. La perte de 53BP1 induit donc une diminution du taux de réplication et de la survie cellulaire, ainsi qu'une accumulation d'aberrations chromosomiques. De plus, 53BP1 est impliquée dans la protection des fourches régressées. Similaire à sa fonction anti-résection dans le choix des voies de réparation des cassures double-brin, 53BP1 favorise une voie sans clivage pour le redémarrage des fourches bloquées.

Les interactions que 53BP1 établit, même si elles ne sont pas toutes connues, sont médiées par ses domaines protéiques. C'est une protéine de 1972 acides aminés dont la structure peut être divisée en trois parties : Une région N-terminal contenant 28 sites Sérine/Thréonine/Glutamine impliqués dans les interactions avec RIF1 pour l'activité anti-résection de 53BP1, une région centrale, qui est la région minimale de

formation de foyers aux sites de lésion, contenant un domaine d'oligomérisation (OD), un domaine GAR, deux domaines Tudor, un signal de localisation nucléaire (NLS), un domaine de recrutement dépendant de l'ubiquitine (UDR) et une partie C-terminal composée de deux domaines BRCT essentiels pour la liaison avec son partenaire p53. Le domaine GAR est une répétition de motif Glycines et Arginines, dont les arginines sont méthylées par les enzymes PRMT1 et PRMT5, qui est connu pour lier l'ARN et est retrouvé dans des RBP canoniques. Cette découverte soulève alors la question : est-ce que 53BP1 a une capacité de liaison à l'ARN et est-ce que cette protéine peut être caractérisée comme une RBP ?

Plusieurs études se sont intéressées à cette potentielle activité et ont démontré que les fonctions de 53BP1 dans les dommages de l'ADN sont médiées par une composante ARN. En effet, les traitements RNase A, qui clive l'ARN simple-brin, dans les cellules empêchent le recrutement de 53BP1 aux sites de cassures. Cependant, ces observations ne démontrent pas d'une interaction directe entre 53BP1 et l'ARN. De plus, l'inhibition de Dicer et Drosha, responsables de la production de petits ARN double-brin, diminue également le recrutement de 53BP1, indiquant alors que de l'ARN est produit au niveau des sites de lésion et est clivé par Dicer/Drosha. Quelques années après ces découvertes, une équipe a démontré qu'au niveau des cassures, l'ARN polymérase II est recrutée et produit de longs ARN non codants, de part et d'autre de la cassure, qui seront par la suite clivés par Dicer/Drosha. Cependant, le mécanisme de passage entre le long ARN non codant et un ARN double-brin reconnu par Dicer/Drosha n'est pas encore bien compris. La transcription d'ARN par l'ARN polymérase II permet également d'activer 53BP1 en dissociant le complexe 53BP1-TIRR. TIRR est une RBP qui régule négativement 53BP1 en interagissant avec ses domaines Tudor, masquant ainsi la surface de liaison aux histones et empêchant alors le recrutement de 53BP1 à la chromatine. Néanmoins, une liaison directe entre 53BP1 et l'ARN n'est pas encore démontrée et par ailleurs, le rôle du domaine GAR n'est pas clairement défini. La délétion ou la mutation de ce domaine n'affectent pas les fonctions de 53BP1, dans la signalisation du DDR ou dans la recombinaison de classe. Cependant, l'altération du domaine GAR n'a pas été étudiée dans un contexte de réplication de l'ADN, donc un potentiel rôle de ce domaine dans ces fonctions n'est pas à exclure. Enfin, même si 53BP1 n'a pas encore été caractérisée comme étant une RBP, deux études protéomiques récentes l'ont suggérée comme un réel candidat.

OBJECTIFS DE LA THESE

Le laboratoire s'est donc intéressé à la caractérisation d'une interaction directe entre 53BP1 et l'ARN. Pour détecter une activité de liaison à l'ARN, des techniques *in cellulo* de CLIP et 2C, basées sur un lien covalent entre protéine et ARN induit par les UV-C, sont utilisées. Ces techniques sont complémentaires puisque dans le CLIP, la protéine est immunoprécipitée puis les ARN liés sont marqués radioactivement à l'extrémité 5' alors que dans le 2C, les ARN sont retenus sur une colonne de silice suivie d'une analyse en western blot pour analyser les RBP liées aux ARN. Ces deux approches ont permis de détecter une activité de liaison à l'ARN de 53BP1 (endogène et transfectée) dans plusieurs lignées cellulaires, cancéreuses et épithéliales. Par ailleurs, des données *in vitro* ont montré que le fragment GAR-Tudor de 53BP1 possédait cette capacité de liaison à l'ARN. Par la suite, pour déterminer la nature de l'ARN lié par 53BP1, la membrane, contenant les complexes ARN-protéines après la procédure de CLIP, est découpée pour extraire les acides nucléiques, qui sont alors déposés sur un gel dénaturant. Il a été observé que 53BP1 interagit directement avec des chimères ARN-ADN, avec une courte partie ARN en 5' et une longue partie ADN en 3', caractéristiques des amorces ARN-ADN générées par PRIM1-Pol α au niveau des fragments d'Okazaki.

L'objectif de cette thèse est donc de déterminer si le recrutement de 53BP1 à la fourche dépend de l'amorce ARN des fragments d'Okazaki, de comprendre le rôle de cette interaction dans le mécanisme de répllication de l'ADN et enfin, de mieux caractériser l'interaction entre 53BP1 et les fragments d'Okazaki en définissant les domaines protéiques impliquées et en identifiant un mutant associé.

RÉSULTATS

Nous avons démontré par des expériences de SIRF, permettant l'étude des interactions entre protéines et ADN naissant, que le recrutement de 53BP1 est inhibé si les cellules sont traitées préalablement avec la RNase A, qui clive l'ARN simple-brin, et la RNase H, qui clive l'ARN contenu dans des hybrides ARN:ADN. Par ailleurs, la déplétion de PRIM1, qui génère l'amorce ARN des fragments d'Okazaki, induit une diminution significative de 53BP1 à la fourche. À l'inverse la perte de FEN1, qui clive l'amorce ARN lors de la maturation des fragments d'Okazaki et accumule des flaps non-clivés, augmente l'association de 53BP1 à l'ADN naissant. Ces données indiquent donc que 53BP1 interagit avec l'amorce ARN des fragments d'Okazaki, sous la forme hybridée à l'ADN, au début de l'élongation, ainsi que sous la forme simple-brin, pendant la maturation, et que cette interaction est essentielle pour le recrutement de 53BP1 à la fourche.

Nous avons alors supposé que le rôle de 53BP1 était d'aider la maturation des fragments d'Okazaki. Si tel est le cas, la déplétion de 53BP1 devrait induire un défaut dans le traitement du brin retard. Ces défauts sont visibles par la présence de chaînes PAR car ils résultent en la formation d'un espace d'ADN simple-brin, entre deux fragments d'Okazaki non ligaturés, qui est reconnu par PARP conduisant alors à une cascade de PARylation. En utilisant une approche quantitative de cytométrie, le QIBC, permettant de déterminer l'abondance d'une protéine à la chromatine selon la phase du cycle cellulaire, nous avons observé que la déplétion de 53BP1 accumulait les chaînes PAR en phase S, traduisant ainsi par un défaut de maturation du brin retard. De plus, la perte de 53BP1 diminue l'association à l'ADN naissant de RPA et DNA2, qui sont les acteurs de la voie du long flap. Cela indique donc que 53BP1 agit en amont de ces protéines et pourrait promouvoir la voie du long flap pour maturer les fragments d'Okazaki. Ces résultats ayant été obtenus dans un contexte non stressé de la fourche, il serait intéressant de déterminer si le rôle connu de 53BP1 dans la gestion du stress répliatif est aussi médié par l'amorce ARN.

Nous avons ensuite tenté d'identifier un mutant de 53BP1 qui perd la liaison à l'ARN et donc potentiellement qui altère ses fonctions dans la réplication de l'ADN. En première intention, nous nous sommes concentrés sur le domaine GAR qui un domaine putatif de liaison à l'ARN. Ce domaine contient une répétition d'arginines et de glycines dont les arginines sont méthylées par PRMT1 et 5. Nous avons observé que le recrutement de 53BP1, à la fourche de réplication, est dépendant de cette signalisation de méthylation médiée par les enzymes PRMT. En outre, en utilisant la technique de CLIP et 2C, nous avons démontré que la région GAR-Tudor de 53BP1 n'est pas suffisante pour lier les amorces ARN-ADN, et que la région C-terminal est la région minimale de liaison aux fragments d'Okazaki. Ces données *in cellulo* sont en contradiction avec les données *in vitro* où le fragment GAR-Tudor suffisait pour lier des amorces ARN-ADN. Cela indique que cette région seule peut adopter une structure *in cellulo* qui affecte la liaison aux fragments d'Okazaki, ou que d'autres domaines protéiques de 53BP1 sont nécessaires pour son recrutement à la fourche de réplication.

La délétion de la région GAR-Tudor sur la protéine 53BP1 ne semble pas affecter la liaison aux fragments d'Okazaki par CLIP. Cependant, en réalisant des expériences de QIBC, la délétion du domaine GAR seul semble suffire à diminuer l'association de 53BP1 à la chromatine. L'implication du domaine GAR dans l'interaction 53BP1-fragments d'Okazaki n'est donc pas encore claire et une étude de biologie structurale plus approfondie aiderait à la caractérisation de l'interaction.

DISCUSSION ET PERSPECTIVES

Ces données ont donc permis de définir un nouveau rôle de 53BP1 et de l'identifier comme une RBP. Similaire à son rôle connu dans le choix des voies de réparation, 53BP1 pourrait être impliquée dans le choix des voies de maturation des fragments d'Okazaki. La voie que 53BP1 semble favoriser est la voie du long flap, qui paraît être une voie moins mutagène et plutôt choisie dans des zones transcriptionnellement actives. Par ailleurs, 53BP1 lie des hybrides ARN:ADN qui pourraient être la conséquence des conflits réplication-transcription présents au niveau de la chromatine active (R-loop). 53BP1 serait alors recrutée dans ces régions et ainsi jouer deux rôles distincts : induire un long flap sur le brin retard et prévenir de la formation des R-loop. Il serait alors intéressant de séquencer les fragments d'Okazaki liés à 53BP1 pour définir la localisation de cette interaction.

De plus, cette nouvelle liaison que 53BP1 établie, dans la réplication de l'ADN, pourrait être étudiée dans un contexte cancéreux puisque la délétion de 53BP1 est associée à des pathologies humaines ou à des résistances thérapeutiques. En effet, dans des cancers déficients pour BRCA, la perte de 53BP1 est connue pour induire une résistance aux inhibiteurs de PARP. Comprendre l'interaction 53BP1-ARN, dans ces situations, pourrait donc s'avérer important pour mieux appréhender les mécanismes de progression tumorale, déterminer des prédispositions pour certaines maladies et ainsi conduire à de meilleurs traitements thérapeutiques.

Par ailleurs, l'interaction 53BP1-ARN est la première interaction décrite entre une RBP et les fragments d'Okazaki. Il serait donc intéressant de caractériser d'autres RBP liant ces amorces ARN dans la réplication de l'ADN, par des approches à grande échelle d'identification de protéines à la fourche, tel que l'iPOND, couplées à des approches d'identification de RBP, tel que l'OOPS. Cette étude permettrait potentiellement de découvrir de nouvelles interactions protéines-ARN impliquées dans la réplication de l'ADN.

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LIST OF ABBREVIATIONS

2C	Complex Capture
4-SU	4-thiouridine
53BP1	p53-binding protein 1
ADAR	Adenosine Deaminase acting on RNA
AGPC	Acid Guanidinium Thiocyanate-Phenol-Chloroform
Alt-NHEJ	Alternative NHEJ
ARE	Adenylate/Uridylate-rich element
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine Triphosphate
ATR	ATM- and Rad3-related protein
BARD1	BRCA1 Associated RING Domain 1
BER	Base Excision Repair
BIR	Break-Induced Replication
BirA	Biotin ligase
BLM	Bloom syndrome protein
BRCA	Breast Cancer gene (BRCA1; BRCA2)
BRCT	BRCA1 C-terminus
CARIC	Click-chemistry Assisted RIC
CDC	Cell Division Cycle (CDC6; CDC45)
CDK	Cyclin-Dependent Kinase
cDNA	complementary DNA
CDT1	Chromatin licensing and DNA Replication factor 1
CHART	Capture Hybridization Analysis of RNA Targets
ChIRP	Chromatin Isolation by RNA Purification
CHK1	Checkpoint kinase 1
CldU	5-chloro-2'-deoxyuridine
CLIP	Crosslinking and Immunoprecipitation
CMG	CDC45-MCM-GINS complex
c-NHEJ	canonical NHEJ

CpG island	Cytosine-phosphate-Guanine island
CPSF	Cleavage and Polyadenylation Specificity Factor
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRUIS	CRISPR-based RNA-United Interacting System
CSR	Class Switch Recombination
CstF	Cleavage stimulatory Factor
CtIP	C-terminal-binding protein-interacting protein
DAPI	4', 6-diaminidino-2-phenylindole
DDK	Dbf4-dependent kinase
DDR	DNA Damage Response
DDRBP	DNA Damage RNA-binding protein
DDRNA	DNA Damage RNA
diIncRNA	damage-induced lncRNA
DNA2	DNA replication helicase/nuclease 2
DNA	Deoxyribonucleic acid
DNA-PKcs	catalytic subunit of DNA-dependent Protein Kinase
DNA Pol	DNA Polymerase (Pol α ; Pol ϵ ; Pol δ)
DNase	Desoxyribonuclease
dNTP	Deoxyribonucleoside triphosphate
DSB	Double-strand break
dsDNA	double-strand DNA
dsRBD	Double-strand RNA-binding domain
dsRNA	double-strand RNA
DYNLL1	LC8 Dynein Light chain
e. Coli	Escherichia coli
ECT2	Epithelial Cell Transforming 2
EdU	5-ethynyl-2-deoxyuridine
eiF4F	eukaryotic initiation Factor 4F complex (eiF4A; E; G)
EMSA	Electrophoretic Mobility Shift Assay
EXO1	Exonuclease 1
FA	Fanconi Anemia
FBH1	F-Box DNA Helicase 1
FEN1	Flap structure-specific Endonuclease 1

FMRP	Fragile X Mental Retardation Protein
FXS	Fragile X Syndrome
GAR	Glycin-Argining Rich
GFP	Green Fluorescent Protein
GINS	Go-ichi-ni-san complex
Gloe-seq	Genome-wide ligation of 3'-OH ends followed by sequencing
G-phase	Gap phase (G1; G2)
G-quadruplex	Guanine quadruplexes
hnRNP	heterogenous nuclear Ribonucleoprotein (hnRNP A1; C; F; G; H; U)
HR	Homologous Recombination
HTLF	Helicase-like Transcription Factor
HU	Hydroxyurea
HuR	Human antigen R
IDR	Intrinsically Disordered Region
IdU	5-iodo-2'-deoxyuridine
IR	Ionizing Radiation
IRES	Internal Ribosome Entry Site
KASRIC	Kethoxal Assisted Single-strand RIC
KH	Lysine Homology
LIG	DNA Ligase (LIG1; LIG3; LIG4)
LINP1	lncRNA in NHEJ pathway 1
lncRNA	long non-coding RNA
MCM2-7	Minichromosome maintenance 2-7
MDM	Murine Double Minute (MDM2; MDMX or 4)
METTL	Methyltransferase-like Enzyme
MMR	Mismatch Repair
M-phase	Mitosis phase
MRE11	Meiotic Recombination protein 11
MRN	MRE11-RAD50-NBS1
mRNA	messenger RNA
Ms2-BioTRAP	MS2 in vivo Biotin-tagged RAP
NBS1	Nijmegen Breakage Syndrome 1
NER	Nucleotide Excision Repair

NHEJ	Non-Homologous End-Joining
NLS	Nuclear Localisation Signal
NonO	Non-POU domain-containing octamer-binding protein
OD	Oligomerization domain
Ok-seq	Okazaki fragments sequencing
OOPS	Orthogonal Organic Phase Separation
ORC	Origin Recognition Complex
ORF	Open Reading Frame
ORI	Origin of Replication
PAIR	Peptide-nucleic-acid-Assisted Identification of RBP
PALB2	Partner and Localizer of BRCA2
PAP	Poly(A) Polymerase (PAP; PAPD5)
PAR	Poly(ADP-ribose)
PAR-crosslinking	Photoactivatable-ribonucleoside-enhanced crosslinking
PARG	Poly(ADP-ribose) Glycohydrolase
PARN	Poly(A)-Specific Ribonuclease
PARP1	Poly(ADP-ribose) Polymerase 1
PARPi	PARP1 inhibitors
PAZ	Piwi/Argonaute/Zwille
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PLA	Proximity Ligation Assay
PNK	Polynucleotide Kinase
poly(A)	poly(Adenine)
PP	Protein Phosphatase (PP1; PP4C)
PRIM	Primase (PRIM1; PRIM2)
PRMT	Protein arginine Methyltransferase
PRP19	pre-mRNA Processing Factor 19
PTIP	PAX Transactivation domain Interacting Protein
QIBC	Quantitative Image-Based Cytometry
RAP	RNA Affinity Purification
RaPID	RNA Proximal Protein Interaction Detection
RBD	RNA-binding domain

RBMX	RNA-binding motif protein X-linked
RBP	RNA-binding protein
RBPome	RNA-Bound Proteome
RecQ	RecQ DNA helicase (RecQ1 ; RecQL4)
RFC	Replication Factor C
RGG	Arginine/Glycine-rich repeats
RIC	RNA Interactome Capture
RIF1	Replication Timing Regulatory Factor 1
RIP	RNA Immunoprecipitation
R/K patch	Arginine- or Lysine-rich patch
RNA	Ribonucleic Acid
RNA Pol II	RNA Polymerase II
RNase	Ribonuclease
RNF	Ring Finger Protein (RNF8; RNF168)
RPA	Replication protein A
RRM	RNA Recognition Motif
rRNA	ribosomal RNA
RT-qPCR	Reverse Transcription-quantitative PCR
SAMHD1	SAM domain- and HD domain-containing protein 1
s. Cerevisiae	saccharomyces Cerevisiae
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide-gel electrophoresis
serIC	serial RIC
SFPQ	Splicing Factor Proline- and Glutamine-rich
SIRF	<i>in situ</i> protein Interaction with nascent DNA Replication Forks
SMARCAL1	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1
SMN	Survival Motor Neuron protein
S-phase	Synthesis phase
SR	Serine/Arginine repeats
SRSF1	Serine/Arginine-rich Splicing Factor 1
SSB	Single-strand break
ssDNA	single-strand DNA
ssRNA	single-strand RNA
STQ sites	Serine/Threonine/Glutamine sites

SYF2	SYF2 pre-mRNA Splicing Factor
TERRA	long non-coding Telomeric Repeat-containing RNA
TERT	Telomerase Reverse Transcriptase
TIRR	Tudor-Interacting Repair Regulator
TLS	Translesion DNA Synthesis
TOP1	DNA Topoisomerase I
TOPBP1	DNA Topoisomerase II-binding protein 1
Trael-seq	Transferase-Activated End Ligation sequencing
TRAPP	Total RNA-associated Protein Purification
TRIBE	Targets of RBP Identified By Editing
TRIP	Tandem RNA Isolation Procedure
TS	Template Switching
UDR	Ubiquitin-dependent Recruitment
UPF1	ATPase up-frameshift suppressor 1
USP28	Ubiquitin Specific Peptidase 28
UTR	Untranslated Region
UV	Ultraviolet
V(D)J	Variable, (Diversity), Joining gene segments
WRN	Werner syndrome helicase
XRCC	X-ray Repair Cross Complementing (XRCC1; XRCC4)
XRMAX	Protein Crosslink RNA Extraction
YB1	Y-box binding Protein 1
YTH	YT521-B Homology
ZnF	Zinc Finger
ZRANB	Zinc Finger Ran-binding domain-containing protein (ZRANB2; ZRANB3)

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INTRODUCTION

I – RNA-protein interactions

During its life, RNA is associated with RNA-binding proteins (RBPs) forming ribonucleoprotein complexes that control its metabolism. This dynamic interaction between proteins and RNAs is crucial for numerous cellular processes, from post-transcriptional regulation of gene expression to maintenance of genome integrity (Dutertre and Vagner, 2017; Dutertre *et al.*, 2014). Therefore, the section I-1 of the introduction aims to describe the canonical and non-canonical functions of RBPs.

RBPs are found across all biological kingdoms, and some are highly conserved (**Fig. 1**). In the past few years, approximately 1400 RBPs have been identified in human cells (Hentze *et al.*, 2018). These RBPs are commonly defined as proteins that directly bind RNA through one or more RNA-binding domains (RBDs), described in section I-2, which may impact the fate of the RNA molecules they bind.

Nowadays, a plethora of methods exist to study RNA-protein interactions, detailed in I-3, and those techniques have highlighted the existence of unconventional RBPs that do not harbour specific domains. These RBPs are often composed of disordered regions capable of binding RNA, also described in section I-2.

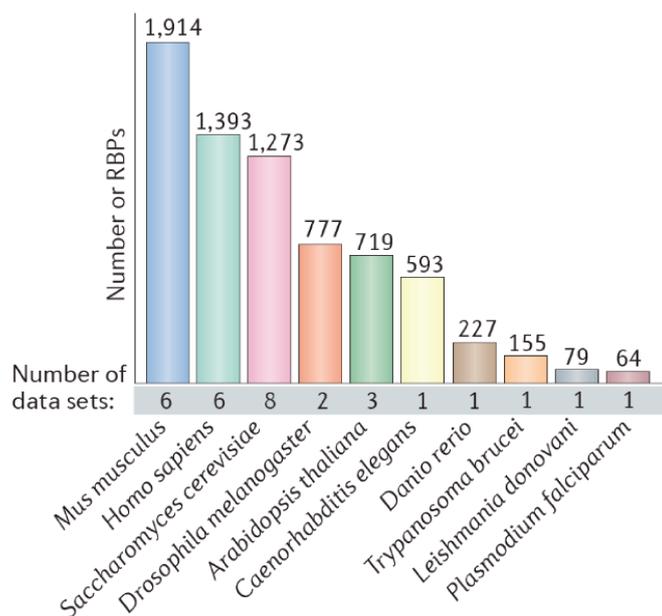


Figure 1 | Number of identified RNA-binding proteins in different organisms. Supersets of RBPs identified by the combination of different RBP detection studies in different cell lines and organisms. From Hentze *et al.*, 2018.

1. Roles in maintenance of genome integrity

1.1. Control of the expression of DNA damage response genes

RBPs are crucial regulators of mRNA metabolism, responsible for controlling co- and post-transcriptional events in the cell. They play essential roles in mRNA processing such as capping, splicing and polyadenylation (Gebauer *et al.*, 2021) (**Fig. 2**). These canonical functions will be detailed below, focusing more specifically on RBPs that act in the maintenance of genome stability.

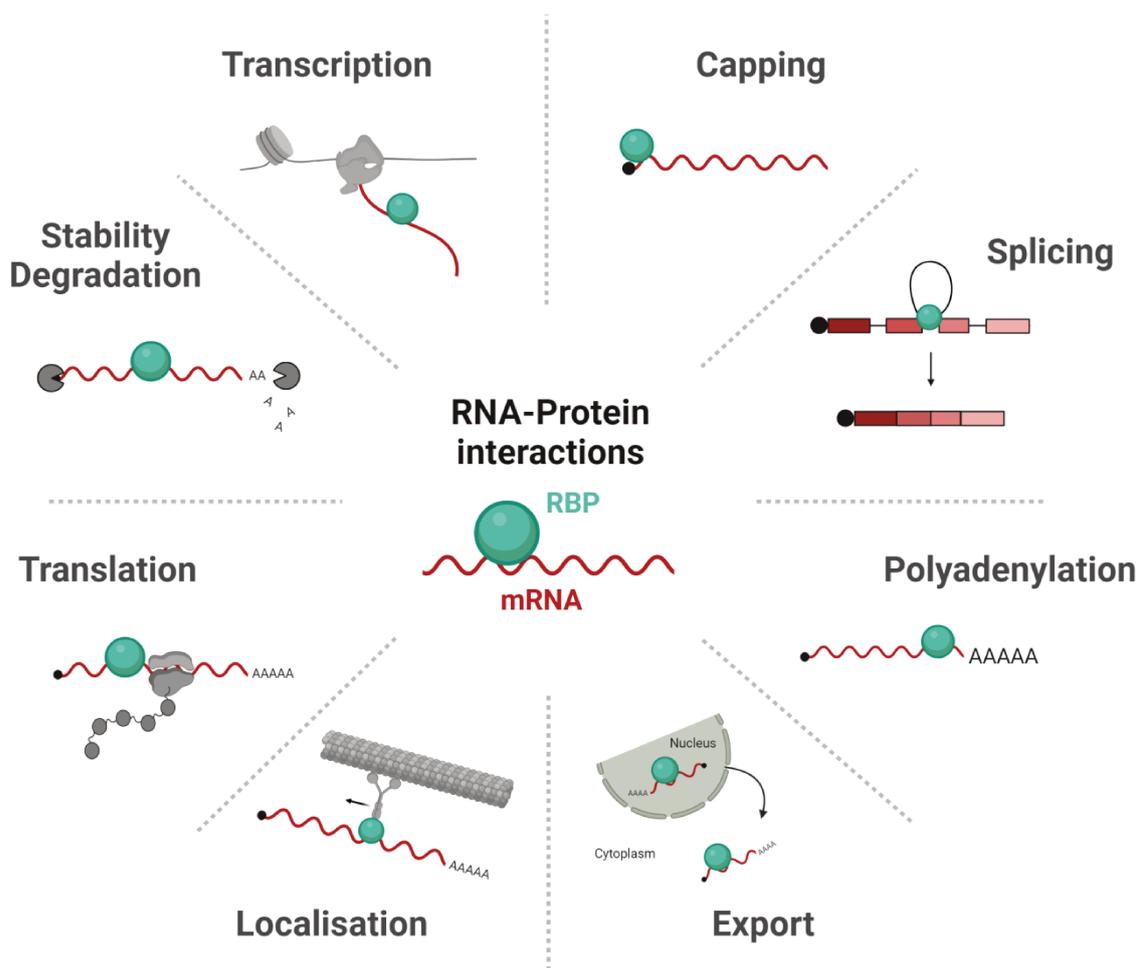


Figure 2 | Canonical roles of RNA-binding proteins. RNA-binding proteins (RBPs) are involved in every steps of mRNA metabolism, acting both in the nucleus (transcription, capping, splicing, polyadenylation) and in the cytoplasm (export, localisation, translation, stability, degradation).

Alternative splicing

Alternative splicing is a critical process which contributes to protein diversity. The heterogenous nuclear ribonucleoprotein (hnRNP) and Serine/Arginine proteins are the main players in this mechanism. Splicing factors bind to specific sequences on pre-mRNA molecules to regulate exon skipping, intron retention or the use of alternative splice sites (Chen and Manley, 2009). For instance, in 2020, a study conducted by the Vagner laboratory have demonstrated that the splicing factors zinc finger ran-binding domain-containing protein 2 (ZRANB2) and the SYF2 pre-mRNA splicing factor (SYF2) both associate with the *epithelial cell transforming 2* (ECT2) mRNA to favour the production of a variant that includes the exon 5. Interestingly, in breast cancer, depletion of this ECT2 variant increases sensitivity to doxorubicin, a drug that inhibits topoisomerase II and induces DNA damages (Tanaka *et al.*, 2020).

Polyadenylation

RBPs are responsible for the process of polyadenylation, which consists of the addition of stretches of Adenines at the 3' end of mRNAs. First the pre-mRNA is cleaved by the cleavage and polyadenylation specificity factor (CPSF) and the cleavage stimulatory factor (CstF). The poly(A) tail is then added by the poly(A) polymerase (PAP) allowing the stabilisation of the mRNA (Proudfoot *et al.*, 2002). Alteration in the polyadenylation affects the transcript at several levels including its export in the cytoplasm, its translation, and its decay rate. These mechanisms (cleavage and polyadenylation) are regulated differently depending on the cellular context. In damaged cells, CstF is sequestered in complexes with various factors, including RNA polymerase II (RNA Pol II), the tumour suppressors BARD1/BRCA1 and PARN, which inhibits 3' processing. However, Adrien Decorsière *et al.* have shown that following DNA damage, the RBPs hnRNP H and F prevent this sequestration to maintain the 3' maturation of *p53* mRNA. These RBPs interact with a G-quadruplex structure downstream of the polyadenylation site of *p53* resulting in the recruitment of Cstf and PAP (Decorsière *et al.*, 2011). Since, the *p53* protein is considered as “the guardian of the genome”, the *p53* mRNA must be tightly regulated by RBPs at all levels, mentioned above, to properly respond to potential cellular stresses (Haronikova *et al.*, 2019).

mRNA stability

Along with the poly(A) tail, the 3' untranslated region (UTR) plays an important role in transcript stability. Among the various 3'UTR-interacting proteins, the human antigen R (HuR) is known to bind a multitude of mRNAs to enhance their translation. For instance, in response to genotoxic agents, HuR stabilises the *poly(ADP-ribose) glycohydrolase* (PARG) mRNA, thereby upregulating PARG expression to facilitate DNA repair through the removal of poly(ADP-ribose) (PAR) chains and the modulation of the interaction between poly(ADP-ribose) polymerase 1 (PARP1) and chromatin (Chand *et al.*, 2017). The *p53* mRNA is also targeted by HuR. In colorectal carcinoma cells, HuR binds to *p53* through the AU-rich elements (AREs), in a UV-dependent manner to stabilise it and thus favour its translation (Mazan-Mamczarz *et al.*, 2003). Moreover, in response to exposure to ionizing radiation HuR can dissociate from its target mRNA hence promoting the cell survival (Masuda *et al.*, 2011).

Translation

Once the mRNA is stabilised, translation can begin, and this post-transcriptional mechanism is also controlled by RBPs. The translation of *p53* mRNA is regulated by numerous RBPs, such as murine double minute 2 (MDM2). Under normal conditions, the E3 ubiquitin ligase MDM2 interacts with the p53 protein to induce its degradation. Following DNA damage, MDM2 gets phosphorylated by the Serine/Threonine kinase Ataxia-telangiectasia mutated (ATM), enabling its interaction with *p53* mRNA, and thereby stimulating its translation. Indeed, phosphorylated MDM2 binds *p53* mRNA through its RING domain and this association results in an increase in synthesis and a decrease in MDM2-dependent degradation of p53 (Gajjar *et al.*, 2012). The murine double minute 4 (MDMX), an MDM2 homolog also phosphorylated by ATM, facilitates this interaction by serving as a chaperone for *p53* mRNA and providing an MDM2 binding platform (Haronikova *et al.*, 2019). Furthermore, disturbances in the translation process are a major event leading to the development of cancer (For review: Fabbri *et al.*, 2021). When oncogenic signalling pathways are active, this can induce the activation of the eukaryotic translation initiation complex eIF4F (composed of eIF4A, eIF4E and eIF4G) and thus promoting translation. RBPs can then

act downstream of these signalling pathways to control the translation of specific mRNAs. For example, HuR increases the stability of eIF4E transcripts in certain cancers, inducing the expression, among others, of *MYC*. The oncoprotein MYC is involved in cell proliferation, and it enhances the expression of components of the eIF4F complex.

Localisation

Many RBPs are also involved in the transport of RNA molecules to specific subcellular compartments. For instance, the fragile X mental retardation protein (FMRP) associates with G-quadruplexes forming in the 3'UTR of its mRNAs target to promote their localisation throughout neuronal projections. FMRP interacts with RNA through its Glycine-Arginine-rich domain (GAR domain composed of a RGG motif) and the deletion of this domain results in a defect in transcript transport. These defects are explained by a reduced ability of FMRP to bind mRNAs, and not by a reduced capacity of the protein to be transported into neurites (Goering *et al.*, 2020). Moreover, FMRP is an essential protein for proper brain development and is involved, among other functions, in neuronal activity and synaptic plasticity. Importantly, the absence of FMRP leads to Fragile X Syndrome (FXS), a genetic disorder that often causes cognitive impairment, behavioural disorders and physical abnormalities. This syndrome is one of the most common forms of hereditary intellectual disability. Additionally, a mutation found in patients with FXS involves an insertion within the RGG box, causing an ORF frameshift, disrupting the RGG box and leading to the production of a short C-terminal sequence. The novel C-terminal peptide encodes a functional nuclear localisation signal altering the subcellular localisation of FMRP (Okray *et al.*, 2015).

As described above RBPs act as co- and post-transcriptional regulators, and may participate in genome stability by targeting DNA damage response (DDR) genes (Dutertre *et al.*, 2014). Furthermore, several DDR proteins have been characterised as having RNA-binding capabilities (**Fig. 3**) (Audouinaud *et al.*, 2021). For example, the NHEJ factor Ku is an RBP that directly binds to RNA with a hairpin structure possessing a bulge. One target of Ku is the *p53* mRNA and Ku interacts with the 3'UTR region of *p53* to repress its translation under normal conditions. When DNA is damaged, this interaction is inhibited to allow the expression of p53 (Lamaa *et al.*, 2016). Another example is the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) which interacts with the long non-coding RNA *LINP1* to promote the NHEJ pathway. *LINP1* also acts as a scaffold in the Ku/DNA-PKcs complex (Zhang *et al.*, 2016). One last example of a DDR protein identified as an RBP is the PARP1 protein. PARP1 recognizes and binds single- and double-strand breaks and enables the recruitment of DDR factors to damage sites through PARylation. Additionally, if the damage is too severe, PARP1 promotes apoptosis. In 2022, Karla F. Meza-Sosa and colleagues demonstrated using RNA affinity purification (RAP) followed by western blot experiments, that PARP1 is bound by *SPARCLE*, a long non-coding RNA induced by p53. *SPARCLE* is induced after one day of DNA damage and does not impact gene expression, but it interacts with PARP1 to stimulate its cleavage mediated by caspase 3, and thus inhibits DNA repair to promote apoptosis (Meza-Sosa *et al.*, 2022).

1.2. Direct players in DNA repair signalling

Apart from RBPs with defined functions in RNA biology, these past years have seen the discovery of an increasing number of RBPs localised at DNA damage sites and therefore playing a more direct role in DDR (**Fig. 3**). These RBPs constitute a new class of DDR players and are now known as DNA Damage RNA-binding proteins (DDRBP) (Dutertre and Vagner, 2017). However, although it is becoming increasingly evident that RNA itself is involved at damage sites (Audouinaud *et al.*, 2021; Michelini *et al.*, 2017, 2018; Zong *et al.*, 2020), it is not yet clearly defined whether the function of RBPs in DNA repair signalling depends on their RNA-binding activity.

When DNA damage occurs, the cell initiates an intricate signalling cascade to facilitate their repair. The human body is composed of approximately $3.7 \cdot 10^{13}$ cells, and each cell undergoes tens of thousands of lesions on average per day. These damages to DNA can originate from the external environment, through the action of physical or chemical genotoxic agents, or from cellular processes themselves (**Fig. 3**).

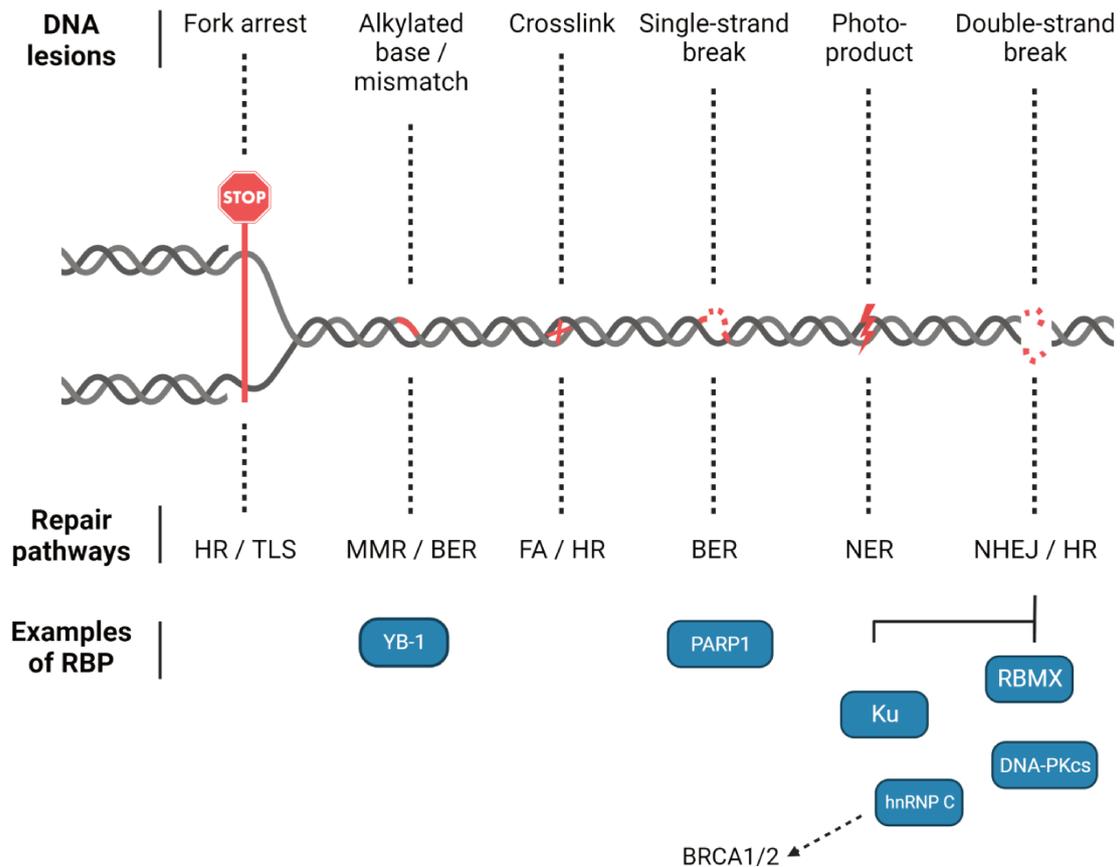


Figure 3 | RNA-binding proteins help to maintain genome stability. DNA damage can originate from endogenous (replication errors, metabolism) or exogenous (ionising radiation, genotoxic agents) sources and can result in alkylated base, mismatch, crosslink, photo-product, single-strand or double-strand DNA break. As indicated, these lesions are repaired by several pathways: homologous recombination (HR), translesion DNA synthesis (TLS), mismatch repair (MMR), base excision repair (BER), fanconi anemia (FA) pathway, nucleotide excision repair (NER) and non-homologous end joining (NHEJ). RBPs (in blue) are involved in these DNA repair pathways. Adapted from Dutertre *et al.*, 2014.

Physical agents are the ionizing radiations and ultraviolet (UV) rays. Chemical agents are typically agents used in chemotherapy, such as alkylating agents (methyl methanesulfonate), cross-linking agents (cisplatin) and topoisomerase inhibitors (etoposide) but they can also be environmental pollutants such as cigarette smoke (Ciccia and Elledge, 2010). Endogenous sources include replication stress (errors, stalled forks), products of cellular metabolism (free radicals) and spontaneous chemical modifications (depurination, deamination) (Tubbs and Nussenzweig, 2017). These endogenous and exogenous lesions generally result in base modifications, mismatches, inter- or intra-strand crosslinks and single- or double-strand breaks.

RBPs prevent DNA damage by controlling R-loop formation, DNA topology and DNA metabolism

RBPs intervene to help prevent the occurrence of the lesions described above (**Fig. 3**) (Dutertre *et al.*, 2014). As mentioned, an important endogenous source of DNA damage is the collision between transcription and replication leading to stalled replication forks during the S-phase. The nascent RNA transcribed by the RNA polymerase II hybridizes with the template DNA strand thus forming RNA:DNA hybrids, displacing the complementary DNA strand and creating a three-strand structure called an R-loop. The formation of R-loops is favoured at Guanine-rich DNA repeats and topologically constraint regions and can be controlled by multiple RBPs (Aguilera and García-Muse, 2012). For instance, depletion of the RBP Serine/Arginine-rich splicing factor 1 (SRSF1 or ASF/SF2) results in an accumulation of R-loops and a hypermutation phenotype. SRSF1 interacts with the nascent pre-mRNA to promote its splicing, facilitated by the carboxy-terminal domain of RNA Pol II which recruits SRSF1 to the transcript, preventing it from associating with the template DNA and thus inhibiting the formation of R-loops (Li and Manley, 2005). SRSF1 also interacts with DNA topoisomerase I (TOP1) to inhibit the occurrence of R-loops (Tuduri *et al.*, 2009). TOP1 resolves topological constraints by relaxing DNA, and interactions between RBPs and TOP1 contribute to safeguarding the integrity of the DNA (Czubaty *et al.*, 2005).

Several RBPs are also being identified as playing roles in DNA metabolism such as DNA replication and telomere biogenesis, which can be a source of DNA damage. Indeed, telomeres are a favourable site for the formation of R-loops. The long non-coding telomeric repeat-containing RNA (*TERRA*) is prone to form this RNA:DNA hybrids resulting in telomere instability. The NonO and SFPQ proteins which bind *TERRA*, suppress RNA:DNA hybrids formation and replication defects at telomeres to ensure their integrity (Petti *et al.*, 2019). In addition, the hnRNP family is also strongly involved in telomere regulation. The length of telomeres is regulated by hnRNP C and U by associating with telomerase reverse transcriptase (TERT). The hnRNP F protein interacts with *TERRA* to control its localisation, thereby impacting the telomere length as well. The hnRNP A1 protein interacts with *TERRA*, enabling the regulation of telomere coating by the Shelterin complex during DNA replication (Nishida *et al.*, 2017). HnRNP A1 also interacts with the flap structure-specific endonuclease 1 (FEN1) to stimulate its activity and therefore to assist in the maturation of Okazaki fragments during the replication process (see section II-3) (Chai *et al.*, 2003). Furthermore, in response to DNA damage, the RNA helicase UPF1 associates with DNA polymerase δ to mediate DNA replication and DNA repair in S-phase (Azzalin and Lingner, 2006).

RBPs can be directly involved in DNA repair pathways

Despite all the existed mechanisms that prevent DNA damage, lesions can still occur. The repair of these lesions is essential for the cell survival and to avoid the accumulation of DNA damage. If the repair is optimal, the cell will proceed with its regular division cycle but if the damage is not properly repaired, it will disrupt the cell cycle. There are several repair pathways with specific actors that depend on the type of lesions and the cell cycle phase (**Fig. 3**). For example, the removal and replacement of damaged bases are handled by the base excision repair (BER) pathway, mismatched bases by the mismatch repair (MMR) pathway, intra-strand crosslinks by the nucleotide excision repair (NER) pathway, while inter-strand crosslinks, as well as double-strand breaks, are repaired of by the homologous recombination (HR) pathway in S-phase or by the non-homologous end joining (NHEJ) pathway in G1-phase (Hakem, 2008; Huang and Zhou, 2021).

In the past few years, the co-localisation between DNA damage sites and certain RBPs has been observed (Dutertre *et al.*, 2014). RBPs are recruited to the damaged DNA in a manner that may or may not be dependent on their RNA-binding activity. Indeed, once recruited, RBPs can interact, independently of RNA, with DDR factors, DNA or PAR chains. PAR chains generated by poly(ADP-ribose) polymerase (PARP) are necessary for recruiting certain RBPs, mainly from the splicing factors family such as RNA-binding motif protein X-linked (RBMX). Indeed, Britt Adamson and colleagues demonstrated in 2012 that RBMX, also known as heterogenous nuclear ribonucleoprotein G (hnRNP G), accumulates at double-strand break sites in a PARP-dependent manner to promote HR (Adamson *et al.*, 2012). Moreover, RBMX is capable of binding to damaged DNA to protect the ends (Shin *et al.*, 2007). Indeed, RBPs can be recruited to damage sites by interacting with the DNA to regulate the signalling of repair pathways. For example, the RBP Y-box binding protein 1 (YB-1) interacts with mismatched DNA and can interfere with the MMR repair pathway (Chang *et al.*, 2014). Another example includes the pre-mRNA processing factor 19 (PRP19) E3 ubiquitin ligase whose ubiquitination favours its binding to double-strand DNA to regulate DNA repair pathways (Idrissou and Maréchal, 2022). PRP19 is recruited to single-strand DNA, formed following damage, by interacting directly with Replication Protein A (RPA) to facilitate the recruitment of other DDR factors (Maréchal *et al.*, 2014). RBPs can also be recruited to damage sites through direct protein-protein interaction. For instance, the splicing factor heterogenous nuclear ribonucleoprotein C (hnRNP C) is part of the complex containing the breast cancer gene 1 and 2 (BRCA1/2) and the partner and localizer of BRCA2 (PALB2) to assist in HR (Anantha *et al.*, 2013).

These examples demonstrate that, in addition to their function in the expression of DDR coding genes, RBPs have a more direct role in the repair pathways that often relies on protein-protein interactions. However, it remains unclear whether it also depends on their RNA-binding activity.

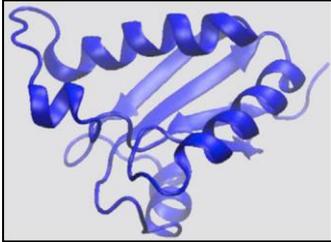
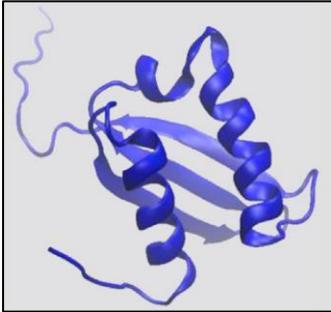
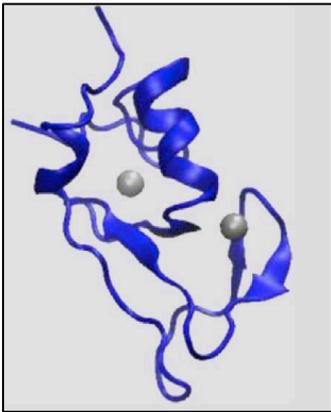
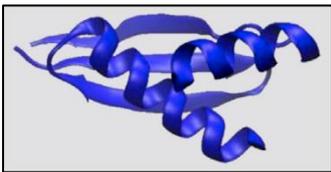
In summary, RNA-protein interactions are crucial for many cellular processes including mRNA processing, regulation of gene expression, and the maintenance of telomere integrity and genome stability. Mutations in RBPs, such as FMRP whose mutations are associated with FXS, can therefore alter RNA-RBP interactions and, consequently, cause human diseases. For this reason, the aberrant expression or altered function of RBPs in pathologies are being increasingly studied (Hong, 2017; Mohibi *et al.*, 2019).

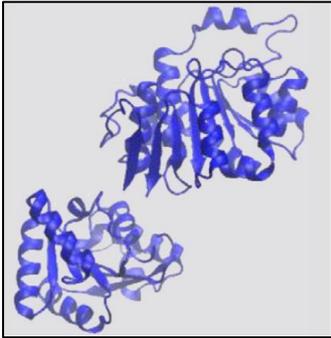
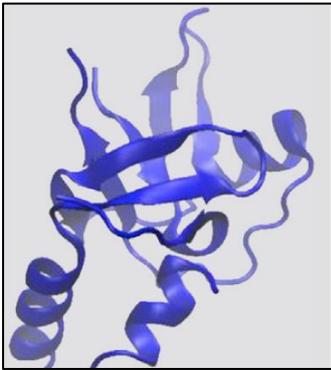
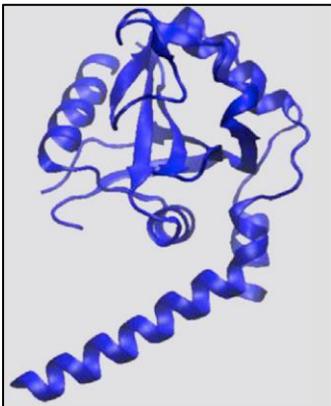
2. RNA-binding domains

As mentioned above, RBPs are major regulators of gene expression due to their direct interaction with mRNAs. Recent approaches have enabled the classification of RBPs into two categories: canonical and unconventional. Canonical RBPs possess one or more RBDs, while unconventional RBPs may present disordered regions or others unconventional ways to interact with RNAs. Furthermore, most RBDs are conserved and homologous to human RBPs are found in other organisms, such as yeast (Gerstberger *et al.*, 2014a). Among the unconventional RBPs, some are also involved in biological processes distinct from mRNA processing (Baltz *et al.*, 2012; Castello *et al.*, 2012; Hentze *et al.*, 2018). Indeed, 27% of identified RBPs in human and 40% in yeast have no known function related to RNA biology and these RBPs are called “enigmRBP” (Beckmann *et al.*, 2015). Many of these RBPs exhibit enzymatic activities and are involved in classical metabolic pathways (Beckmann *et al.*, 2016; Castello *et al.*, 2012; Perez-Perri *et al.*, 2023).

There are several characterised types of RBDs, each exhibiting unique structural and functional properties and a few examples of RBDs are succinctly described in **Table 1** (Corley *et al.*, 2020). It is noteworthy that RBPs can possess both canonical and non-canonical domains. Additionally, a considerable number of RBPs still lack a precisely defined RBD.

Table 1 | Examples of canonical and unconventional RNA-binding domains. Adapted from Corley *et al.*, 2020.

RNA-binding domains	Structure	Examples of RBPs
RNA recognition motif (RRM)	<p>90 amino acids</p> 	<p>Splicing factors Present in 13% of RBPs</p>
K-homology (KH)	<p>70 amino acids</p> 	<p>Translation regulator Present in 3% of RBPs</p>
Zinc finger (ZnF)	<p>30 amino acids</p> 	<p>Transcription factors, METTL enzymes</p>
Double-strand RNA-binding domain (dsRBD)	<p>65-70 amino acids</p> 	<p>RNAses, ADAR, Dicer</p>

<p>Dead box helicases domain</p>	<p>350-400 amino acids</p> 	<p>RNA-binding helicases Present in 4% of RBPs</p>
<p>Piwi/Argonaute/Zwille (PAZ)</p>	<p>170 amino acids</p> 	<p>Argonaute proteins, Dicer</p>
<p>Intrinsically disordered regions (IDR)</p>	<p>Variable</p>	<p>Most RBP (<i>e.g.</i> SRSF1, FMRP, hnRNP, PAPD5) Present in 55% of RBPs</p>
<p>YT521-B Homology (YTH)</p>	<p>100-150 amino acids</p> 	<p>m⁶A readers</p>

2.1. Canonical domains

Multiple RBDs can be found within a single RBP. This increases the affinity and specificity of the RNA-proteins interaction. Currently, the best characterised RBDs include the RNA recognition motif (RRM), the K-homology (KH) domain, the zinc finger (ZnF) domain and the double-strand RNA-binding domain (dsRBD).

RNA recognition motif

The RRM is the most abundant domain in higher eukaryotes (Gerstberger *et al.*, 2014b). This common type of RBDs is found in many RBPs, mainly in splicing factors such as the hnRNP A1 protein. In general, the RRM is a 90 amino acids domain that encompasses two conserved sequences known as RNP1 and RNP2. These sequences are composed of 8 and 6 amino acids, respectively. The RRM adopts a characteristic structure, called $\beta_1\alpha_1\beta_2\beta_3\alpha_2\beta_4$ topology, involving a four-strand antiparallel β -sheet supported by two α -helices. The RNA-binding sequences RNP1 and RNP2 are positioned in the central β -sheet β_3 and β_1 , respectively. They expose three conserved aromatic residues on the surface of the β -sheet, which serve as the main binding surface for RNA molecules (Cléry and Allain, 2011; Daubner *et al.*, 2013). Due to their structure, RRM domains interact with 2-8 nucleotides of single-strand RNAs, and the accumulation of RRMs in an RBP enhances the specificity of the RNA-protein interaction (Maris *et al.*, 2005).

K-homology domain

Historically, the KH domain was discovered in the hnRNP K protein in the 1990s and has been identified in numerous other RBPs ever since, such as translation regulators. It is a domain composed of 70 amino acids that has the ability to bind both single-strand RNAs and single-strand DNAs (Valverde *et al.*, 2008). Generally, the RNA-binding surface of the KH domain includes two α_1 and α_2 helices, connected by a GXXG loop (X being any amino acid) and a β -sheet. The conserved GXXG motif can recognize 4 nucleotides (Cléry and Allain, 2011). Similar to RRM domains, KH domains are often repeated to enhance the efficiency of RNA-binding (Corley *et al.*, 2020).

Zinc finger domain

While zinc finger proteins are most known for their role as DNA-binding transcription factors, there are certain classes of zinc finger proteins that also function as RNA-binding proteins, like the methyltransferase-like (METTL) enzymes. The zinc finger domain contains 30 amino acids and forms a $\beta\beta\alpha$ topology coordinated by a zinc ion. There are several types of zinc finger domain capable of binding RNA, classified based on the amino acids that interact with this ion (*e.g.* CCHC, CCCH, CCCC or CCHH zinc fingers) (Cléry and Allain, 2011; Hall, 2005).

Double-strand RNA-binding domain

Double-strand RNA-binding domains are characterised by a conserved $\alpha\beta\beta\alpha$ topology and typically consist of around 70 amino acids. They are found in various proteins across different organisms, such as RNAses or adenosine deaminases acting on RNA (ADAR), and play important roles in RNA processing, RNA editing and RNA interference. It is often considered that these domains recognize the shape of RNA rather than the sequence. However, it has been shown that *in vivo*, proteins containing the dsRBD bind to specific double-strand RNAs (Masliah *et al.*, 2013).

There are also other classical sequence-specific RBDs that have been characterised over the years, and not described here, such as the dead box helicases domain and the Piwi/Argonaute/Zwille (PAZ) domain (Corley *et al.*, 2020).

2.2. Intrinsically disordered regions

Among the large family of RBPs, more than half do not possess known RBDs. For comparison, the RRM domain, which is the most common, is only present in 13% of RBPs (Beckmann *et al.*, 2016). These non-canonical RBDs identified in RBPs do not have the same structural features as the canonical RBDs, but they still play important roles in RNA-binding and processing.

Recent techniques for studying RBDs have revealed that most RBPs actually possess intrinsically disordered regions (IDRs) (Castello *et al.*, 2016). An IDR is a region of at least 30 amino acids that lacks stable tertiary structure (Uversky, 2019). This instability is due to low sequence complexity of IDRs. Indeed, IDRs are depleted in hydrophobic residues and enriched in amino acids that promote disorder, such as charged or polar residues (Glycine, Proline, Lysine, Arginine, Aspartate, Glutamate, Serine), as well as Tyrosine, which is an amino acid found in surfaces interacting with RNAs. There are different types of IDRs based on the repetition of the disordered motif: Serine/Arginine (SR repeats), Arginine/Glycine (RGG repeats) and Arginine- or Lysine-rich patches (R/K basic patches).

The lack of a defined structure in IDRs enables them to recognize RNA sequences and structures that are not accessible to more structured RBDs. Furthermore, the binding to RNAs stabilizes the disordered structure of these regions (Järvelin *et al.*, 2016; Varadi *et al.*, 2015). RBPs with IDRs typically have multiple binding sites that interact with RNAs in a flexible and dynamic manner. However, a very recent study (Ray *et al.*, 2023) appears to show that many unconventional RBPs do not bind to RNAs in a specific manner, which could be precisely associated with the abundance of IDRs within the protein.

SR repeats

Serine and Arginine-rich repetitions are predominantly found in SR proteins, known as splicing factors for the pre-mRNA, such as Serine/Arginine-rich splicing factor 1 (SRSF1). The splicing efficiency depends on the number of repeats and phosphorylation of the SR motif. These proteins contain an RRM domain at the N-terminal end, which recognizes the RNA, and an SR-rich domain at the C-terminal end,

which plays a role in protein-protein and RNA-protein interactions. In addition proteins containing SR motifs are also involved in various processes, such as mRNA export, translation and genome stability maintenance. For example, SRSF3 acts as a post-transcriptional regulator by controlling internal ribosome entry site (IRES)-mediated translation (Jeong, 2017).

RGG repeats

The RGG repetitions, also known as GAR, are a motif frequently found in RBPs, but their RNA-binding properties are still poorly defined. The interaction between RNA and RGG repeats varies depending on the sequence and structure of the target RNA. For example, the RGG motif of the FMRP interacts with its target, the *sc1* RNA, through the recognition of a G-quadruplex structure (Phan *et al.*, 2011). In addition, proteins with RGG repeats play key roles in many cellular processes such as transcription, DNA damage signalling and regulation of apoptosis. For example, meiotic recombination 11 (MRE11), which is the first player in the signalling of DNA repair pathways, possesses a GAR domain and this motif is essential for the recruitment of MRE11 to DNA damage sites (Yu *et al.*, 2012).

The activity of the RGG motif in these mechanisms is often regulated by Arginine methylation by protein arginine methyltransferase (PRMT) (Thandapani *et al.*, 2013). This post-translational modification influences the localisation, activity and function of proteins with GAR domains, as well as the interaction of these proteins with their partners (Chong *et al.*, 2018). For instance MRE11 is methylated by the enzyme PRMT1, important for its localisation at DNA damage sites and also for the DNA damage checkpoint response mediated by MRE11 (Boisvert *et al.*, 2005a, 2005b; Déry *et al.*, 2008). The 53BP1 protein, a crucial protein in double-strand break repair, also possesses an RGG motif (described below in III-2) that is methylated by PRMT enzymes and this methylation is important for stabilizing 53BP1 (Boisvert *et al.*, 2005c; Hwang *et al.*, 2020)

R/K basic patches

Basic patches are composed of 4 to 8 Lysines or, less frequently, Arginines forming a highly positive surface that mediates molecular interactions. These motifs are very common among RBPs. Basic patches can be found anywhere in the protein structure and often form islands that flank globular domains, indicating the cooperation between structured and disordered regions (Järvelin *et al.*, 2016). An example of a protein with a basic patch is the poly(A) RNA polymerase D5 (PAPD5), involved in the degradation of aberrant rRNA. At its C-terminal end, PAPD5 possesses a basic patch, containing Lysines, that is directly involved in its RNA-binding activity (Rammelt *et al.*, 2011).

In summary, disordered regions as well as canonical RNA-binding domains play an important role in biological processes through their RNA-binding activity. The domains detailed above constitute only a fraction of the existing RBDs. Research on RNA-protein interactions continues to expand, and system-wide approaches (see section I-3) have led to the discovery of new domains and domains that are yet to be annotated. For example, the YT521-B homology (YTH) was identified in 2010 as a novel RBD that binds modified RNAs (m⁶A mark) (Zhang *et al.*, 2010). Overall, these additional RBDs enrich the repertoire of RBDs in RBPs and highlight the diverse ways in which RBPs interact with RNAs.

3. Methods to monitor RNA-protein interactions

The precise mechanisms by which an RBP recognizes and selectively interacts with its target RNA are not always well understood, but various crystallography and structural biology techniques have provided some insights. Several methods are available to characterise RNA-protein interactions *in vivo*, both from a protein-centric or RNA-centric perspectives. In other words there are techniques to identify the RNAs bound to a specific RBP or to determine the RNA-bound proteome (RBPome), *i.e.* the entire set of proteins interacting with RNA at a given time (Van Ende *et al.*, 2020; Ramanathan *et al.*, 2019) (**Table 2**).

Historically, RNA-protein interactions have been described using methods based on UV-C irradiation at 254 nm, creating irreversible covalent bond within living cells, known as cross-linking. Cross-linking enables the interactions to be maintained and the RNA-protein complexes to be purified under stringent conditions. The UV-C cross-linking requires direct contact (“zero” distance) between the protein and the nucleic acids and does not favour protein-protein cross-linking (Greenberg, 1979; Pashev *et al.*, 1991). UV-C cross-linking depends on the structure of the RNA and the protein, which implies that overall UV-C cross-linking has low efficiency. Indeed, UV-C has a preference for crosslinking uridines (Hockensmith *et al.*, 1993) and also certain amino acids (Cysteine, Lysine, Phenylalanine, Tryptophane and Tyrosine). In addition, even though UV-C crosslinks RNA-protein interactions more efficiently (mainly single-strand RNA), it has also been used to crosslink interactions between protein and single-strand DNA (Steen *et al.*, 2001).

Furthermore, there are other crosslinking methods used to study RNA-protein interactions, such as crosslinking by formaldehyde treatment which is not specific to RNA and can also crosslink protein-protein interactions (Niranjanakumari *et al.*, 2002), or photoactivatable-ribonucleoside-enhanced crosslinking (PAR-crosslinking) with incorporation of 4-thiouridine (4-SU), an uridine analogue (Hafner *et al.*, 2010). In the case of PAR-crosslinking, 4-SU is incorporated into nascent RNA and UV-A at 365 nm is used to crosslink interactions between protein and RNA containing 4-SU. These crosslinking alternatives have disadvantages (protein-protein crosslinking for formaldehyde and cellular toxicity for PAR-crosslinking) which makes UV-C the preferred choice in many techniques for studying RNA-protein interactions.

Table 2 | Examples of methods to monitor RNA-protein interactions. Adapted from Ramanathan *et al.*, 2019 and Van Ende *et al.*, 2020.

	Methods	Advantages	Disadvantages	References
RNA-centric techniques	RAP	<i>In vivo</i> High specificity with UV cross-linking and long oligonucleotide probes (120 nts)	High Input cell numbers	McHugh and Guttman, 2018
	PAIR	<i>In vivo</i> High specificity with UV cross-linking	Cost and effort for production of peptide nucleic acid	Zeng <i>et al.</i> , 2006
	MS2-BioTRAP	<i>In vivo</i> High specificity with UV cross-linking	Requires MS2 conjugation to RNA Transfection/infection of RNA and labeller protein High input cell numbers	Tsai <i>et al.</i> , 2011
	TRIP	<i>In vivo</i> High specificity with UV cross-linking	Two capture steps with poly(A) and biotinylated ASO capture decrease efficiency	Matia-González <i>et al.</i> , 2017
	CHART	<i>In vivo</i>	Additional RNase H step to identify accessible sites for probes High input cell numbers	Simon <i>et al.</i> , 2011
	ChIRP	<i>In vivo</i> No prior knowledge of RNA accessibility required for probe design	Short probes may pull down similar sequence fragments High input cell numbers	Chu <i>et al.</i> , 2011
	RaPID	<i>In vivo</i> Low number of cells required Direct labelling of protein	Requires BoxB link to RNA Short sequence limits Transfection/infection of RNA and labeller protein	Ramanathan <i>et al.</i> , 2018
	RIC	<i>In vivo</i> Specifically poly(A) RNA associated Widely used protocol	Isolates only mRNA complexes Co-purification of free RNA	Baltz <i>et al.</i> , 2012; Castello <i>et al.</i> , 2012
	CARIC	All RNA types	Use of nucleoside analogues Co-purification of naturally biotinylated proteins	Huang <i>et al.</i> , 2018

	2C	Fast and cost-effective method	Contamination of both free protein and free RNA Dependent on the scale of the silica columns	Asencio <i>et al.</i> , 2018
	TRAPP	Cost-effective method Scalable protocol	DNA is co-eluted Co-purification of free RNA	Shchepachev <i>et al.</i> , 2019
	OOPS	All RNA type Cost-effective method	Technically challenging Cannot be used as a starting point	Queiroz <i>et al.</i> , 2019
	XRNAX	All RNA types Little free RNA Cost-effective method Good starting point	Glycoproteins and RNA-protein adducts cannot be distinguished Technically challenging	Trendel <i>et al.</i> , 2019
Protein-centric techniques	RIP-seq	No knowledge of the RNA is required Low signal-to-noise ratio	Non-specific antibodies will precipitate non-specific complexes Lack of cross-linking may lead to false negatives	Zhao <i>et al.</i> , 2010
	CLIP-seq	<i>In vivo</i> Well-established method Can identify interactions at the 3' end of RNA	Long protocol UV cross-linking may be poor Reverse transcriptase must bypass the cross-linked nucleic acids	Licatalosi <i>et al.</i> , 2008; Ule <i>et al.</i> , 2005
	TRIBE	No need to purify the protein of interest No dependence on UV cross-linking RBP interaction may occur anywhere in RNA	Less extensive examples of effective use of the method	McMahon <i>et al.</i> , 2016
	RNA tagging	No need to purify the protein of interest No dependence on UV cross-linking: straightforward protocol	Has not been demonstrated outside <i>Saccharomyces cerevisiae</i> Might not work on RBPs distant from the 3' end of RNA	Lapointe <i>et al.</i> , 2015

3.1. RNA-centric techniques

RNA-centric methods are used to investigate proteins that bind to a particular RNA. These methods consist of initiating the study with a specific RNA of interest, followed by the characterisation of the associated RBPs, mainly through mass spectrometry or western blot analysis.

Methods with cross-linking

These methods involve a cross-linking step either UV or formaldehyde. In UV-based approaches, examples include RNA affinity purification (RAP), peptide-nucleic-acid-assisted identification of RBP (PAIR), MS2 *in vivo* biotin-tagged RAP (MS2-BioTRAP) and tandem RNA isolation procedure (TRIP). These techniques share the common step of cross-linking, but they differ in the methods used for capturing RNA and purifying of RNA-protein complexes. For example, the RAP method uses biotinylated probes containing an antisense oligonucleotide to the target RNA, enabling purification using streptavidin-coated beads. On the other hand, the PAIR technique uses a peptide nucleic acid probe. Furthermore, there are also methods based on formaldehyde cross-linking, such as capture hybridization analysis of RNA targets (CHART) or chromatin isolation by RNA purification (ChIRP) (Ramanathan *et al.*, 2019).

Methods without cross-linking

Additionally, there are methods available that do not require a cross-linking step. For example, the RNA proximal protein interaction detection (RaPID) method relies on the properties of the biotin ligase (BirA) from the bacterium *Escherichia coli*. The RNA of interest is tagged with an aptamer called boxB, enabling the recruitment of BirA which can biotinylate proteins in close proximity of the RNA of interest. The biotinylated proteins are then captured by streptavidin-coated beads and analysed by western blot or mass spectrometry. More recently, new techniques have emerged with the discovery of CRISPR systems. In dCas13-based approaches, such as the CRISPR-based RNA-united interacting system (CRUIS), a guide RNA recruits catalytically dead Cas13

(dCas13) fused to a biotin-labelling enzyme specifically to an RNA of interest. Upon addition of biotin, the biotin-labelling enzyme biotinylates proteins in its proximity (Gräwe *et al.*, 2021).

Methods to study the RBPome

All the methods described above start by targeting an RNA of interest. However, in large-scale studies for the discovery of RBPs and better characterisation of the RBPome, there is no specific RNA. Therefore, it was necessary to develop other techniques capable of capturing a group of RNA.

In 2012, two research teams described the RNA interactome capture (RIC) method, which enabled hundreds of RBPs to be identified in HeLa and HEK human cell lines (Baltz *et al.*, 2012; Castello *et al.*, 2012). This method is based on a cross-linking with UV light (UV-C at 254 nm or UV-A at 365 nm). After irradiation, the polyadenylated RNA (poly(A) RNA) is purified using oligo(dT) columns. Finally, the RBP bound to these poly(A) RNAs are analysed using mass spectrometry. In 2016, Thomas Conrad and colleagues developed the serial interactome capture (serIC), a derivative of the RIC, which allows the investigation of RNA-protein interactions specific to the nucleus (Conrad *et al.*, 2016).

The main limitation of RIC and its derivatives is that these techniques focus on poly(A) RNA associated with RBPs. In 2018, Xing Chen's group developed a click-chemistry-assisted RNA interactome capture (CARIC) strategy, which allows the identification of RBP independent of the polyadenylation (Huang *et al.*, 2018). The same year, Matthias Hentze's group developed the complex capture (2C) (**Fig. 4**) technique to address this limitation (Asencio *et al.*, 2018). After UV-C cross-linking, cells are lysed and the lysate is loaded on a silica column. Thanks to its physico-chemical properties, the silica column retains RNA, whether free or associated with proteins. It is then possible to analyse, through western blot or mass spectrometry, the RBPs associated with both poly(A) and non-poly(A) RNA. A year later, David Tollervey's team developed a similar technique to 2C called total RNA-associated protein purification (TRAPP), which use silica beads to identify RBPs in the yeast *Saccharomyces cerevisiae* and the bacterium *E. coli* (Shchepachev *et al.*, 2019).

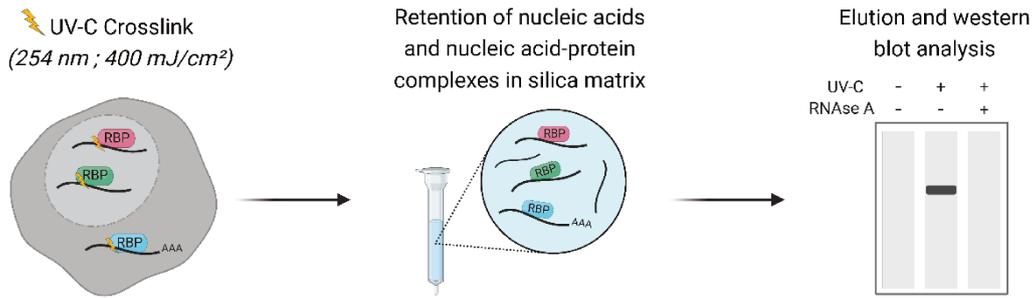


Figure 4 | Complex capture (2C) procedure. First, a covalent bond between protein and RNA is induced by a UV-C crosslink. Then the cells are lysed and the protein lysate is loaded on a silica column which will retain the RNAs and the RNA-protein complexes. Next, proteins are eluted and a western blot is performed.

In 2019, two teams developed the orthogonal organic phase separation (OOPS) method and the protein crosslink RNA extraction (XRNAX) method, both based on the properties of the acid guanidinium thiocyanate-phenol-chloroform (AGPC) in inducing phase separation of a cell lysate. The upper aqueous phase contains RNA while the lower organic phase contains proteins. RNA-protein complexes are found at the interphase and the purification of this interphase allows for the recovery of these complexes (Queiroz *et al.*, 2019; Trendel *et al.*, 2019).

All these system-wide approaches have uncovered many novel RBPs, mostly unconventional RBPs, lacking canonical RNA-binding domains. However, the list of known RBPs to date is certainly underestimated. Furthermore, the study model may lead to different results. Indeed, a recent study adapted the RIC method to organs, which led to the discovery of several hundred new RBPs that had not been identified in cultured cells (Perez-Perri *et al.*, 2023). New RBPs are therefore regularly identified both in cultured human cells and in organs, and probably others have yet to be discovered.

3.2. Protein-centric techniques

Protein-centric approaches are used to identify the RNA targets of a protein of interest. These approaches mostly rely on immunoprecipitation techniques followed by the detection of RNA interacting with the protein of interest, typically through sequencing or RT-qPCR.

Immunoprecipitation-dependent methods

A commonly used approach to analyse RBPs in cells is the RIP (RNA Immunoprecipitation). This method involves immunoprecipitating a protein of interest along with the associated RNA. The RNAs are then precipitated, purified and sequenced. The identified RNAs need to be further validated by RT-qPCR. To preserve RNA-RBP complexes, immunoprecipitation is performed using low-stringency washes, which may potentially induce non-specific interactions.

To overcome this specificity issue, CLIP (Cross-linking and Immunoprecipitation) was developed by the team of Robert B Darnell in 2003 and is now the most widely used method for identifying and characterising *in vivo* RNA-RBP interactions (Ule *et al.*, 2003) (**Fig. 5**). This technique is based on the cross-linking between protein and RNA using UV-C light, allowing the use of highly stringent washes unlike RIP. After cross-linking, cells are lysed, followed by RNase treatment. The protein of interest, along with the associated RNA, is immunoprecipitated using a specific antibody against the protein of interest. The protein-RNA complexes are then radioactively labelled at the 5' end of the RNA and migrated on a denaturing SDS-PAGE. The complexes are transferred onto a nitrocellulose membrane, allowing the visualization of the RNA-RBP complex through autoradiography. In cases like CLIP-seq, the RNA bound to the RBP of interest, can also be extracted. For this purpose, the membrane is cut, proteins are digested and the bound RNAs are purified. After creating a cDNA library, the nucleic acids can be sequenced to identify the RNA target interacting with the RBP of interest (Ule *et al.*, 2005).

Over the years, variations of CLIP have been developed (*e.g.* PAR-CLIP, iCLIP, eCLIP, irCLIP, HITS-CLIP) to optimize and improve different steps of the original CLIP method, such as cross-linking, library construction and sequencing (Lee and Ule, 2018).

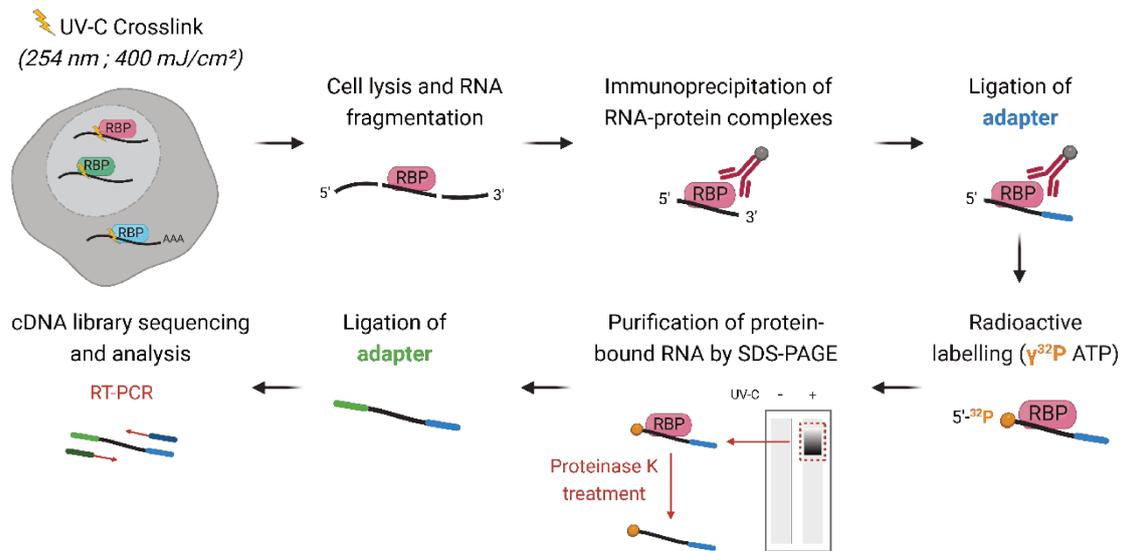


Figure 5 | Cross-linking immunoprecipitation sequencing (CLIP-seq) procedure. First, a covalent bond between protein and RNA is induced by a UV-C crosslink. Then the cells are lysed and the RNA is fragmented by RNase digestion. The protein of interest is immunoprecipitated and after stringent washes, the RNA, bound to the protein of interest, is dephosphorylated, ligated to 3' RNA linker and radioactively labelled at the 5' end using ³²P ATP. The protein-RNA complexes are migrated on a SDS-PAGE and transfer to a nitrocellulose membrane. After transfer and autoradiography, the region corresponding to the protein-RNA complexes is cut. The protein is digested by proteinase K treatment and the RNA is purified and a 5' RNA linker is ligated. Using the linkers, the RNA is then amplified by RT-PCR.

Immunoprecipitation-independent methods

One limitation of CLIP, or RIP, is that they are based on immunoprecipitation of the protein, which requires the use of a specific antibody. Recently immunoprecipitation-independent techniques have emerged. For example, in the targets of RNA-binding proteins identified by editing (TRIBE) technic, the target protein is fused to the catalytic domain of the RNA editing enzyme ADAR. ADAR deaminates neighbouring adenosines and the resulting deaminated bases can be identified through sequencing (McMahon *et al.*, 2016). In addition to RNA editing, RNA tagging methods exist, where the RBP is fused to an enzyme that covalently adds uridines to the end of the RNA, which can also be identified through sequencing (Lapointe *et al.*, 2015).

In summary, the methods used to study RNA-protein associations provide valuable insights into characterising interactions and understanding their roles within the cell. These powerful tools are developed at all levels: *in silico*, *in vitro*, *in cellulo* and *in vivo*. New methods or derivatives of original methods are constantly emerging to search for more specificity. For example, a very recent study has introduced a new technique specifically designed to identify single-strand RBPs: the kethoxal assisted single-strand RNA interactome capture (KASRIC) (Zhao *et al.*, 2023). In addition, alongside these experimental approaches, numerous bioinformatics methods have been developed to predict RNA-protein interactions (Corley *et al.*, 2020). For instance, RNAbindPlus is a web server designed to predict RNA-binding residues within a given protein sequence. On the contrary, RBPmap searches for RBP-binding motifs within a given RNA sequence. These *in silico* tools help to determine the binding sites between protein and RNA. The development of the modelling methods and their reliability takes time and is not always straightforward to implement (Wei *et al.*, 2022). However, an increasing number of computational techniques are emerging, and recently, AlphaFold and RoseTTAFold that enable the accurate prediction of protein and nucleic acid structure, has revolutionized the field of structural biology (Baek *et al.*, 2021; Jumper *et al.*, 2021).

II – DNA replication: Focus on the lagging strand

DNA replication is an essential, highly conserved and tightly regulated process that follows orderly steps occurring in the S-phase of the cell cycle. This semi-conservative process ensures the accurate duplication of the genetic material, from the mother cell to the daughter cell containing one original and one newly synthesized strand, prior cell division. The fidelity of this mechanism is therefore a crucial step in maintaining genome integrity. Indeed, this process involves many proteins, and any errors or defects can lead to genomic instability and disease (Burgers and Kunkel, 2017; Chai *et al.*, 2003; Gaillard *et al.*, 2015; Zeman and Cimprich, 2014).

In eukaryotes, DNA replication is bidirectional, with replication occurring at two replication forks moving in opposite directions. One strand, the leading strand, is synthesized continuously in the direction of replication fork movement. The other strand, the lagging strand, is synthesized discontinuously in the opposite direction, creating Okazaki fragments that are later joined to form a continuous strand. The replication of the lagging strand is a mechanism that is still not fully understood in human. DNA replication process can be divided into three main stages: initiation at replication origins, synthesis of DNA at replication forks known as elongation, and termination at the site where two converging forks meet (Dewar and Walter, 2017; Fragkos *et al.*, 2015; Kang *et al.*, 2018; Zaher *et al.*, 2018). Each stage will be described below, with a particular focus on the lagging strand.

1. Initiation

To start DNA replication, an origin of replication (ORI) is required. In mammals, several ORIs are distributed along the chromosomes. Their nature is poorly defined, as ORIs do not seem to have consensus sequences. However, a genome-wide study in 2008 localized ORIs in regions close to CpG islands (Cadoret *et al.*, 2008). Only 20 to 30% of ORIs will be activated during the S-phase. Indeed, several ORIs are dormant and used to rescue stalled replication forks during replicative stress (Courtot *et al.*, 2018).

In early G1-phase, initiation sites are first recognized and bound by the 6 proteins of the origin recognition complex (ORC) family. The ORC complex will then recruit chromatin licensing and DNA replication factor 1 (CDT1) and cell division cycle gene 6 (CDC6) allowing the subsequent loading of two minichromosome maintenance 2-7 (MCM2-7) replicative helicases which surround the double-strand DNA. These proteins then form the pre-replication complex which is inactive (Fragkos *et al.*, 2015). This mechanism is called origin licensing (**Fig. 6**).

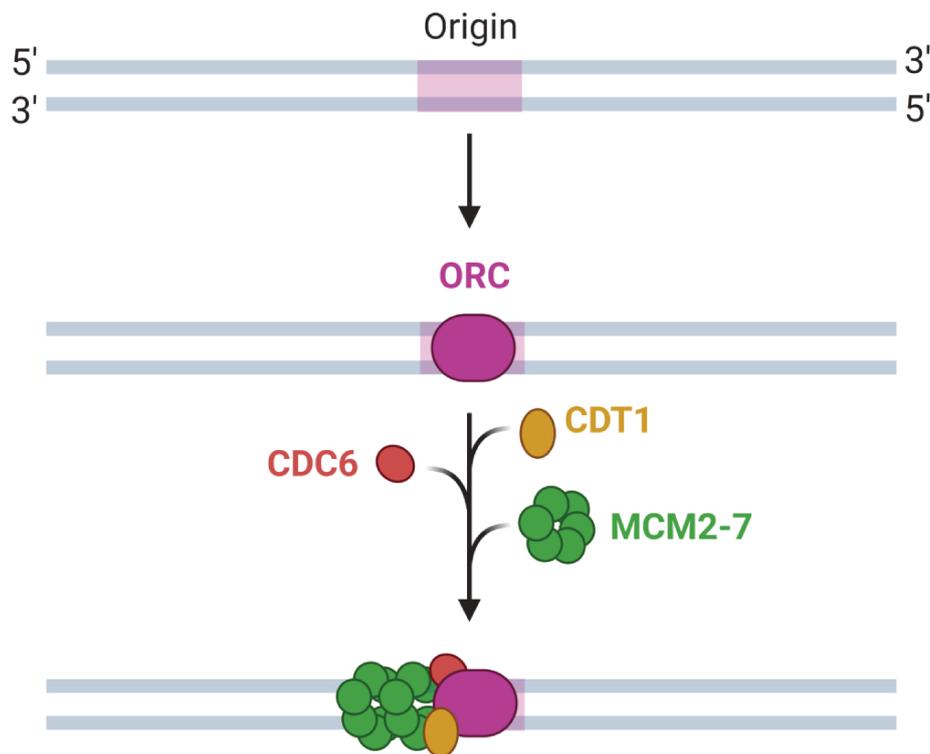


Figure 6 | Origin licensing. Origin licensing is the phase of recognition of potential replication origins. First the origin recognition complex (ORC) is recruited. Next CDC6-CDT1 and the minichromosome maintenance (MCM) helicases complex are loaded sequentially, forming the pre-replication complex. Adapted from Fragkos *et al.*, 2015.

The firing phase follows the licensing phase and enables DNA synthesis to be activated. During the G1/S-phase transition, the MCM2-7 complex is phosphorylated by the Dbf4-dependent kinase (DDK) and cyclin-dependent kinase (CDK). This leads to the activation of the MCM7 double hexamer and the binding of the cell division cycle 45 protein (CDC45) and GINS factors (from the Japanese “go-ichi-ni-san” meaning “5-1-2-3”, after the four related subunits of the complex Sld5, Psf1, Psf2 and Psf3) to the MCM complex, forming the CMG complex (Heller *et al.*, 2011). The formation and activation of the CMG complex are also favoured and mediated by the phosphorylation of the RecQL4 helicase, DNA polymerase ϵ and treslin, by CDKs. The origins of replication are then activated, and the pre-replication complexes are converted into pre-initiation complexes. These complexes will unwind the double-strand DNA and replication factors will be recruited to form the replisome, such as replication protein A (RPA) which bind the single-strand DNA, replicative polymerases, replication factor C (RFC) and the proliferating cell nuclear antigen (PCNA) that acts as a processivity factor for DNA polymerases. Then, the replisome starts converting the pre-initiation complex into two replication forks moving in opposite directions (**Fig. 7**).

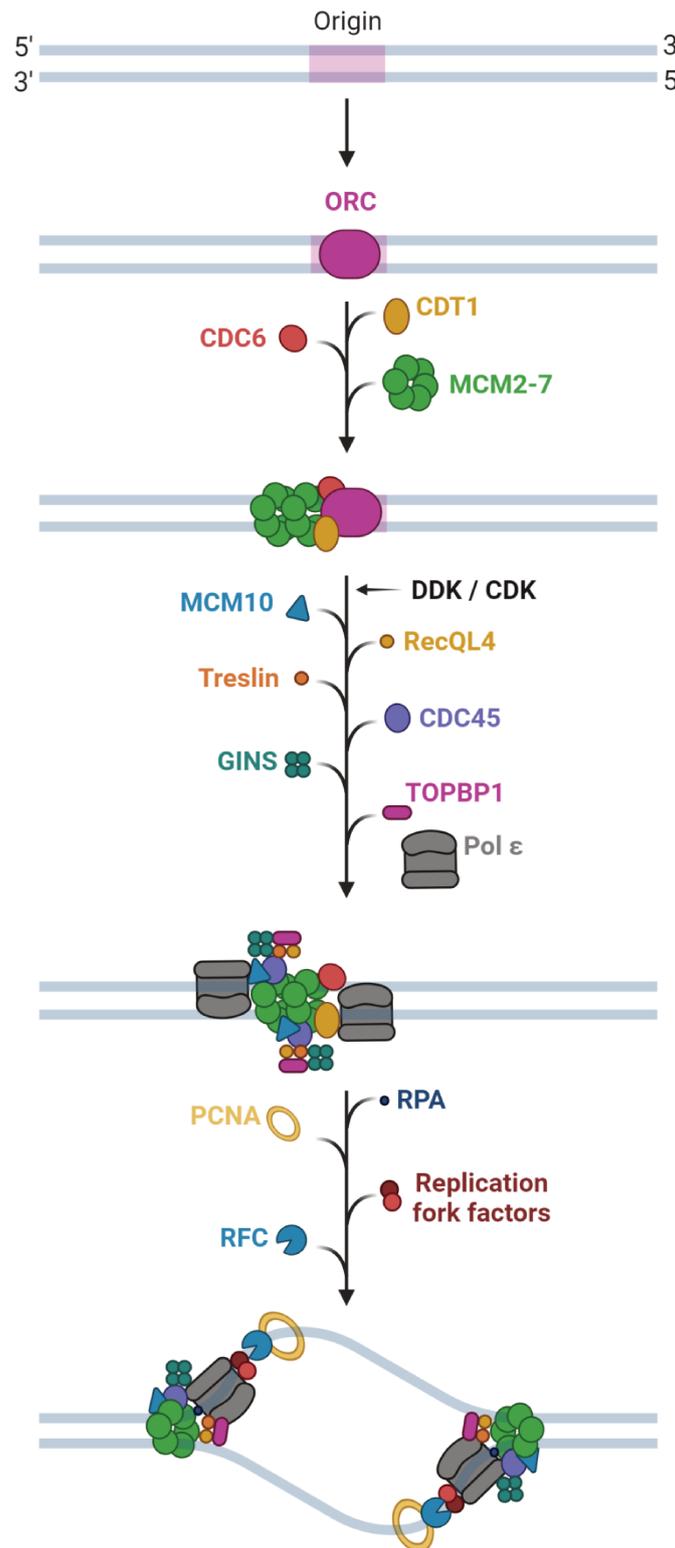


Figure 7 | Origin firing. Origin firing is the step of DNA synthesis activation. First, the MCM helicase complex is activated at the G1/S-phase transition, triggered by the phosphorylation of several factors (MCM10, CDC45, RecQL4, treslin, GINS, TOPBP1 and Pol ϵ) by Dbf4-dependent kinases (DDKs) and cyclin-dependent kinases (CDKs). In S-phase the MCM-CDC45-GINS (CMG) complex is activated resulting in DNA unwinding and inducing the recruitment of other proteins such as replication factor C (RFC), proliferation cell nuclear antigen (PCNA), replication protein A (RPA) and DNA polymerases. This will lead to two functional replication forks that move in opposite directions from the activated origin, with the replisome at each forks. Adapted from Fragkos *et al.*, 2015.

2. Elongation

In the 1960s, Okazaki and colleagues demonstrated the existence of a leading strand and a lagging strand (Okazaki *et al.*, 1968). The leading strand is synthesized in the same direction as the fork progression, while the lagging strand is synthesized in the opposite direction, hence discontinuously (Kang *et al.*, 2018).

The first step of DNA synthesis is common to both strands and begins with the action of Primase-DNA polymerase α (PRIM-Pol α) complex. The primase, containing a catalytic subunit PRIM1 and a regulatory subunit PRIM2, initiates DNA synthesis by generating a 5' RNA primer of about 7 to 12 ribonucleotides in length. Subsequently, the DNA polymerase Pol α , incorporates 20 to 25 nucleotides, in the 5' to 3' direction, following this RNA primer. The RNA-DNA primer enables replication elongation on both strands (Jain *et al.*, 2018; Sun *et al.*, 2022) (**Fig. 8**).

On the leading strand, the PRIM-Pol α complex is then replaced by DNA polymerase ϵ (Pol ϵ) to continue DNA synthesis. On the other hand, on the lagging strand, DNA polymerase δ (Pol δ) synthesizes, discontinuously as the polarity of the parental strand is inverted, approximately 100-200 nucleotides, forming the Okazaki fragments (Burgers and Kunkel, 2017; Nick McElhinny *et al.*, 2008). To form a functional double-strand DNA, these Okazaki fragments need to be processed and RNA primers to be removed. Several mechanisms for maturing Okazaki fragments exist and are described below.

During each division of a human cell, tens of millions of Okazaki fragments are produced and ligated. DNA combing analyses, based on double incorporation of thymidine analogues followed by fiber stretching (Quinet *et al.*, 2017a), have shown that forks travel approximately at 1-3 kb per min, depending on cell type (Conti *et al.*, 2007). This means that it takes only a few seconds to synthesize a single Okazaki fragment (Burgers, 2019; Zheng and Shen, 2011).

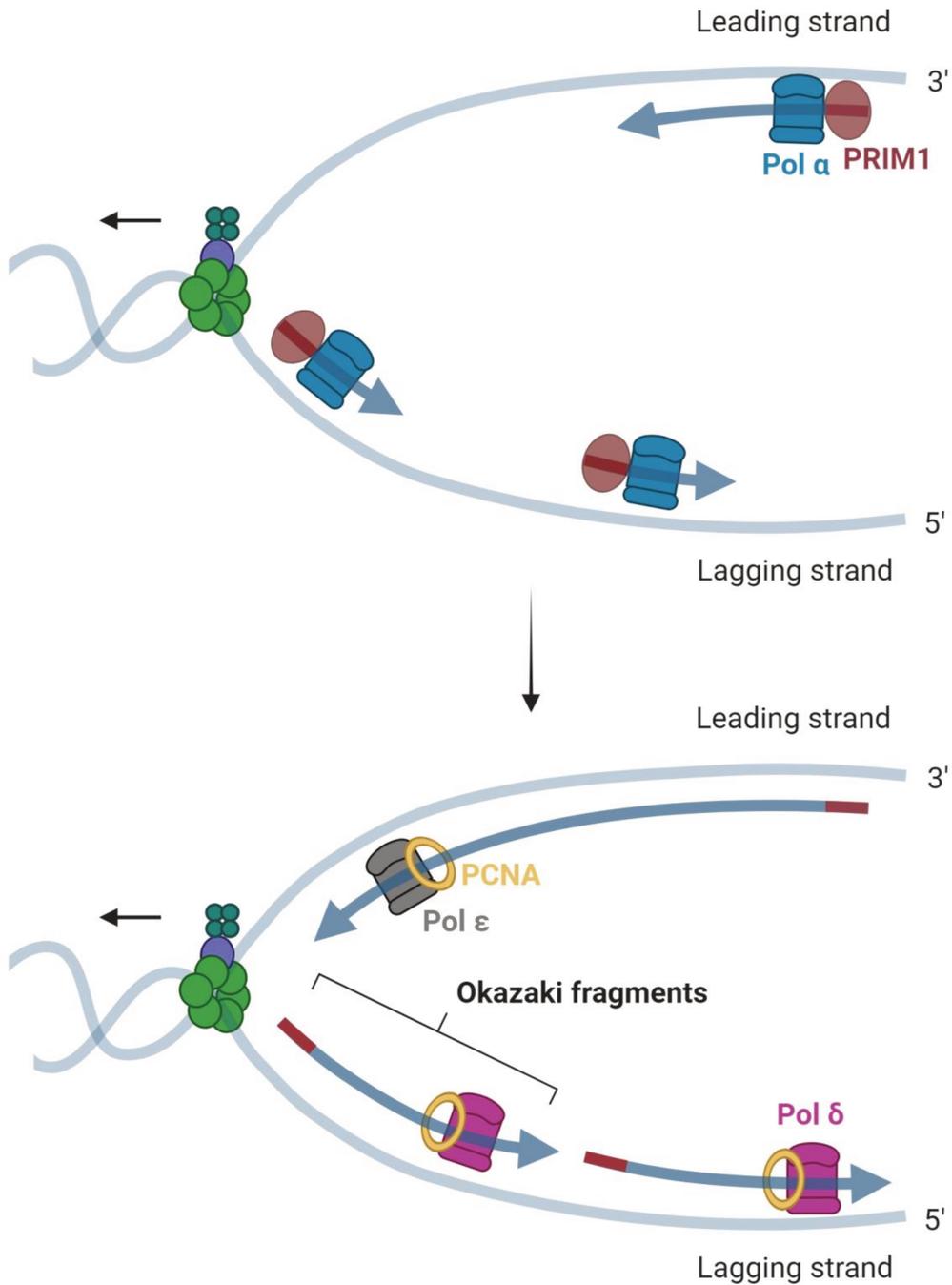


Figure 8 | DNA elongation. After initiation of the replication, the catalytic subunit of the primase (PRIM1) generates a short 5' RNA primer. The DNA polymerase α ($\text{Pol}\alpha$), included in the PRIM-Pol complex, incorporates about 25 nucleotides after the RNA primer. From this RNA-DNA primer, Pol ϵ synthesizes the DNA on the leading strand and Pol δ on the lagging strand, discontinuously.

DNA replication in human cells and the fidelity of this process rely on three different polymerases: Pol α , Pol δ and Pol ϵ . There are notable differences in fidelity among these three polymerases. Unlike Pol δ and Pol ϵ , Pol α lacks 3'-exonuclease activity, and is therefore considered to be an error-prone polymerase, that particularly generates insertions and deletions (Guilliam *et al.*, 2015). Pol α synthesizes approximately 10% of the genome during DNA replication, and the errors potentially generated are therefore deemed "acceptable". Furthermore, considering all polymerases together, it is estimated that there is one error per 10^4 - 10^5 nucleotides incorporated (Ganai and Johansson, 2016). Moreover, Pol δ can displace the strand, an activity necessary for the maturation of Okazaki fragments, whereas Pol ϵ has a very limited capacity and can only move one or two nucleotides. The potential strand-displacement activity of Pol α is not well defined. It seems that this polymerase has the ability to displace the DNA strand but only in the presence of manganese ions (Mn^{2+}) (Boldinova *et al.*, 2020) **(Table 3)**.

Table 3 | Major characteristics of eukaryotic DNA polymerases.

	Polα	Polδ	Polϵ
Activities	Polymerase	Polymerase 3'-exonuclease	Polymerase 3'-exonuclease
Fidelity	Error-prone	Error-free	Error-free
Strand displacement	Conditional	Yes	Limited

3. Okazaki fragments maturation

3.1. Flap processing pathways

The mechanism of Okazaki fragments processing and all the players involved are still not well described. However, *in vitro* and recent *in vivo* data have enabled to model this process, especially in yeast. It is accepted that the removal of the primer and ligation of the Okazaki fragments can occur through two pathways: the short flap and the long flap pathway (**Fig. 9**) (Rossi and Bambara, 2006; Sun *et al.*, 2022; Zaher *et al.*, 2018).

Short flap pathway

Okazaki fragment synthesis is an extremely rapid process, meaning that Pol δ , in complex with PCNA, frequently encounters the downstream Okazaki fragment, forcing it to displace the RNA primer. The RNA primer displacement induces a 5' single-strand RNA structure, known as the flap. This flap is short and is approximately 2 to 10 nucleotides. Pol δ does not have an endonuclease activity, therefore the Flap endonuclease 1 (FEN1) cleaves the flap nucleotide by nucleotide, inducing a DNA nick. The two adjacent Okazaki fragments are joined by the DNA ligase 1 (LIG1) to produce a functional double-strand DNA (Raducanu *et al.*, 2022).

Long flap pathway

In some cases, cleavage by FEN1 is delayed, resulting in the formation of a long flap, whose length is greater than 20 nucleotides. The exact reason for the delayed cleavage by FEN1 is still not well defined. It could be due to a FEN1 protein defect or the presence of RPA. Indeed, RPA binds to the single-strand DNA part of the flap, preventing the action of FEN1. The DNA replication helicase/nuclease 2 (DNA2) is then recruited and its nuclease activity is stimulated by RPA. DNA2 cleaves the long flap in the middle to shorten it and thus obtaining a 5' short flap of less than 10

nucleotides, which is further supported by the short flap pathway mediated by FEN1 (Bae *et al.*, 2001; Duxin *et al.*, 2012; Stewart *et al.*, 2009).

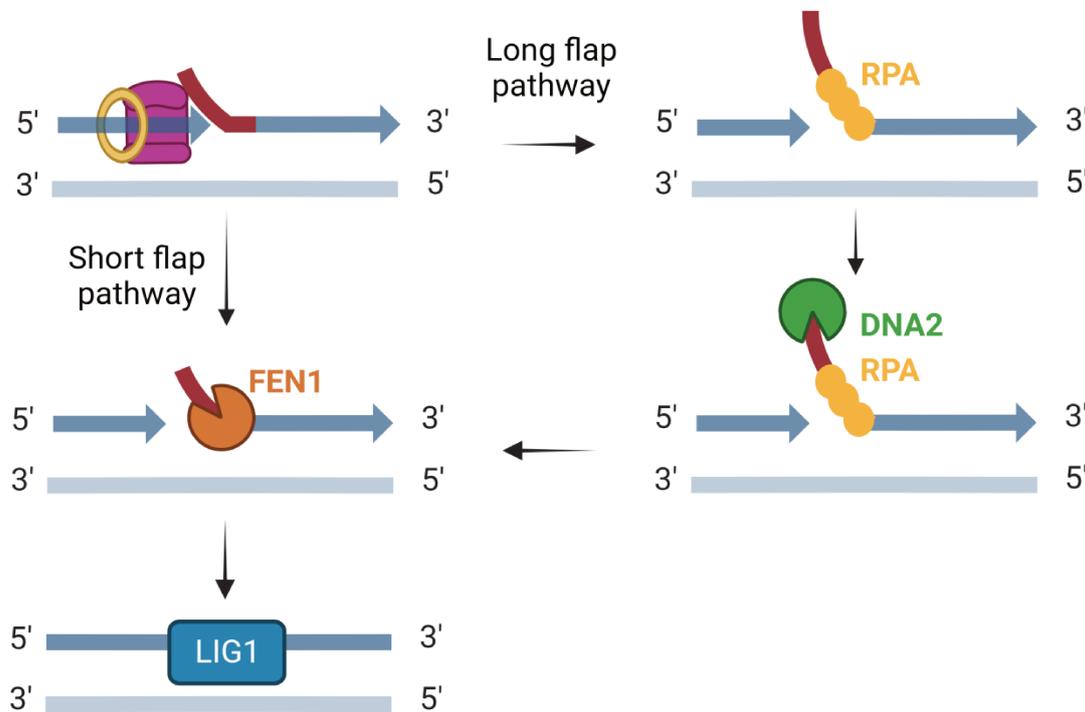


Figure 9 | Short and long flap pathways to mature Okazaki fragments. When it reaches the downstream Okazaki fragments, Pol δ displaces the RNA primer and creates a short 5' flap, from 2 to 10 nts in length. This 5' flap is recognized and cleaved by FEN1, resulting in the creation of a nick that is sealed by DNA ligase 1 (short flap pathway). In minor cases, the short flap escapes cleavage by FEN1 leading to the formation of a long flap. RPA will bind to the ssDNA part of the long flap which inhibits the activity of FEN1. In such cases, DNA2 displaces RPA from the long flap and cleaves it until the flap become too short to maintain DNA2 binding. At this point, the flap shorten by DNA2 becomes a substrate for FEN1 cleavage in the short flap pathway.

The switch between short and long flap is still not fully understood. However, a hypothesis has emerged in recent years suggesting that the long flap may be less mutagenic than the short flap. Indeed, Pol α is an error-prone polymerase, as described above, which lacks proofreading activity. It is proposed that the long flap contains both the RNA primer generated by PRIM1 and the DNA primer generated by Pol α . Removal of the Pol α DNA primer, and its subsequent resynthesis by the error-free Pol δ , would prevent mutations and maintain genome integrity.

The long flap pathway would thus be favoured in transcriptionally active regions. In 2010, Lata Balakrishnan and colleagues demonstrated *in vitro* that the histone acetyltransferase p300 plays a role in the switch between the long and short flap pathways. Specifically, p300 acetylates FEN1, leading to its inhibition, and also acetylates DNA2, stimulating its activity. Thereby these two simultaneous actions of p300 promote the long flap pathway. Furthermore, p300 is known to be enriched in an active chromatin environment to assist the transcription machinery in accessing DNA. In addition to this role, p300 would therefore ensure replication fidelity in these transcriptionally active regions, by promoting the long flap pathway (Balakrishnan and Bambara, 2011; Balakrishnan *et al.*, 2010).

3.2. Defects in Okazaki fragments maturation

It is essential for the cell to properly mature the Okazaki fragments. Indeed, alterations in Okazaki fragments processing lead to genetic instability and are often associated with human pathologies or cancers. For example, mutations in FEN1, leading to a dysfunction in its nuclease activity, are associated with autoimmunity, chronic inflammation or cancers with aberrant chromosome numbers (Zheng *et al.*, 2007, 2011, 2019).

In case of a defect, the cell has several mechanisms for the recognition and the processing to maintain trouble-free replication. These defects can be induced by the loss of maturation factors or by genotoxic agents, resulting in an ssDNA gap between two unligated Okazaki fragments. PARP1 recognises these gaps on the lagging strand and binds to the resulting single-strand DNA, leading to PARylation of PARP1 and surrounding proteins. In human cells, Hana Hanzlikova and colleagues demonstrated that this increase in S-phase PAR, led by the depletion of FEN1 or LIG1, is not related to DNA damage or replication stress. Moreover, the PARP activity facilitates the recruitment of the single-strand break repair protein XRCC1 and its partner LIG3, resulting in a backup processing of Okazaki fragments. Therefore the accumulation of PAR in S-phase reflects a defect in the lagging strand maturation (Hanzlikova *et al.*, 2018).

PARP1 inhibitors (PARPi) are commonly used in the clinic, such as olaparib which induces PARP1 trapping on DNA and therefore inhibits the ssDNA break repair signalling. These inhibitors can also disrupt nascent DNA maturation, particularly when FEN1 is depleted (Vaitsiankova *et al.*, 2022). For example, the synthetic lethality using PARPi is frequently employed to target BRCA-deficient cancers. Indeed, loss of BRCA leads to an increase in the ssDNA replication gaps targeted by PARPi (Cong *et al.*, 2021; Panzarino *et al.*, 2021). In these genetic backgrounds, the loss of 53BP1 leads to resistance against PARPi. To overcome this resistance, depleting LIG3 restores the PARPi-sensitive phenotype (Paes Dias *et al.*, 2021).

4. Termination

When two converging forks meet, the termination step can begin (**Fig. 10**).

The first step is to resolve the topological tensions. The topological stress is resolved by the production of pre-catenanes and the dissolution of these catenated replication products by the action of topoisomerases.

Then it is necessary to fill and ligate the remaining gaps. The CMG complex from the leading strand of one fork passes over the 5' end of the last Okazaki fragment on the lagging strand of the converging fork and moves along the double-strand DNA (Dewar and Walter, 2017).

The last step is the dissociation of the CMG complex from chromatin, by poly-ubiquitination of the MCM7 subunit. This dissociation notably allows for the avoidance of inappropriate protein recruitment and thus promotes a correct progression of cell cycle. The proteins responsible for poly-ubiquitination are not clearly identified in mammals (Kang *et al.*, 2018). Overall, the termination process is not yet fully understood, and many questions remain to be answered.

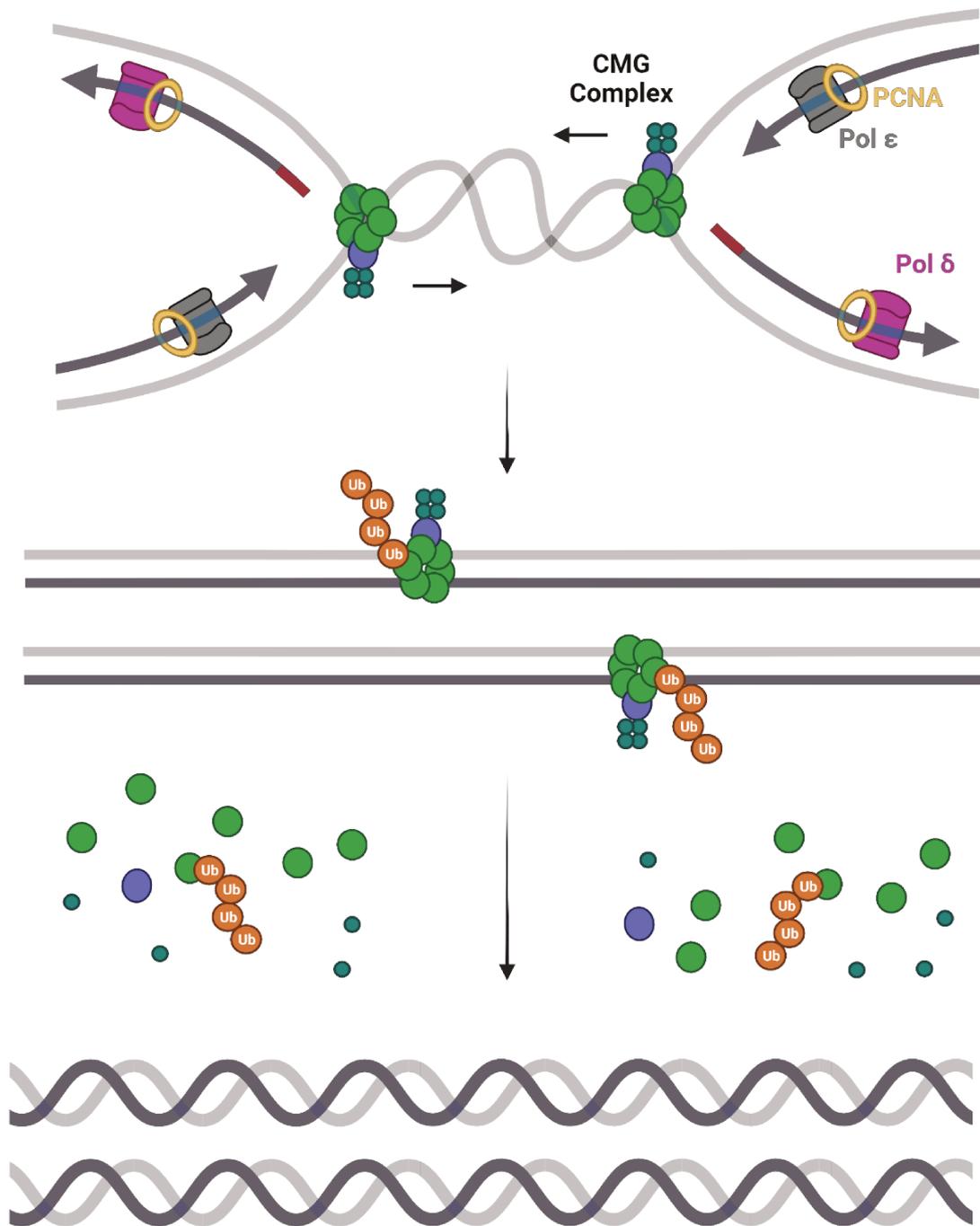


Figure 10 | Termination of the replication. This process involves 3 major steps: resolving topological tensions, filling in the remaining gaps and dissociating the replisome.

5. Replication stress

DNA replication is therefore a complex and highly regulated process. It is important for the cell to maintain faultless, stress-free replication before continuing its progression through the cell cycle. Indeed, defects during DNA replication are a major source of genetic instability, which is also a hallmark of cancer (Gaillard *et al.*, 2015; Hanahan and Weinberg, 2011; Macheret and Halazonetis, 2015).

5.1. Cause of replication stress

The cell is constantly subjected to stress that must be resolved. When this stress occurs during the S-phase of the cell cycle, it results in a slowdown or halt of replication forks. When a fork is stalled, it temporarily paused to remove the stress, and then it either restarts or collapses. There are several sources of replicative stress, both endogenous and exogenous to the cell (**Fig. 11**).

Endogenous sources include DNA damage (*e.g.* double-strand breaks, single-strand DNA gaps), incorporation of ribonucleotide, unusual DNA structures (*e.g.* G-quadruplexes) and transcription-replication conflicts (*e.g.* RNA:DNA hybrids) (Zeman and Cimprich, 2014). A deficiency in replication factors, the presence of fragile sites, an insufficiency of the pool of deoxyribonucleotide triphosphates (dNTPs) or a lack of activation of replication origins can also be a source of stress (Briu *et al.*, 2021; Willaume *et al.*, 2021).

Exogenous sources correspond to genotoxic agents, used in chemotherapy or in research laboratories. There are several types of these drugs, such as molecules that block the synthesis of deoxyribonucleotide by targeting the nucleotide metabolism (*e.g.* hydroxyurea) or activity of polymerases (*e.g.* aphidicolin), those that cause DNA damage or interfere with DNA repair signalling (*e.g.* cisplatin, olaparib) and those that interfere with topoisomerases (*e.g.* etoposide, camptothecin) (Vesela *et al.*, 2017). The cell must therefore implement appropriate responses to manage these sources of stress effectively.

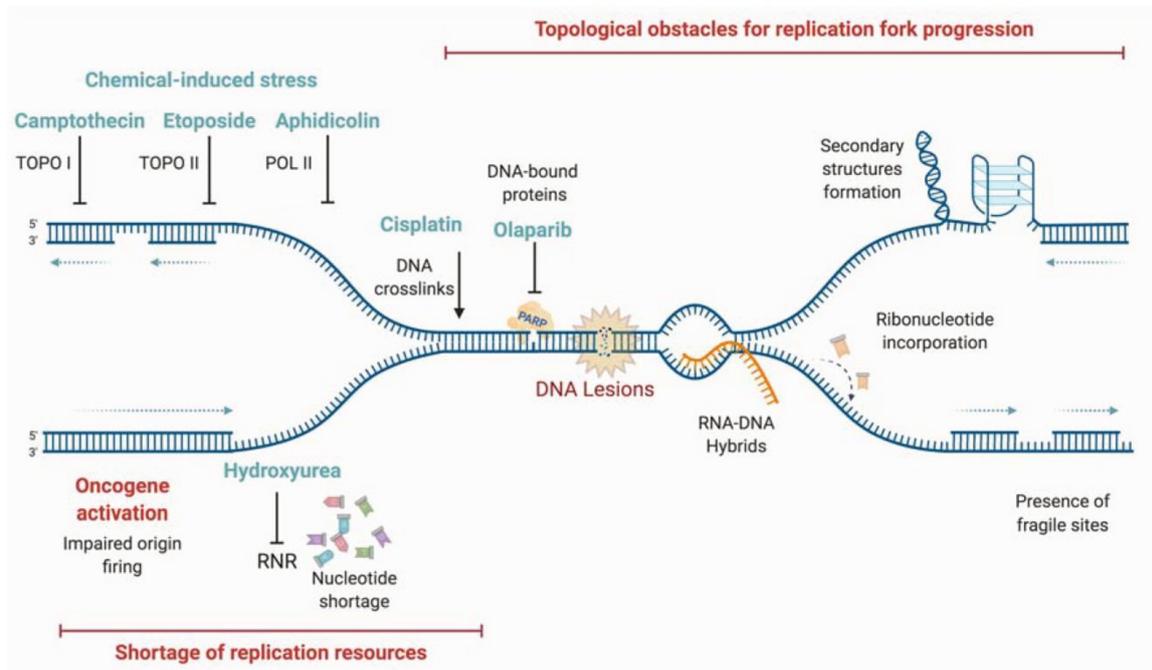


Figure 11 | Main sources of replication stress. DNA replication progression is constantly challenged. Replication stress, a major source of genome instability, is defined as the temporary slowing or stalling of replication forks due to the encountered obstacles or DNA lesions induced by both endogenous and exogenous agents. Among them are topological obstacles such as G-quadruplex, the shortage of replication resources, oncogene activation, DNA protein crosslinks, and DNA:RNA hybrids. Additionally, several chemicals can induce replication stress by the inhibition of key enzymes for replication or generation of blocking lesions. From Willaume *et al.*, 2021.

5.2. Replication fork recovery

Several mechanisms have been put in place to manage a stressed fork to prevent it from collapsing, which is an irreversible state where the fork cannot resume replication.

When replicative stress occurs, the replication fork is stalled, and the cell cycle halted to allow time for the fork to be repaired. This fork arrest generates single-strand DNA gaps. Subsequently, ATM- and Rad3- related (ATR) protein is recruited, activated and initiates Checkpoint kinase 1 (CHK1)-mediated signalling. The activation of CHK1 notably inhibits the activation of replication origins. The ATR-mediated response helps to stabilize the fork to facilitate its repair and restart (Nam and Cortez, 2011; Saldivar *et al.*, 2017; Zeman and Cimprich, 2014).

Once the fork is stabilized, several mechanisms can be employed to restart it (**Fig. 12**), such as the activation of dormant origins, the regression of the fork or the activation of DNA damage tolerance pathways (Neelsen and Lopes, 2015; Pasero and Vindigni, 2017; Quinet *et al.*, 2017b). Conversely, if there is a defect in fork stabilization, the fork collapses as proteins from the replisome dissociate, leading to a double-strand break. Therefore, the players of homologous recombination come into play to repair the DNA.

DNA damage tolerance

To avoid the consequences of a blocked fork, there are mechanisms of DNA damage tolerance. These mechanisms stabilize the fork and allow it to continue progression, even in the presence of lesions (**Fig. 12**). Three mechanisms are defined. First is template switching (TS), which uses the newly synthesized sister chromatid and is thus an error-free mechanism. In contrast, translesion synthesis (TLS) is an error-prone pathway based on the ability of a DNA polymerase to replicate while bypassing a DNA lesion. TLS is often associated with a risk of mutagenesis. Finally, the last mechanism is the repriming mechanism by PRIM-Pol α complex, which involves re-initiating replication downstream of the lesion using its DNA primase activity, leaving a ssDNA gap to be filled later (Berti and Vindigni, 2016; Pasero and Vindigni, 2017; Quinet *et al.*, 2021; Willaume *et al.*, 2021).

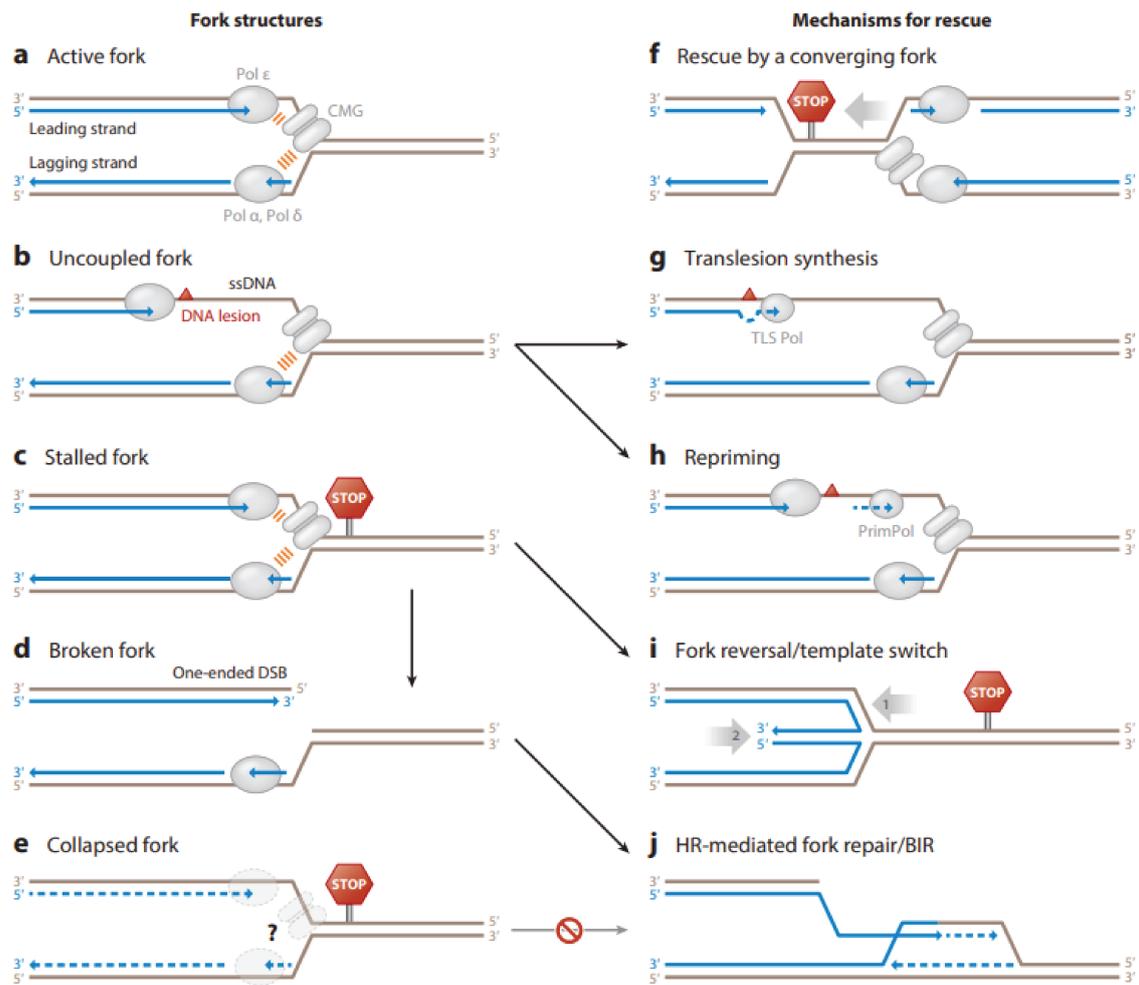


Figure 12 | Recovery of stressed replication forks. This figure illustrates the different types of fork structure (*a-e*) observed under replication stress conditions and the mechanisms used by eukaryotic cells to rescue them (*f-j*). (*a*) Simplified representation of a eukaryotic replication fork showing the unwinding of parental DNA strands by the Cdc45/MCM2-7/GINS (CMG) helicase complex and the DNA polymerases acting on the leading and lagging strands. CMG and DNA polymerases associate with many additional factors at replication forks to form the replisome. Orange lines represent physical interactions between the CMG complex and DNA polymerases. (*b*) DNA lesions that are small enough to pass through the CMG channel block the progression of DNA polymerases, leading to uncoupling of polymerase and helicase activities and increased single-strand DNA (ssDNA) at the fork. (*c*) Replication forks stall when they encounter obstacles impeding the progression of the replisome. (*d*) Replication forks break when they encounter a DNA lesion or an ssDNA gap, and when a stalled fork is cleaved by a structure-specific endonuclease. This leads to the formation of a one-ended DNA double-strand break (DSB) and to replisome run-off. (*e*) Forks that cannot be restarted or rescued by dormant origins in a timely fashion eventually collapse. Fork collapse corresponds to an irreversible inactivation of the fork, with or without loss of replisome components. (*f*) A stalled fork can be rescued by an active fork progressing from a downstream initiation event (*g*) DNA lesions can be bypassed by translesion DNA synthesis (TLS). (*h*) Lesions on the leading strand can also be bypassed by repriming downstream of the lesion with the human primase PRIM-Pol. (*i*) Newly replicated DNA strands can also anneal at stalled forks, leading to fork reversal or template switching events. (*j*) Broken forks can be repaired by homologous recombination (HR)-mediated mechanisms, including break-induced replication (BIR). From Pasero and Vindigni, 2017.

Fork reversal

The fork reversal process is an alternative DNA damage tolerance mechanism. A stalled fork can regress or reverse by rewinding the template DNA forming a structure known as “chicken-foot”. This is a protective process that prevents chromosomal breakage and stabilizes the fork while awaiting repair. Although not fully understood, key factors have been identified (**Fig. 13**). Initially, RPAs bind to the single-strand DNA region. Then RAD51 will replace RPA to initiate fork regression. DNA translocases SMARCAL1, ZRANB3 and HTLF are involved in stabilizing the fork during the reversal process. The second step is the restart of the reversed fork, which occurs in two ways. The first one is mediated by the helicase RecQ1. The second relies on the action of WRN and DNA2, whose activity is inhibited by RecQ1. These enzymes promote the resection of nascent strands which could lead to restart via homologous recombination (Berti *et al.*, 2020; Neelsen and Lopes, 2015; Quinet *et al.*, 2017b).

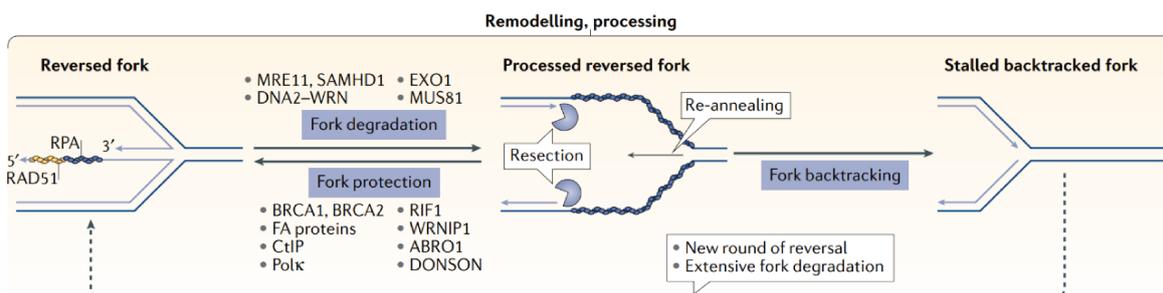


Figure 13 | Fork reversal mechanism. Nascent strands at reversed forks are susceptible to degradation by nucleases such as MRE11 (and its activator SAMHD1), DNA replication helicase/nuclease 2 (DNA2)-Werner syndrome helicase (WRN), exonuclease 1 (EXO1) and MUS81. Replication fork protection generally requires the formation of stable RAD51 filaments, which is promoted by homologous recombination and Fanconi anemia (FA) factors. However, many fork accessory factors - including CtIP, replication timing regulatory factor 1 (RIF1), WRN helicase-interacting protein 1 (WRNIP1), ABRO1 and DONSON - prevent fork degradation independently of RAD51 loading. Backtracking of stalled replication forks likely requires extensive re-annealing of the parental DNA strands after nascent-strand degradation, as well as multiple rounds of fork reversal and nucleolytic attack. Modified from Berti *et al.*, 2020.

Homologous recombination-mediated replication fork restart

A stalled replication fork can result in a double-strand break (DSB). There are several pathways for the repair of double-strand breaks, depending on the cell cycle (**Fig. 14**). Homologous recombination (HR) is favoured in post-replication and the non-homologous end joining (NHEJ) pathway is promoted in pre-replication. Unlike NHEJ, HR is a high-fidelity DSB repair pathway that functions in G2/S-phase because this pathway requires the availability of a homologous copy of the DSB-containing region, *i.e.* the sister chromatid. In this pathway, DSBs are recognized by the MRE11/RAD50/NBS1 complex (MRN). MRE11 is responsible for the resection of the DSB end. To achieve this, MRE11 binds RAD50, which is associated with DNA, to maintain the DNA resected by MRE11. It also binds NBS1, which recruits the ATM kinase and the CtIP protein. The recruitment of CtIP will activate the endonuclease activity of MRE11 and thus initiate the resection (Daley *et al.*, 2015; Sun *et al.*, 2020). To extend the resection, other enzymes are required such as the exonuclease EXO1. The resulting single-strand DNA end is bound and stabilized by RPA. The BRCA1/BRCA2 complex will allow the exchange of RPA by RAD51 (Carreira *et al.*, 2009). Subsequently, a RAD51 filament is obtained and stretched to look for sequence homology (Renkawitz *et al.*, 2014). Once the homology is found, a double Holliday junction is formed. The repaired DNA will then contain or not crossovers (Wright *et al.*, 2018).

A defect in HR decreases the replication fork progression speed (Daboussi *et al.*, 2008). Indeed, HR is necessary to protect and repair the replication forks. When the fork is stalled, RAD51 bound to single-strand DNA protects the nascent DNA from excessive degradation by nucleases like MRE11. BRCA2 also plays a role in protection as it stabilizes RAD51 to the DNA (Liu *et al.*, 2020).

When the stalled fork fails to restart or when the processing of the fork is prolonged, it becomes a substrate for structure-specific endonucleases which generate single-strand DSBs (Dehé and Gaillard, 2017). Subsequently, these breaks undergo repaired by the break induced replication (BIR) pathway, which is a highly mutagenic HR mechanism (Costantino *et al.*, 2014).

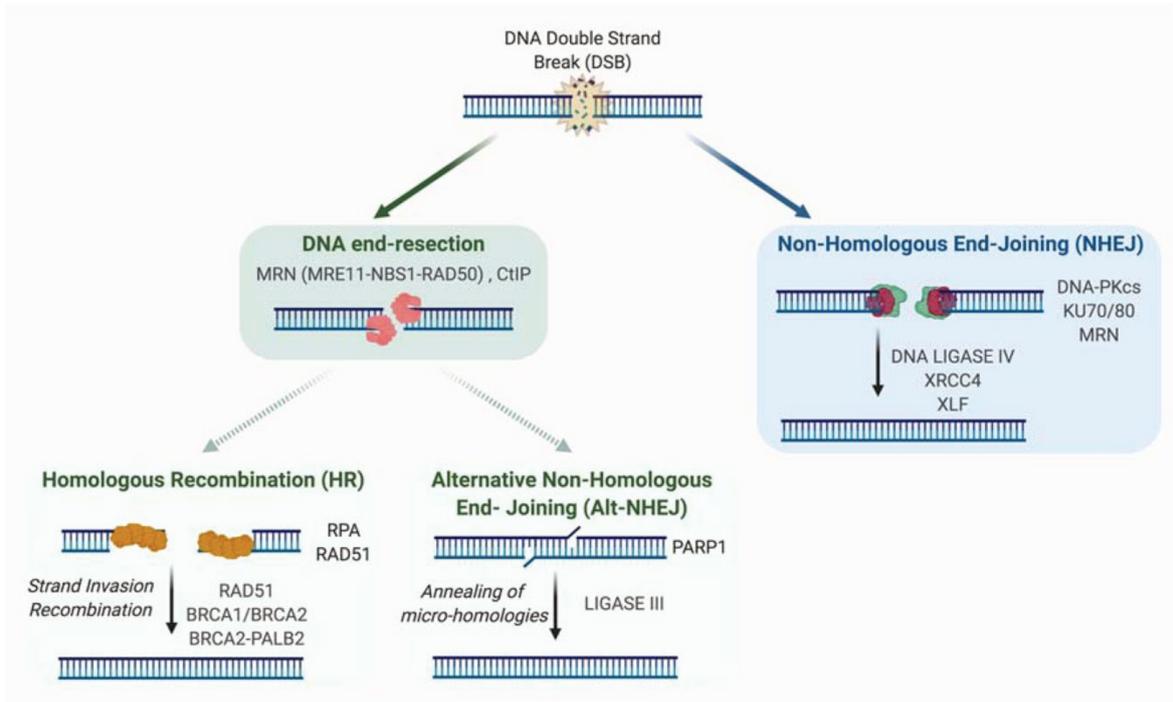


Figure 14 | Double-strand break repair mechanisms. A deficient repair of DSB has detrimental consequences, including genomic instability and cell death. There are two main mechanisms to repair DSBs: HR and canonical NHEJ (c-NHEJ). A pivotal process in the choice of DNA repair pathways is the DNA end-resection. Indeed, if DNA end-resection is blocked, c-NHEJ is favoured to the detriment of HR. c-NHEJ involves the ligation of DNA extremities without the requirement for homology and does not require resection. An alternative pathway of NHEJ (Alt-NHEJ) exists and unlike c-NHEJ, it is highly inaccurate and requires short DNA end-resection. Unlike Alt-NHEJ, HR requires extensive DNA end-resection and requires the presence of an intact homologous template. From Willaume *et al.*, 2021.

Although the HR pathway is favoured to manage replicative stress, some specific factors of NHEJ also play a key role. For instance, the absence of Ku and XRCC4 factors leads to an increased sensitivity to replication inhibitors (Saintigny *et al.*, 2001). 53BP1, a major NHEJ player, also acts to protect nascent DNA (see section III-1.4) (Her *et al.*, 2018; Liu *et al.*, 2020). These NHEJ actors are thus involved in managing replicative stress, but in a way independent of their canonical role in NHEJ.

In conclusion, DNA replication is a common process to all organisms that enables the transmission of genetic material. Disturbances to this process, whether from endogenous or exogenous agents, pose a challenge for the cell that must be addressed correctly to maintain the integrity of the genome. Mechanisms such as DNA damage tolerance, fork reversal and homologous recombination help to stabilize, repair and restart the replication forks. Furthermore, replication errors generated by replicative polymerases are repaired by the mismatch repair (MMR) pathway, which is not detailed in this introduction. In this process, the lesion is first recognized, then the incorrect DNA sequence is excised and finally the DNA is resynthesized without errors. Overall, replication mechanisms, and notably the replication of the lagging strand through Okazaki fragments, in both stressed and unstressed conditions are complex and not yet fully understood. However, an increasing number of new factors are regularly being identified as replication players.

III – The 53BP1 protein: a novel RNA-binding protein in genome integrity?

1. Roles throughout the cell cycle

Historically, the 53BP1 protein (p53-Binding Protein 1), encoded by the *TP53BP1* gene located on chromosome 15 in human, was identified in 1994 as a partner of the protein p53 (Iwabuchi *et al.*, 1994). Over the years, 53BP1 has become best known for its crucial role in double-strand break (DSB) repair, where it promotes the NHEJ (Non-Homologous End-joining) pathway. Today, 53BP1 remains well studied and its biological functions are increasingly characterised (**Fig. 15**) (Mirman and De Lange, 2020; Rass *et al.*, 2022; Zimmermann and De Lange, 2014).

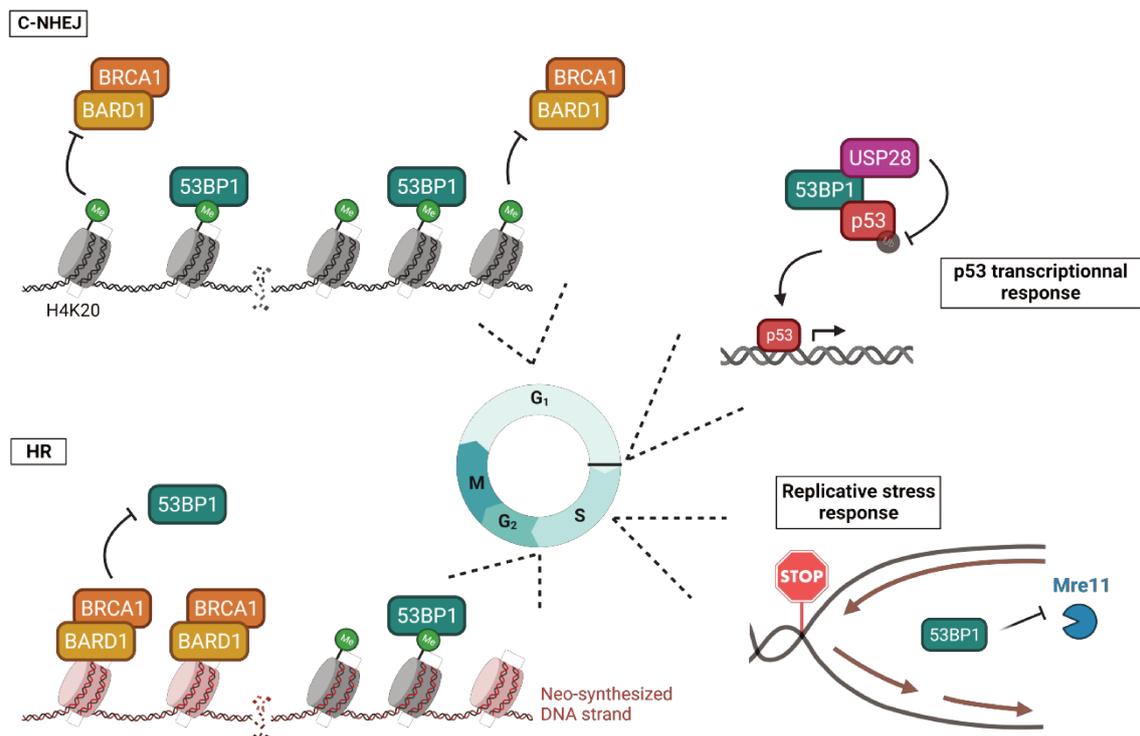


Figure 15 | Roles of 53BP1 during the different phases of the cell cycle. 53BP1 is involved in numerous cellular processes in response to stress. It plays a role in double-strand break repair signalling through the recognition of H4K20me2 histones and participates in pathway choices, promoting the canonical NHEJ (c-NHEJ) pathway in G₁-phase. During the S-phase, the BRCA1/BARD1 complex recognizes newly synthesized histones H4K20me0, thus preventing 53BP1 from acting. Additionally, 53BP1 acts as a partner of p53 contributing to the regulation of cell-cycle checkpoints at the G₁/S transition. Finally, in S-phase, the anti-resection activity of 53BP1 protects nascent DNA from degradation by nucleases such as MRE11, in response to replicative stress.

1.1. p53 transcriptional response

Although the interaction between 53BP1 and p53 has been known for decades, the role of 53BP1 in p53 signalling remained undefined until recently. p53 is a transcription factor that control a multitude of biological mechanisms such as cell-cycle arrest, apoptosis, DNA repair and cellular stress responses (Amelio and Melino, 2020; Vousden and Prives, 2009). 53BP1 mediates p53-dependant cell cycle progression by regulating cell-cycle checkpoint. This 53BP1-p53 pathway is mediated by the ubiquitin specific peptidase 28 (USP28) (**Fig. 15**). For instance, in the event of centrosome loss or prolonged mitosis, 53BP1 interacts with the deubiquitinase USP28 that can deubiquitinate p53 to stabilize it. Therefore, p53 induces the transcription of target genes (*e.g.* p21) to arrest cell growth (Fong *et al.*, 2016; Lambrus *et al.*, 2016). Moreover, in response to ionizing radiation, Raquel Cuella-Martin and colleagues demonstrated that 53BP1 also promotes the p53-dependent transcriptional response (Cuella-Martin *et al.*, 2016). Furthermore, another actor involved in the function of 53BP1 in p53 signalling is the AHNAK protein (also known as desmoyokin meaning “giant” in Hebrew). AHNAK interacts with 53BP1 to suppress p53 activity. Consequently, depletion of AHNAK increases 53BP1-p53 interaction, leading to the activation of p53 and inducing apoptosis in human cancer cells (Ghodke *et al.*, 2021). This regulatory role of 53BP1 on p53 is independent of its role in DNA repair.

1.2. End-joining processes

One of the most studied roles of 53BP1 today is its involvement in the repair of DSBs. DSBs are cytotoxic lesions formed when both DNA strands are broken and the cell must repair this break to survive. The repair can occur optimally through DSB repair mechanisms, enabling the cell to continue its normal cycle of division. However, if the break is not properly repaired, it can lead to genetic instability and chromosomal abnormalities giving rise to numerous diseases, including cancers (Jackson and Bartek, 2009).

DSBs can occur in different context: from an exogenous source (*e.g.* ionizing radiation, UV, genotoxic chemicals) or from a spontaneously endogenous source (*e.g.* replicative stress) (Chapman *et al.*, 2012). The major DSBs repair pathways are HR (homologous recombination) and c-NHEJ (canonical NHEJ).

The c-NHEJ pathway is a conserved process that promotes direct ligation of break ends. It is a fast mechanism but also error prone. Indeed, Zhiyong Mao and colleagues showed in 2008, using chromosomally integrated fluorescent reporter, that NHEJ lasts about 30min, compared with 7h for HR (Mao *et al.*, 2008a). NHEJ is active throughout the cell cycle, favoured in G1-phase and at the G2/M transition (Karanam *et al.*, 2012; Mao *et al.*, 2008b). Inevitably, this speed leads to errors. For instance, the NHEJ pathway can be associated with insertions, deletions, substitutions at the break sites, and translocations if DSBs from different parts of the genome are joined (Chang *et al.*, 2017).

After double-strand break, the first step in the NHEJ pathway is the recognition of the DNA damage site. This recognition is enabled thanks to the binding of the Ku70/80 heterodimer (Soutoglou *et al.*, 2007). Since most DSBs have incompatible ends, nuclease activity is required. Ku70/80, which binds to DNA without sequence preference, serves as a platform for the recruitment of the catalytic DNA-PK subunit (DNA-PKcs). The association of Ku/DNA-PKcs induces the autophosphorylation of DNA-PKcs and the phosphorylation of target proteins such as the endonuclease Artemis. Artemis will therefore generate regions of microhomologies (< 4 nucleotides) to facilitate end joining. Then, polymerases will be recruited to fill in the double-strand DNA. Finally, the DNA Ligase 4 (LIG4) and the X-ray repair cross-complementing protein 4 (XRCC4) complex join the DNA ends (Lieber, 2008; Riballo *et al.*, 2004) (**Fig. 16**).

53BP1 is not considered a core protein of the NHEJ pathway, however it is involved upstream of this pathway and promotes its signalling in certain context. 53BP1 also mediates programmed NHEJ in the context of telomere fusion, V(D)J recombination and CSR (Class Switch Recombination). For example, the oligomerisation of 53BP1 is required for CSR signalling (Bothmer *et al.*, 2011). The depletion of 53BP1 also induces a defect in V(D)J recombination with extensive degradation (Difilippantonio

et al., 2008). Moreover, in a deprotected telomere context, the recruitment of 53BP1 to damaged chromosome end is essential to facilitate repair by NHEJ (Dimitrova *et al.*, 2008).

1.3. Double-strand break repair choice

53BP1 has therefore become known for its significant function in DSB repair through the NHEJ pathway, and also for its role in the choice of DSB repair signalling (Daley and Sung, 2014; Lei *et al.*, 2022) (**Fig. 16**). 53BP1 possesses an anti-resection activity, thus protecting damaged ends from resection by nucleases, an essential step for HR. The antagonist of 53BP1 is BRCA1, associated with its partner BARD1, which promotes this DNA resection. The deletion of BRCA1 is a driver event in the development of breast cancers as it induces HR deficiency. The same applies to BARD1. In these genetic backgrounds, the loss of 53BP1 rescues HR, leading to resistance of cancer cells to anti-cancer agents (Becker *et al.*, 2021; Bouwman *et al.*, 2010; Bunting *et al.*, 2010; Jaspers *et al.*, 2013).

The NHEJ pathway is the primary choice for repairing DSBs as it can be activated during any phase of the cell cycle, triggered by phosphorylation of 53BP1. In the S-phase, BRCA1 promotes the HR pathway by facilitating the dephosphorylation of 53BP1 through the phosphatase PP4C leading to the release of RIF1 (Isono *et al.*, 2017). In fact, as soon as 53BP1 is phosphorylated by ATM, it recruits RIF1 and the phosphatase PP1, thus forming a barrier to DNA end resection in G1-phase by inhibiting the recruitment of MRN and CtIP (Chapman *et al.*, 2013; He *et al.*, 2018). Moreover, 53BP1 can also interact with DYNLL1 that physically inhibits the resection machinery (He *et al.*, 2018). 53BP1 therefore prevents early resection, however if DNA resection has already been initiated, 53BP1 can also act to inhibit extended resection by interacting with RIF1, PTIP and Shieldin. Subsequently, Shieldin recruits CTC1-STN1-TEN1(CST)/Pol α /Primase inducing PRIM-Pol α -dependent 3' end fill-in DNA synthesis (Gupta *et al.*, 2018; Mirman *et al.*, 2022; Noordermeer *et al.*, 2018).

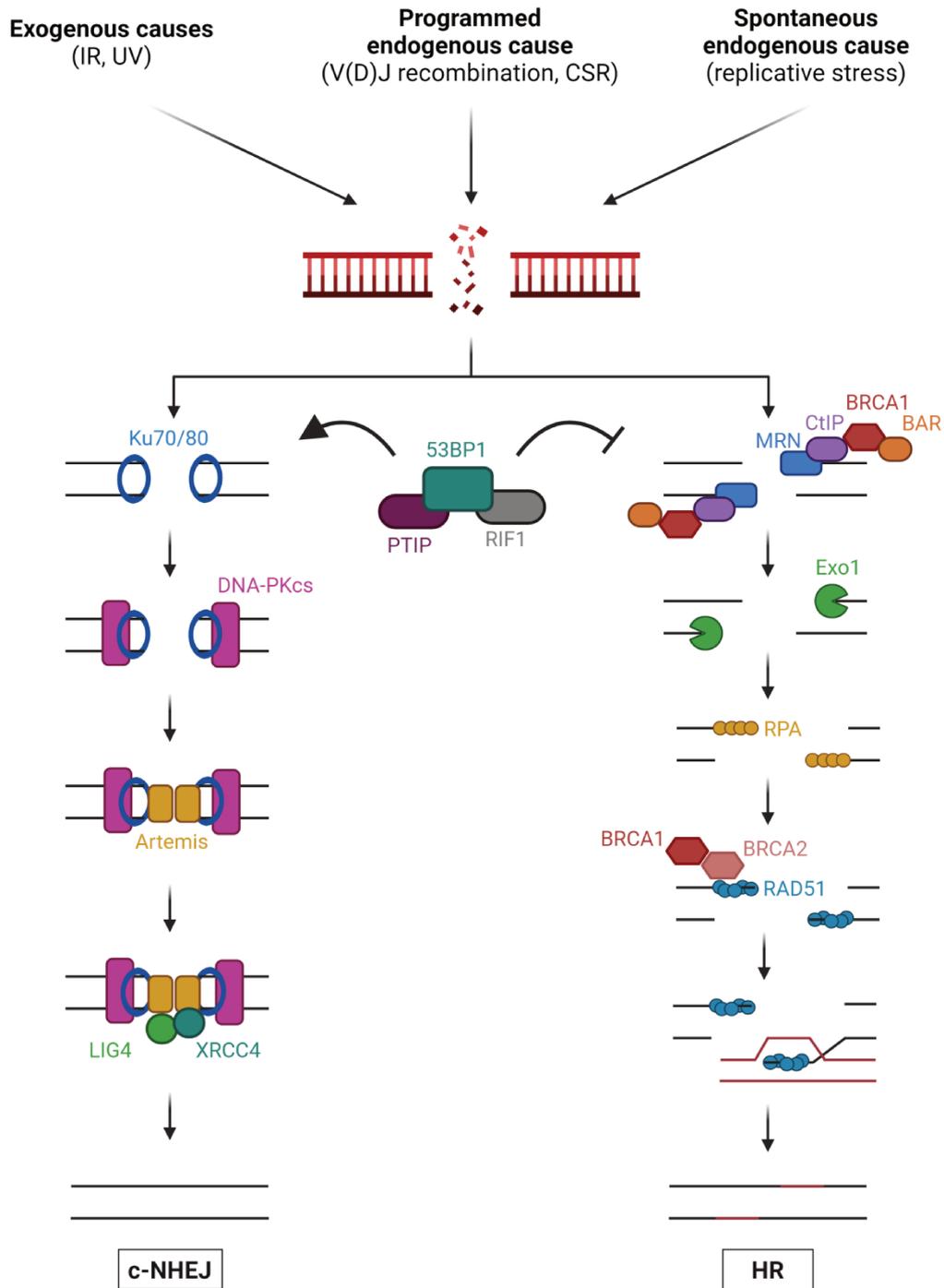


Figure 16 | Double-strand break repair pathways. Double-strand breaks can arise from exogenous sources (ionizing radiation (IR) or ultraviolet (UV)), endogenous sources (replicative stress) or can even be programmed to participate in diversity within antigen receptor genes (class switch recombination (CSR) or variable (V), diversity (D), joining (J) recombination). 53BP1 plays a crucial role in the choice of double-strand break repair pathways. It functions as an anti-resection factor and thus promotes the canonical NHEJ (c-NHEJ) rather than the HR pathway, where the first step involves DNA resection. When double-strand breaks are repaired by the NHEJ pathway, the DNA ends are bound by the Ku70/80 heterodimers, inducing the recruitment of DNA-PKcs and Artemis enabling the creation of microhomology regions. These regions are used by XRCC4 and LIG4 to join DNA ends. Alternatively, double-strand breaks can also be repaired by the HR pathway. In this case, DNA is degraded by nucleases, such as MRE1 and Exo1. The resulting ssDNA is bound by RPA, after which RAD51 replaces RPA to initiate homology search on the sister chromatid, facilitating the repair of DSBs. HR is therefore a pathway favoured in the S-phase of the cell cycle.

The choice of DSB repair pathways also relies on chromatin structure and histone marks. 53BP1 recognizes histone marks H4K20me₂, methylated by MMSET, and H2AK15ub, ubiquitinated by the RNF8-RNF168 pathway, at double-strand breaks, enabling it to play its role in inhibiting resection (Fradet-Turcotte *et al.*, 2013; Pei *et al.*, 2011). In S-phase, however, the H4K20me₂ motif is less present at chromatin because it is diluted after the synthesis of new nucleosomes incorporating into replicated DNA. The recognition of H4K20me₀ by BRCA1/BARD1 therefore prevents the anti-resection activity of 53BP1 and promotes HR (Nakamura *et al.*, 2019) (**Fig. 15**).

1.4. Replication stress response

Recent proteomic studies have identified NHEJ factors as being active during DNA replication, when replicative stress is induced (Dungrawala *et al.*, 2015; Mukherjee *et al.*, 2019; Ribeyre *et al.*, 2016). This is indeed the case for 53BP1 which also plays a role in the S-phase during the response to replication stress (**Fig. 15**).

In 2004, Sagar Sengupta and colleagues demonstrated that following HU treatment, which reduces the dNTPs pool and thus induces replicative stress, 53BP1 is recruited to nascent DNA. Once recruited, 53BP1 facilitates the accumulation of BLM and p53 at stalled forks, in a CHK1- and ATR-dependent manner (Sengupta *et al.*, 2004). Moreover, the recruitment of 53BP1 to the fork is a fast process, approximately 5min after the induced stress (Dungrawala *et al.*, 2015).

The role of 53BP1 at the stalled fork is actually also based on its anti-resection activity. In 2018, Joonyoung Her and colleagues observed that in primary B cells, the depletion of 53BP1 resulted in the degradation of nascent DNA upon HU treatment. However, the interactions that 53BP1 establishes to mediate its functions in DNA replication are still unclear. Her *et al.* propose that 53BP1 interacts with histones H4K20me₂ and H2AK15Ub at the stalled fork to activate ATR-mediated CHK1 phosphorylation and thus stabilize the fork. Moreover, 53BP1 protects the fork by limiting access to the MRE11 exonuclease, thereby inhibiting its activity (Her *et al.*, 2018).

Furthermore, following a replicative stress, the loss of 53BP1 reduces replication rates, the cell survival and increases chromosomal aberration (Tripathi *et al.*, 2007; Villa *et al.*, 2018).

53BP1 is also involved in the fork reversal mechanism. There are 2 pathways to protect the regressed fork end: one is 53BP1-dependent and the other is independent of 53BP1. 53BP1 protects specifically the substrate generated by the F-Box DNA helicase 1 (FBH1), associated with BRCA2 and RAD51 (Liu *et al.*, 2020).

Additionally, similar to the mechanism of choosing the appropriate DSB repair pathways, 53BP1 competes with BRCA1 in selecting the pathway for the restart of stalled replication fork. 53BP1 in association with RIF1 promotes a pathway without cleavage, whereas BRCA1 favours a slower pathway with cleavage, known as the break-induced replication (BIR) pathway (Xu *et al.*, 2017).

Taken together, these studies show that 53BP1, like its anti-resection role in DSB repair, controls nascent DNA degradation by acting as a nuclease barrier and thus preventing resection of stalled replication forks after induction of replication stress.

2. Structure

Human 53BP1 is a protein, with no enzymatic activity, of 1972 amino acids. It contains several domains (**Fig. 17**) that can be divided into 3 main parts (Panier and Boulton, 2014; Rass *et al.*, 2022):

- The N-terminal part which plays a role in 3' overhangs control following DNA damage. This region is composed of 28 Serine-Threonine-Glutamine (STQ) sites that can be phosphorylated by ATM/ATR and are known to interact with RIF1 and PTIP proteins to control resection and promote the NHEJ repair pathway.
- A central part which is the minimum focus forming region and therefore is involved in the recruitment of 53BP1 to double-strand break sites. This region includes the Oligomerization Domain (OD), the Glycine-Arginine Rich (GAR) domain, two Tudor domains, the Ubiquitin-Dependent Recruitment (UDR) motif and the Nuclear

Localisation Sequence (NLS). This central region is crucial for the DNA repair functions of 53BP1. Histone marks play a significant role in 53BP1 signalling, and the interaction with these marks is mediated by its Tudor domains, recognising mono- and di-methylated H4K20, and UDR domain, recognising H2AK15ub. Furthermore, this binding occurs when 53BP1 is dimerised, facilitated by the OD domain. The Tudor domain of 53BP1 has been defined by the presence of conserved residues found in other Tudor proteins, such as the SMN protein (Alpha-Bazin *et al.*, 2005). The Tudor sequence of 53BP1, for instance, share similarity with the Tudor domain of the JMJD2A protein, but unlike this protein, the Tudor domain of 53BP1 forms two independently folded structures (Lu and Wang, 2013). Moreover, the sequences of the two Tudor domains of 53BP1 differ. Indeed, in the first Tudor domain, a surface capable of binding DNA and RG peptides is found, not present in the second Tudor domain (Charier *et al.*, 2004; Côté and Richard, 2005). However, the Tudor domains of 53BP1 have not been shown to interact with the GAR domain of 53BP1. As for the GAR domain, it is an RNA-binding domain found in other RBPs (as described in section I-2.2, paragraph RGG) where Arginines are methylated by PRMT1 and 5 (Boisvert *et al.*, 2005c; Hwang *et al.*, 2020). Its role is not well-defined, however an *in vitro* study demonstrated that 53BP1 can bind DNA (single- and double-strand) through the GAR domain (Boisvert *et al.*, 2005c).

– The C-terminal region containing two BRCA1 C-Terminus (BRCT) domains, essential for p53 and USP28 binding. Moreover, the BRCT domains are dispensable for most of the activities of 53BP1 in double-strand break repair.



Figure 17 | Structure of the 53BP1 protein. The N-terminal part of 53BP1 contains 28 Serine/Threonine/Glutamine (Ser/Thr/Gln) sites. In its central part, 53BP1 is composed of an oligomerization domain (OD), a Glycine/Arginine-rich (GAR) domain, a tandem Tudor motif, an ubiquitination-dependent recruitment (UDR) domain and a nuclear localisation signal (NLS). Finally, the C-terminal of 53BP1 consists of two BRCA1 carboxy-terminal (BRCT) domains.

3. 53BP1 and RNA

As described in the section I-1, numerous studies have shown that RBPs and RNAs play a role in the regulation of the response to DNA damage. Furthermore, some proteins in the DNA damage response, such as Ku, have been characterised as having the ability to bind RNA. As mentioned above, since 53BP1 contains a putative RNA-binding domain with GAR motifs (RGRGRRGR) (Thandapani *et al.*, 2013) (**Fig. 17**), it therefore raises the question of whether 53BP1 also possesses an RNA-binding activity. Over the past few years, several studies have attempted to provide answers to this question.

In 2005, Fiona Pryde and colleagues have observed that the formation of 53BP1 foci at DNA damage sites is mediated by an RNA component. After induction of double-strand breaks in murine (NIH3T3) and human (HeLa) cells, by exposure to ionizing radiation, RNase A treatment, that cleaves single-strand RNA, decreased the recruitment of 53BP1 at DNA break sites (Pryde *et al.*, 2005).

These results were also obtained in another study in 2012 where Francia *et al.*, showed that after irradiation in human cells (HeLa), the RNase A treatment leads to a decrease in 53BP1 foci (Francia *et al.*, 2012). Moreover, the inhibition of the ribonucleases Dicer and Drosha, that are responsible for the production of small double-strand RNAs in cells, also decreased 53BP1 recruitment to DNA damage. The team therefore concluded that at DNA damage sites RNAs are produced and cleaved by Dicer and Drosha and they play a role in the activation of DNA repair signalling.

The production of DNA damage response RNA (DDRNA) at DNA damage sites was subsequently confirmed (Michellini *et al.*, 2017, 2018). After DNA break, MRN is recruited, triggering the transcription by RNA polymerase II (RNA Pol II) on each side of the break. The RNA Pol II then synthesizes damage-induced long non-coding RNAs (dilncRNAs) (Pessina *et al.*, 2019). These RNAs contribute to the formation of double-strand RNAs, the DDRNA precursors. These precursors are subsequently cleaved by Dicer and Drosha to generate DDRNAs involved in DDR signalling, by recruiting factors such as 53BP1. The mechanism of obtaining DDRNA precursors is still poorly described (Audouinaud *et al.*, 2021) (**Fig. 18**).

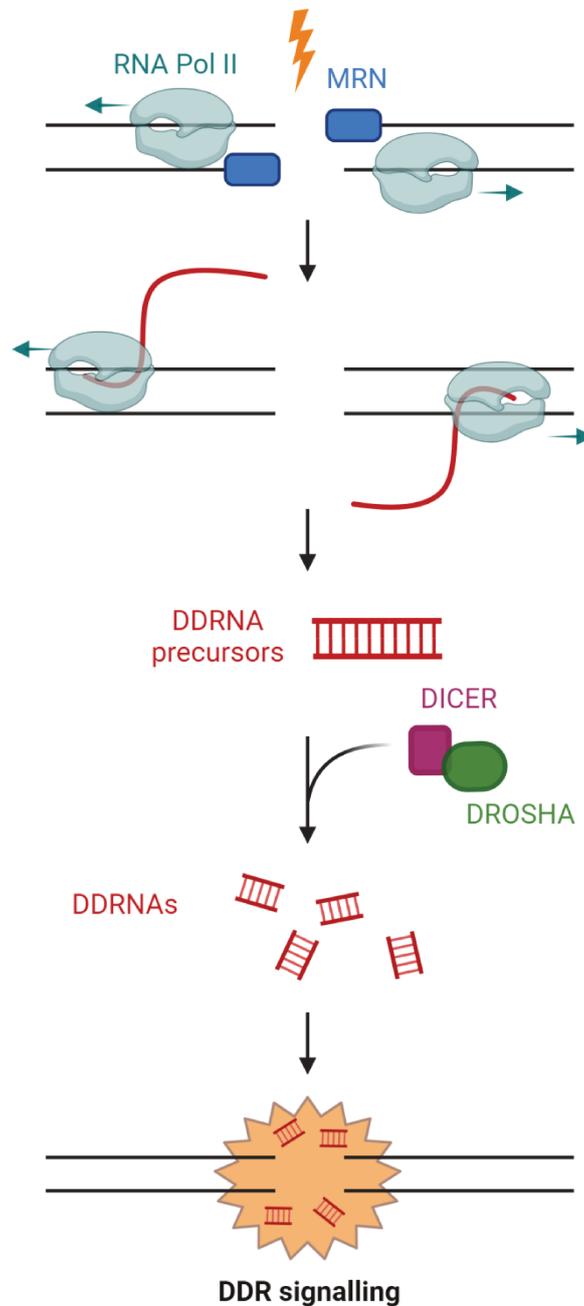


Figure 18 | Production of RNA at DNA damage sites. After a double-strand break, the MRN complex recruits RNA polymerase II (RNA Pol II), which will synthesize non-coding RNA *de novo* at the ends of the damaged DNA. In transcriptionally active regions, the damage-induced RNA is complementary to pre-existing transcripts, thus forming long non-coding RNAs. These long double-strand RNAs are then cleaved by Dicer and Drosha, generating small non-coding RNAs, known as DNA damage response RNA (DDRNA). DDRNAs then participate in DNA damage response signalling. Adapted from Audouinaud *et al.*, 2021.

Furthermore, 53BP1 is negatively regulated by the RNA-binding protein Tudor-interacting repair regulator (TIRR). Under normal conditions, TIRR binds to 53BP1 and inhibits its activity. By binding to the Tudor domain of 53BP1, TIRR masks the methylated histone-binding surface, preventing 53BP1 from being recruited and therefore from acting. TIRR also inhibits the formation of the 53BP1-p53 complex and the loss of TIRR leads to an abnormal increase in the transcriptional response mediated by p53 (Botuyan *et al.*, 2018; Drané *et al.*, 2017; Parnandi *et al.*, 2021). Upon damage, ATM phosphorylates 53BP1, recruiting RIF1 which assists in the dissociation of the 53BP1-TIRR complex to release the inhibition of 53BP1 and enables 53BP1 to play its role in DNA damage response. This dissociation is made possible by RNAs transcribed at DSBs by RNA Pol II and is dependent on Dicer and Drosha (Ketley *et al.*, 2022). Moreover, using *in vitro* EMSA assays to visualize the affinity between a fragment of 53BP1 containing only the Tudor region and various substrates (ssRNA, ssDNA and RNA beacon), Ketley *et al.* did not observe a binding of the Tudor domains to any of the tested substrates.

Therefore, the Tudor domains do not seem to have an RNA-binding activity. But what about the GAR domain of 53BP1, the characterised RNA-binding domain? The role of the GAR domain of 53BP1 is still not well understood and a direct binding to RNA has not yet been demonstrated. In 2005 Pryde *et al.*, interestingly demonstrated that the deletion of the GAR domain does not impact 53BP1 foci. After RNase A treatment, the recruitment of 53BP1 deleted from the GAR domain decreases only at early stages (20min after irradiation) and not at late stages (2h after irradiation). This suggests that the GAR domain helps the formation of 53BP1 foci but is not essential for its recruitment. This has been confirmed in a 2013 study where Lottersberger *et al.*, studied the phenotype associated with 53BP1 mutated in the GAR domain (three Arginines substituted in three Lysines). They showed that the GAR mutant had no impact on the various functions of 53BP1, *e.g.* in DNA repair signalling, in class switch recombination and in blocking the resection at dysfunctional telomeres (Lottersberger *et al.*, 2013). The GAR domain is therefore not essential in most functions of 53BP1. However, mutations in the GAR domain have not been studied in the context of DNA replication, so it cannot be excluded that this domain is required in this context.

Even though no direct binding between 53BP1 and RNA has yet been demonstrated, recent proteomic analyses have suggested 53BP1 as a potential RBP. Indeed, using the OOPS assay, it was shown that a 53BP1 peptide could associate with RNA (Queiroz *et al.*, 2019; Trendel *et al.*, 2019). However, these observations do not demonstrate a direct interaction between the 53BP1 protein and RNA.

In conclusion, the studies described above show that the role of 53BP1 in its DNA repair function is dependent on an RNA component and that 53BP1 could be characterised as an RBP. These findings raise two questions: Does 53BP1 have an RNA-binding activity and is 53BP1's function in DNA replication also mediated by RNA? The aim of this thesis was thus to address these questions.

OBJECTIVES

As described in the introduction, RNA-binding proteins are important in maintaining genome stability throughout the cell cycle by participating in DNA damage repair and response to replication stress. Additionally, an increasing number of proteins involved in the NHEJ pathway, a process of repairing double-strand break, have been suggested to exhibit a potential RNA-binding activity (Audoynaud *et al.*, 2021). However, the exact role of this possible activity in the functions of these proteins remains unclear. The laboratory has explored these candidate RBPs with particular emphasis on the 53BP1 protein for several reasons.

Firstly, 53BP1 possesses an RNA-binding domain, the GAR domain rich in Arginine and Glycine where the Arginines are methylated by PRMT1 and 5 (Boisvert *et al.*, 2005c; Hwang *et al.*, 2020; Panier and Boulton, 2014; Thandapani *et al.*, 2013). Furthermore, in the context of DNA damage, the functions of 53BP1 are mediated by an RNA component. Indeed, treating cells with RNase A significantly reduces the formation of 53BP1 foci following ionising irradiation. However, these observations do not demonstrate a direct interaction between 53BP1 and RNA. In addition, deletion or mutation of three Arginines in Lysines in the GAR domain does not affect the recruitment of 53BP1 to the lesion sites, nor does it impact other functions of 53BP1 such as class switch recombination (Francia *et al.*, 2012; Lottersberger *et al.*, 2013; Michelini *et al.*, 2017; Pryde *et al.*, 2005). Nevertheless, the alteration of the GAR domain has not been tested in the functions of 53BP1 related to the response to replication stress, therefore a potential role of this domain in these functions cannot be ruled out. Indeed, in the S-phase 53BP1 is recruited to the stalled replication fork within 5min following the replication stress. This recruitment aims to protect the nascent DNA from degradation by nucleases such as MRE11. Therefore, 53BP1 contributes to the resolution of stalled or regressed forks (Dungrawala *et al.*, 2015; Her *et al.*, 2018; Liu *et al.*, 2020).

To detect and characterise the interaction between a protein and RNA, the laboratory used the cross-linking immunoprecipitation (CLIP) and the complex capture (2C) techniques (Asencio *et al.*, 2018; Ule *et al.*, 2005). These methods are based on UV-C crosslink which induces a covalent bond between RBPs and their associated RNAs. In CLIP, the protein is immunoprecipitated and then the bound RNAs are radioactively labelled, whereas in 2C the RNAs, and thus the bound proteins, are retained on a silica

column followed by a western blot to analyse the RBPs. These two approaches have enabled the laboratory to previously identify the direct RNA-binding activity of 53BP1 (endogenous and transfected) in both cancerous and epithelial cells. Furthermore *in vitro* data have demonstrated that the GAR-Tudor region of 53BP1 possesses this RNA-binding ability (Leriche, Bonnet *et al.*, in revision). By identifying the nature of this RNA, after extracting the nucleic acids bound to 53BP1 following the CLIP procedure, the laboratory found that 53BP1 binds to a specific substrate composed of a 5' RNA part and a 3' DNA part, similar to Okazaki fragments. The Okazaki fragments are discontinuously produced on the lagging strand during S-phase and the RNA-DNA primers are essential for their synthesis. During the replication, ribonucleotides are produced by the action of the primase PRIM1 and, following this RNA primer, the DNA polymerase α can initiate DNA synthesis. The RNA primer (referred to as a flap in its single-strand form) is then processed and cleaved by the endonuclease FEN1, before the ligation of Okazaki fragments by the DNA ligase 1. Up to now, although certain RBPs are present at the replication fork, it has never been demonstrated that these RBPs directly interact with the RNA primers of the Okazaki fragments.

The prior identification by the laboratory of the interaction between 53BP1 and RNA serves as the foundation for this thesis project, which seeks to better characterise this interaction and its implications in DNA replication. Three objectives are thus defined **(Fig. 19)**:

- 1- Determine whether the recruitment of 53BP1 to the replication fork is dependent on the RNA primer present in the Okazaki fragments.
- 2- Understand the role of this RNA-binding in the normal mechanism of DNA replication, without induction of replication stress.
- 3- Characterise how the interaction between 53BP1 and the Okazaki fragments occurs, by identifying the protein domains implicated and an associated mutant.

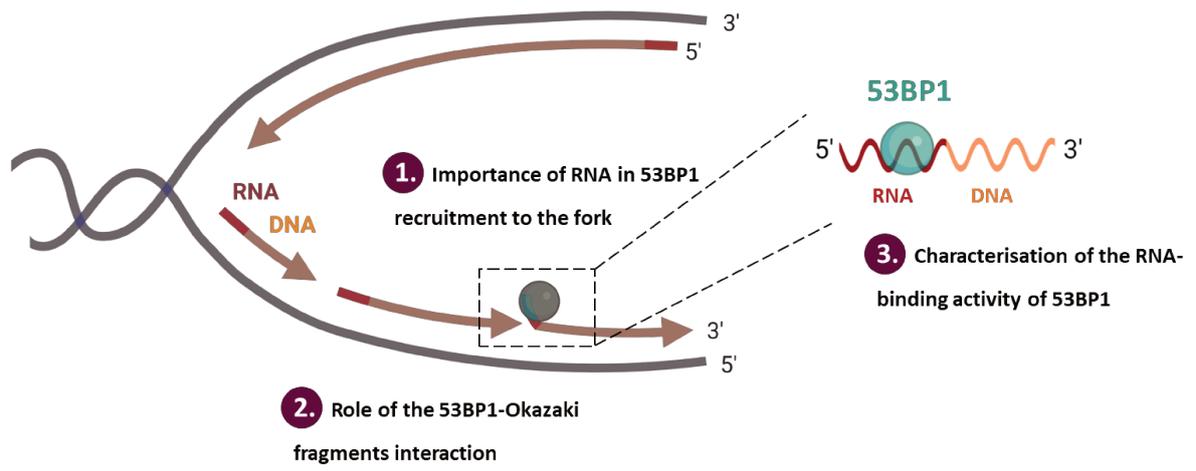


Figure 19 | Objectives of the thesis.

RESULTS

I – Article: 53BP1 interacts with the RNA primer from Okazaki fragments to support their processing during unperturbed DNA replication

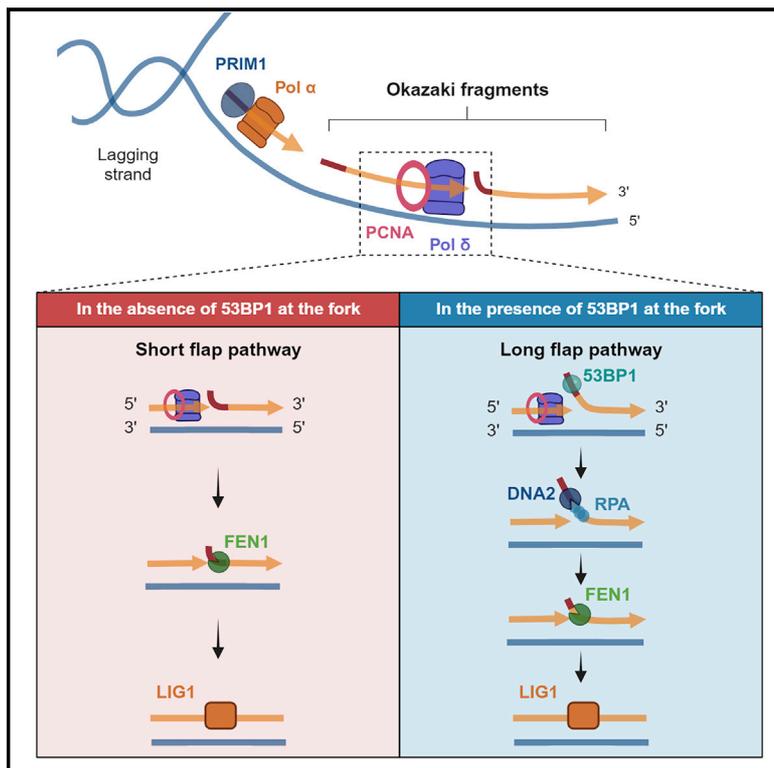
The first objective of my thesis is to understand the role of the interaction between 53BP1 and RNA during DNA replication. To monitor this binding, *in vitro* assays were conducted. Subsequently transitioning to *in cellulo* model, we employed approaches based on the covalent bond between protein and RNA in living cells induced by UV-C: the cross-linking immunoprecipitation (CLIP) and complex capture (2C) techniques. The choice of UV-induced crosslink offers the advantage of irreversible binding, enabling stringent wash conditions. In addition, CLIP and 2C are complementary methods. While the CLIP is protein-centric through immunoprecipitation, the 2C is RNA-centric using a silica column. Moreover, CLIP can be used to extract the nucleic acids bound to the protein and thus determine their nature (chimeras, hybrids, single-strand, double-strand).

Once the interaction between 53BP1 and RNA-DNA primers, similar to the Okazaki fragments, had been identified, we used microscopy approaches to characterise the specific interactions to S-phase and at nascent DNA. Firstly, the quantitative image-based cytometry (QIBC) technique was performed to determine the abundance of a specific protein in chromatin according to the cell cycle. To further elucidate the interaction at nascent DNA, the *in situ* protein interaction with nascent DNA replication forks (SIRF) technique was employed. This method is based upon proximity ligation assay (PLA) procedure and enables the visualisation of the association between 53BP1 and nascent DNA.

Through the application of all these techniques, we have demonstrated that 53BP1 interacts with the RNA primer of Okazaki fragments, with the purpose of facilitating their maturation during unstressed DNA replication.

53BP1 interacts with the RNA primer from Okazaki fragments to support their processing during unperturbed DNA replication

Graphical abstract



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In brief

Leriche et al. show that 53BP1, distinct from its canonical mode of recruitment to chromatin, is an RNA-binding protein that interacts with RNA-DNA chimeric structures constituting Okazaki fragments. 53BP1 acts as a binder of Okazaki fragments and plays a role during unstressed DNA replication.

Highlights

- 53BP1 is an RNA-binding protein that directly interacts with RNA-DNA chimeric structures
- Perturbation of Okazaki fragment processing changes the proximity of 53BP1 with nascent DNA
- Depletion of 53BP1 perturbs the maturation of Okazaki fragments



Report

53BP1 interacts with the RNA primer from Okazaki fragments to support their processing during unperturbed DNA replication

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SUMMARY

RNA-binding proteins (RBPs) are found at replication forks, but their direct interaction with DNA-embedded RNA species remains unexplored. Here, we report that p53-binding protein 1 (53BP1), involved in the DNA damage and replication stress response, is an RBP that directly interacts with Okazaki fragments in the absence of external stress. The recruitment of 53BP1 to nascent DNA shows susceptibility to *in situ* ribonuclease A treatment and is dependent on PRIM1, which synthesizes the RNA primer of Okazaki fragments. Conversely, depletion of FEN1, resulting in the accumulation of uncleaved RNA primers, increases 53BP1 levels at replication forks, suggesting that RNA primers contribute to the recruitment of 53BP1 at the lagging DNA strand. 53BP1 depletion induces an accumulation of S-phase poly(ADP-ribose), which constitutes a sensor of unligated Okazaki fragments. Collectively, our data indicate that 53BP1 is anchored at nascent DNA through its RNA-binding activity, highlighting the role of an RNA-protein interaction at replication forks.

INTRODUCTION

Recent observations highlight the importance of RNA-binding proteins (RBPs) in genome maintenance. RBPs, through their direct and generally specific binding to *cis*-acting elements in mRNAs, usually act as post-transcriptional regulators of gene expression.¹ Importantly, in addition to their proven role in DNA-damage-induced post-transcriptional regulation of DNA damage response (DDR) gene expression, RBPs, and the RNAs to which they bind, may play a more direct role in genome integrity.^{2–4} Several proteins involved in the DDR have been suggested to be RBPs. However, a potential RNA-binding activity of these proteins remains to be evaluated.

The p53-binding protein 1 (53BP1) is a key protein that mediates the signaling of DNA double-strand breaks (DSBs) in G1 and S/G2 phases of the cell cycle. In G1, 53BP1 acts as a molecular scaffold that recruits additional DSB-responsive proteins to damaged chromatin to limit DNA end resection and promote repair by non-homologous end joining (NHEJ).⁵ The accumulation of 53BP1 at DSBs is RNA dependent,^{6–8} and RNA immunoprecipitation (RIP) assays have shown that 53BP1 associates with RNAs.^{7,8} These observations, however, do not demonstrate

a direct interaction between 53BP1 and RNA, even if a recent proteomic analysis has identified 53BP1 as a candidate protein interacting with RNA.⁹ In addition to its role in the DDR, 53BP1 plays an important function in DNA replication. Indeed 53BP1 has been shown to be rapidly recruited to stalled replication forks following a replicative stress induced by hydroxyurea (HU) treatment.¹⁰ Lack of 53BP1 decreases cell survival and enhances chromosomal aberration after replication arrest.¹¹ 53BP1 protects replication forks during the cellular response to replication stress in primary B cells,¹² and the *S. cerevisiae* 53BP1 ortholog (RAD9) protects stalled replication forks from degradation in Mec1 (ATR)-defective cells.¹³ The mechanism of 53BP1 recruitment to replication forks may not be completely similar to the one existing for 53BP1 recruitment to DSBs since depletion of RNF8, RNF168, or 53BP1, but not MDC1, displays a similar replication defect (i.e., delayed fork progression and reversed fork accumulation).¹⁴ It is therefore not clear which interaction(s) 53BP1 establishes to mediate its functions in DNA replication.

During initiation of DNA replication, several ribonucleotides are polymerized by the combined action of an RNA polymerase (DNA primase) and an error-prone DNA polymerase (POL α). DNA POLs are unable to initiate DNA synthesis *de novo* and require



an initiating step to generate an RNA primer. This “priming” role is fulfilled by specialized DNA-dependent RNA POLs, called DNA primases containing a small catalytic subunit (PRIM1) and a large accessory subunit (PRIM2), capable of synthesizing short RNA chains (7–12 ribonucleotides). Primases are also essential throughout the process of lagging strand replication, where they initiate synthesis of the discontinuously synthesized Okazaki fragments.¹⁵ On the lagging strand, the DNA POL δ synthesizes approximately 100 deoxyribonucleotides downstream of this RNA-DNA primers to form the Okazaki fragments.¹⁶ When POL δ displaces the downstream fragment, it creates a short 5' flap single-stranded nucleic acid that is cleaved by the flap structure-specific endonuclease 1 (FEN1) to allow subsequent ligation of the two Okazaki fragments by DNA ligase I (LIG1). In some instances, a long flap is created that is covered with replication protein A (RPA), thereby blocking the access to FEN1. RPA can then recruit the DNA nuclease/helicase DNA2, which cleaves the long flap into a short flap that can be subsequently cleaved by FEN1.^{17–20} Whether RBPs, reported to be present at replication forks,²¹ directly bind RNA primers of Okazaki fragments during unperturbed DNA replication has not been established.

Using UV-C-induced crosslinking IP (CLIP²²) and complex capture (2C²³) experiments in living cells, we provide evidence that 53BP1 directly interacts with RNA. By analyzing the nature of the nucleic acids bound to 53BP1, we found entities of about 20–200 nt, composed of ribonucleotides at the 5' end followed by deoxyribonucleotides at the 3' end, that constitute Okazaki fragments. Consistent with the hypothesis that 53BP1 directly interacts with Okazaki fragments, we found that ribonuclease (RNase) treatment decreases 53BP1 recruitment to the fork (as determined by *in situ* analysis of protein interactions at DNA replication forks [SIRF]²⁴). In addition, while depletion of PRIM1 leads to reduced 53BP1 assembly, loss of FEN1 enhances the 53BP1 association with nascent DNA. Finally, depletion of 53BP1 leads to reduced RPA and DNA2 loading at nascent DNA. Altogether, these results indicate that the interaction between 53BP1 and Okazaki fragments is a key determinant of their processing and, more generally, of the functions of 53BP1 in DNA replication.

RESULTS

53BP1 is an RBP

53BP1 contains a putative intrinsically disordered GAR (glycine-arginine-rich) RNA-binding domain (Figure S1A).²⁵ To investigate a potential RNA-binding activity, we first used an electrophoretic mobility shift assay (EMSA). The migration of the structured SC1 RNA (Figure S1B) (known to interact with the GAR domain of the FMRP protein; Figure S1C) was shifted by a recombinant GAR-Tudor 53BP1 protein (Figures S1D and S1E). Surface plasmon resonance (SPR) experiments indicated that the GAR-Tudor fragment interacts with the SC1 RNA with an affinity of about 8 nM, while a GAR-Tudor fragment mutated in the GAR domain (3R to 3K) completely lost its affinity with the SC1 RNA (Figure S1F). The ability of 53BP1 to directly bind to RNAs *in vivo* was analyzed following irradiation of living cells with high doses of UV-C (254 nm) in a CLIP experiment.²² UV crosslinking requires direct contact (“zero” distance) between protein and RNA and does not promote protein-protein crosslinking.^{26,27} We irradiated hu-

man HEK293T living cells with UV-C and immediately prepared cell lysates. Following 53BP1 IP and stringent washes, the IP was radiolabeled with T4 polynucleotide kinase (PNK), which incorporates ³²P at the 5' end of nucleic acids (Figures 1A and 1B). In UV-exposed cells, we observed a sharp radioactive band at the size of 53BP1 (Figure 1B), indicating that 53BP1 directly interacts with RNAs. A small interfering RNA (siRNA) pool directed against 53BP1 was used to ascertain that the band migrating at the size of 53BP1 corresponds to 53BP1 and that the lower band (labeled with an asterisk [*]) corresponds to a non-specific signal. We obtained very similar results in other cell lines, including HeLa cervical cancer cells, A2058 melanoma cells, RPE1 non-transformed human epithelial cells, and U2OS osteosarcoma cells (Figure S2A). To ascertain that the above CLIP results were not linked to an artifact related to the use of the 53BP1 antibody, we expressed GFP-53BP1 fusion proteins in HEK293T cells and performed the CLIP experiment with a GFP antibody instead of the 53BP1 antibody. A radioactive band was observed at the predicted size of the GFP-53BP1 protein or of the GFP-53BP1-Cter protein containing the carboxy-terminal half of 53BP1 that is still able to be recruited to ionizing radiation-induced damaged DNA (Figures S2B and S2C). Altogether, these data show that an RNA-binding activity can be found in the carboxy-terminal half of 53BP1 containing the GAR-Tudor domain.

Even if UV irradiation is mainly used to monitor RNA-protein interactions,²² it has also been used to establish crosslinks between proteins and single-stranded DNA (ssDNA) (e.g., Steen et al.²⁸). We therefore used an orthogonal approach (2C) that uses the property of silica matrices to strongly and specifically retain nucleic acids and crosslinked nucleic acid-protein complexes based on charges (Figures 1A, 1C, and 1D).²³ 53BP1 was retained on the silica matrix in a UV- and RNA-dependent manner (Figure 1C). The well-characterized RBP HuR and the tubulin were used as positive and negative controls, respectively. Consistent with the EMSA data, the GAR-Tudor region is sufficient to interact with RNAs in living cells (Figure 1D). Altogether, these data show that 53BP1 directly interacts with RNA *in vitro* and *in vivo*.

53BP1 binds RNA-DNA chimeras

To analyze the nature of the nucleic acids bound to 53BP1, we extracted nucleic acids from the ³²P-labeled crosslinked nucleic acid-GFP-53BP1 complexes, loaded them on a denaturing TBE-urea gel, and revealed the radioactive signal by autoradiography. We found that nucleic acids bound to 53BP1 migrate as a smear of about 20–150 nt (Figure 2A). The smear collapsed below 25 nt following treatment with RNase A (which, in our experimental conditions, cleaves both ssRNA and double-stranded RNA, but not DNA or RNA engaged in an RNA:DNA duplex; Figure S3), showing that these nucleic acids possess ribonucleotides at their 5' ends. Strikingly, the size of the smear was also shortened following treatment with deoxyribonuclease I (DNase I) (which specifically cleaves DNA but not RNA; Figure S3), indicating that the nucleic acids interacting with 53BP1 are also composed of deoxyribonucleotides. The remaining DNase I-resistant shorter smear was composed of RNA at its 5' end since it disappeared following treatment with both RNase A and DNase I (Figure 2A). Similar results were found with the endogenous 53BP1 (Figure S4A). DNase I had no effect on nucleic acids

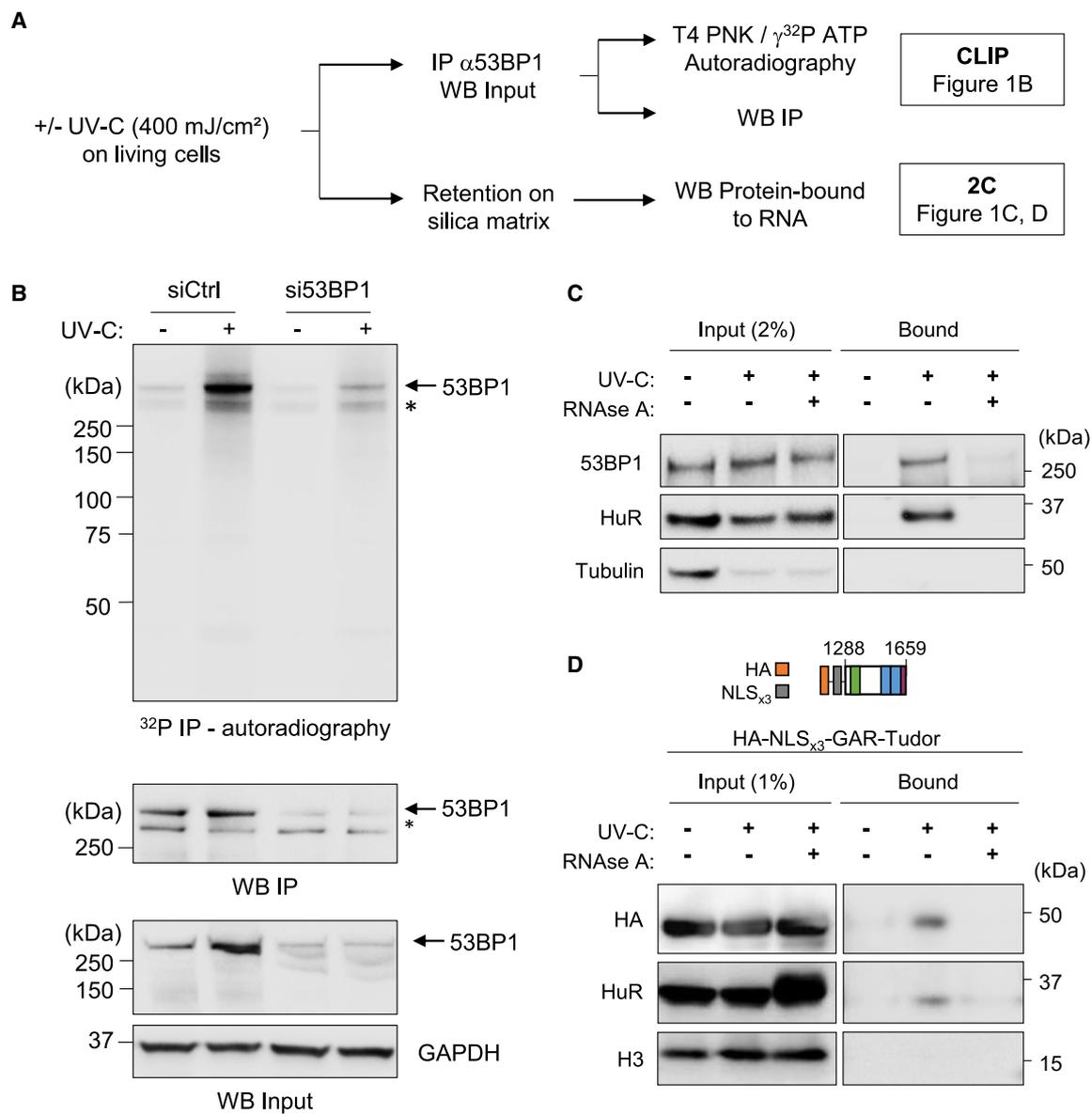


Figure 1. 53BP1 directly interacts with RNA

(A) Schematic representation of the crosslinking immunoprecipitation (CLIP) and complex capture (2C) procedures. WB, western blot; IP, immunoprecipitation; PNK, polynucleotide kinase.

(B) Top: autoradiography of 53BP1-nucleic acid complexes in UV-C-treated (+) or -untreated (-) HEK293T cells depleted of 53BP1 (si53BP1) or left untreated (siControl [siCtrl]). Bottom: IP of 53BP1 and WB of 53BP1 and GAPDH (used as a normalization control); same conditions as on the top. The asterisk indicates a non-specific band (*), and the arrow indicates the position of 53BP1-nucleic acid complexes (n = 3 biological replicates).

(C) WB on 2C experiments performed in A2058 cells (n = 3 biological replicates).

(D) WB on 2C experiments performed in GAR-Tudor-transfected HEK293T cells (n = 3 biological replicates).

bound to the RBP RBMX (Figure 2B), ruling out the possibility that DNase I was contaminated with RNases. We concluded that the cellular nucleic acids interacting with 53BP1 are RNA-DNA chimeras composed of 5'-end RNAs of different sizes (less than 25 nt) followed by deoxyribonucleotides (up to 100 nt) at their 3' ends. Of note, the GAR-Tudor fragment that is sufficient to bind RNA (Figures 1D and S1) cannot bind RNA-DNA chimeras since DNase I treatment did not lead (in contrast to RNase treat-

ment) to a reduction in the size of the smear (Figure S4B), which is, therefore, only composed of ribonucleotides. This indicates that the binding of 53BP1 to Okazaki fragments through the GAR domain requires additional 53BP1 protein domains.

53BP1 interacts with Okazaki fragments

Based on these findings, we hypothesized that Okazaki fragments (the only known RNA-DNA chimeras in human cells) might

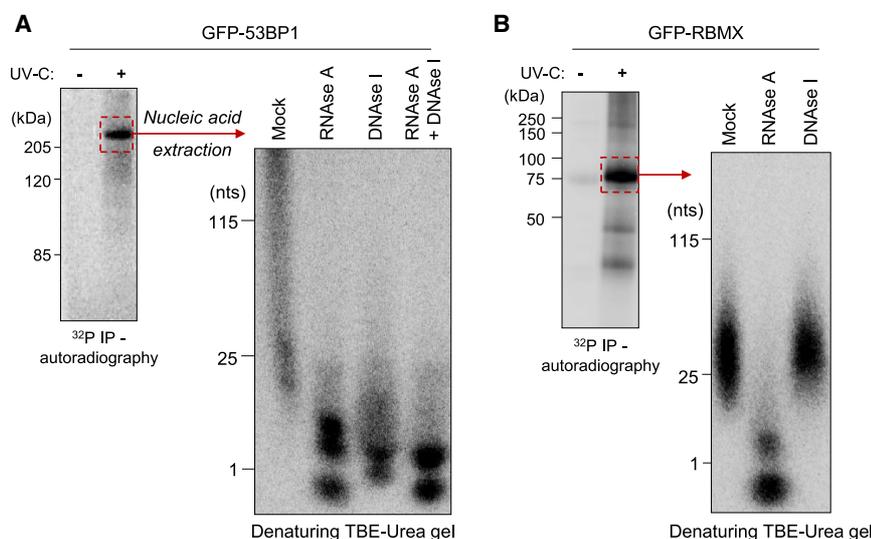


Figure 2. 53BP1 binds an RNA-DNA chimera
CLIP (left) and nucleic acid extraction (right) from GFP-53BP1- (A) or GFP-RBMX (B)-transfected HEK293T cells (n = 3 biological replicates).

Okazaki fragments through depletion of PRIM1/FEN1 or RNA degradation leads to reduced proximity between 53BP1 and nascent DNA.

Depletion of 53BP1 perturbs the lagging strand processing

Okazaki fragment maturation during lagging strand replication involves several layers of nuclease-driven pathways for proper RNA-DNA primer removal. Two pathways exist: the short flap pathway mediated by FEN1 and the long flap pathway mediated by DNA2/RPA. Based

contribute to the recruitment of 53BP1 to DNA replication forks. To test this hypothesis, we depleted the human PRIM1 subunit that synthesizes RNA primers of replication and employed quantitative image-based cytometry (QIBC²⁹) to quantify the chromatin abundance of 53BP1 at the single-cell level. The depletion of PRIM1 with two independent siRNAs led to a decrease of 53BP1 association with chromatin (Figures 3A and S5A) in the different phases of the cell cycle (Figure S5B) without altering the cell-cycle profile (Figures S5C and S5D). Consistent with previous results,³⁰ PRIM1 loss led to DNA replication stress, as seen from the pronounced induction of ssDNA and chromatin loading of RPA2 during the S phase (Figures S5E and S5F). Interestingly, while PRIM1 depletion decreased the global chromatin association of 53BP1 (Figure S6A), it had a modest effect of disturbing spontaneously arising 53BP1 foci assembled on chromatin due to dedicated DDR signaling (Figure S6B). Together, these results suggest that in addition to the well-established histone-modification-based chromatin loading of 53BP1 at DSBs,⁵ RNA primers synthesized by PRIM1 could facilitate the recruitment of 53BP1 on chromatin during DNA replication.

While the QIBC approach allows the determination of the chromatin abundance of proteins, it does not allow the determination of the abundance of proteins at replicating DNA. We therefore used a SIRF procedure that allows for the quantitative assessment of the proximity of proteins with ongoing, stalled, and previously active replication forks using a proximity ligation assay (PLA) approach.²⁴ As expected, 53BP1 could be found associated with nascent DNA (Figure S7). Treatment of cells with RNase A (Figure 3B) led to a decreased proximity between 53BP1, but not the proliferating cell nuclear antigen (PCNA), a component of the replisome, and nascent DNA, without changes in EdU incorporation (Figures S8A and S8B). The same result was observed upon depletion of PRIM1 (Figures 3C and S8C). In contrast, depletion of FEN1, which leads to an accumulation of unligated Okazaki fragments,²⁰ led to an increased proximity between 53BP1 and nascent DNA (Figures 3D and S8D). Taken together, these data indicate that perturbation of the synthesis and maturation of

on our results that 53BP1 associates with Okazaki fragments during active DNA synthesis, we next sought to determine whether 53BP1 binding to RNA-DNA primers plays a role in supporting Okazaki fragment maturation. We found that 53BP1 depletion leads to an increased accumulation of mono- and poly-(ADP-ribose) (MAR and PAR) in chromatin (Figures 4A and S9) that is more pronounced in S and G2 (Figure S9B) and which constitutes a sensor of unligated Okazaki fragments.³¹ Short incubation with PARG inhibitor to preserve nascent PAR further enhanced the levels of ADP ribosylation in 53BP1-depleted cells (Figure 4A). We also found that depleting 53BP1 leads to reduced RPA and DNA2 loading at chromatin (Figures 4B, 4C, 4D, and S10A–S10D), as well as accumulation of ssDNA (Figure S10E). Collectively, these results indicate that 53BP1 binds to long flaps of Okazaki fragments, fosters proper RNA-DNA primer removal, and prevents genotoxic DNA lesions that might arise from the lagging DNA strands in the absence of external stress inducer.

DISCUSSION

Here, we show that 53BP1 directly interacts with RNA *in vitro* and *in vivo* in human cells, revealing an additional activity of 53BP1 as a *bona fide* RBP (Figure 1). Strikingly, the nucleic acids interacting with 53BP1 are RNA-DNA chimeric structures composed of 5'-end ribonucleotides followed by deoxyribonucleotides at their 3' ends (Figure 2) that constitute Okazaki fragments (Figure 3). 53BP1, distinct from its canonical mode of chromatin binding, is anchored at the replication fork via its RNA-binding activity, thereby constituting a direct binder of Okazaki fragments during unperturbed DNA replication. Of note, it has been recently established that, during replication stress in fission yeast, the NHEJ factor Ku, also known as an RBP,⁴ binds to RNA:DNA hybrids originating from Okazaki fragments and establishes a barrier to replication fork degradation.³²

Similar to its role in DNA DSB repair pathway choice,⁵ 53BP1 may contribute to the choice between the short and long flap

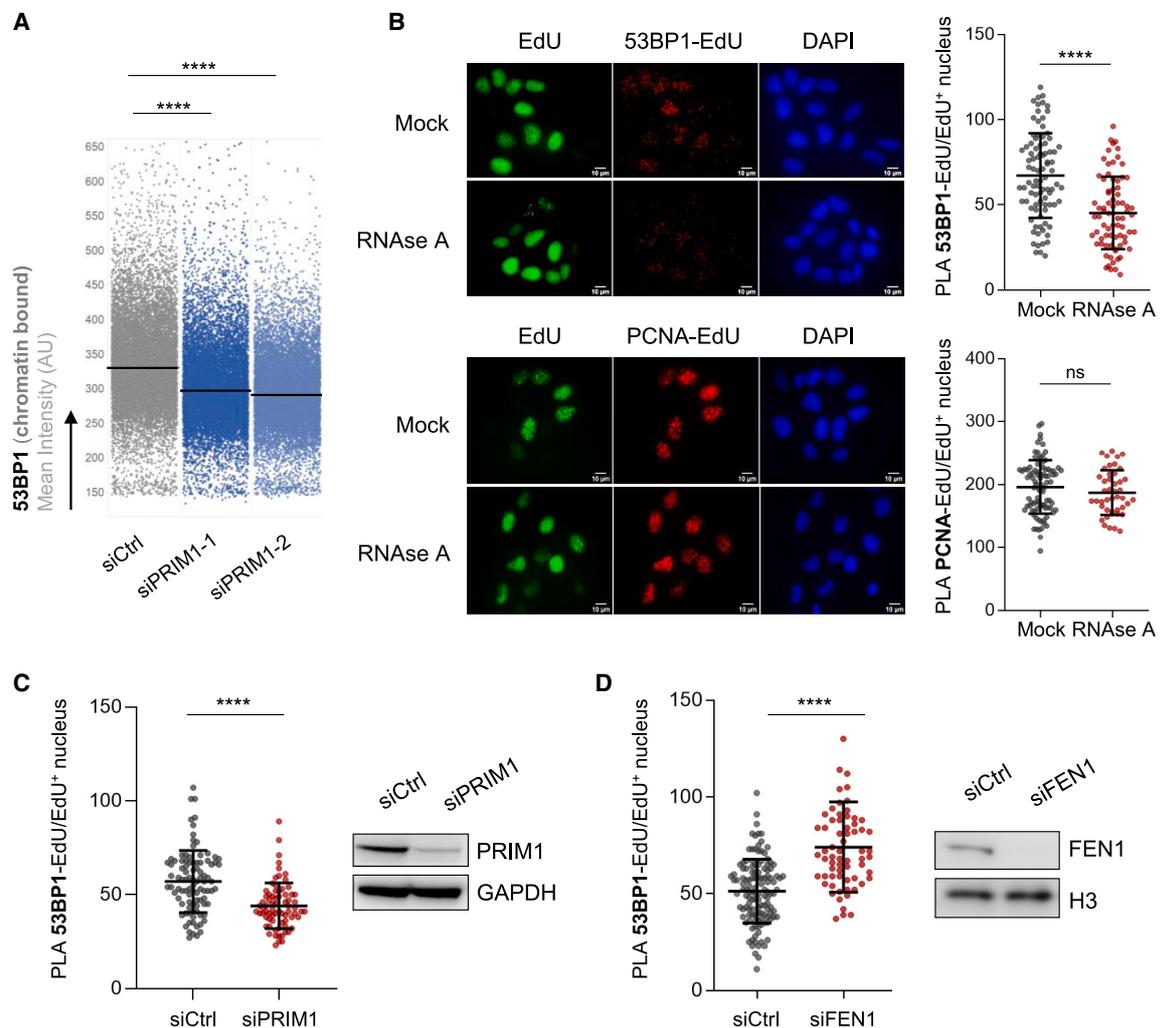


Figure 3. 53BP1 interacts with the RNA primer of Okazaki fragments

(A) QIBC of chromatin-loaded 53BP1 mean intensity. U2OS cells were treated with indicated siRNAs for 48 h (also see Figure S6). The horizontal lines are median values. p values were determined by one-way ANOVA with Tukey's test, ****p < 0.0001 (n > 10,000 cells from 3 biological replicates).

(B) Representative images and/or analysis of 53BP1-Edu and PCNA-Edu SIRF in U2OS cells permeabilized and treated with RNase A (RNase A) or left untreated (Mock); PLA signal (red), Alexa Fluor 488-Edu staining (green), and DAPI staining (blue). The significance for 53BP1-Edu and PCNA-Edu PLA values (shown as a scatterplot) was derived from the Mann-Whitney statistical test. Bars represent the mean ± SD. ****p < 0.0001 (n = 3 biological replicates).

(C and D) Representative scatterplot of U2OS cells depleted of PRIM1 (siPRIM1) (C) or FEN1 (siFEN1) (D) or left untreated (siCtrl) for 48 h. The significance was derived from the Mann-Whitney statistical test. Bars represent the mean ± SD. ****p < 0.0001 (n = 3 biological replicates).

pathways during Okazaki fragment maturation. In yeast, it has been shown that a delay in cleavage by FEN1 can induce a long flap.¹⁸ Our data on the association of RPA and DNA2 with nascent DNA upon 53BP1 depletion (Figure 4) indicate that the interaction between 53BP1 and the long flap may contribute to prevent the action of FEN1, thus supporting the long flap pathway and further recruitment of RPA/DNA2. Maturation through the long flap pathway was shown to limit mutation rates, especially in transcribed regions, because of the replacement of the DNA primer synthesized by the error-prone POL α with a DNA strand synthesized by the error-free POL δ .^{33,34} In this scenario, 53BP1 may contribute to maintain genome integrity during DNA replication.

This role of 53BP1 in the vicinity of replication fork might be of prime importance in human health. Indeed, defects in the maturation of Okazaki fragments lead to increased genetic instability, which causes certain cancers.^{35–37} Moreover, several studies have indicated that *TP53BP1* mutation might be associated with cancer risk.^{38,39} Hence, the evaluation of 53BP1 mutations should be extended, beyond their effects on DSB signaling, to replication defects. In addition, it will be interesting to analyze the role of the RNA-binding activity of 53BP1 in the context of the sensitivity to PAR POL inhibitors (PARPi) of tumor cells, which are deficient in hereditary breast cancer (BRCA) genes (BRCA1 and BRCA2). PARPi, which has anticancer activity in BRCA-deficient cancers, perturbs the processing of Okazaki

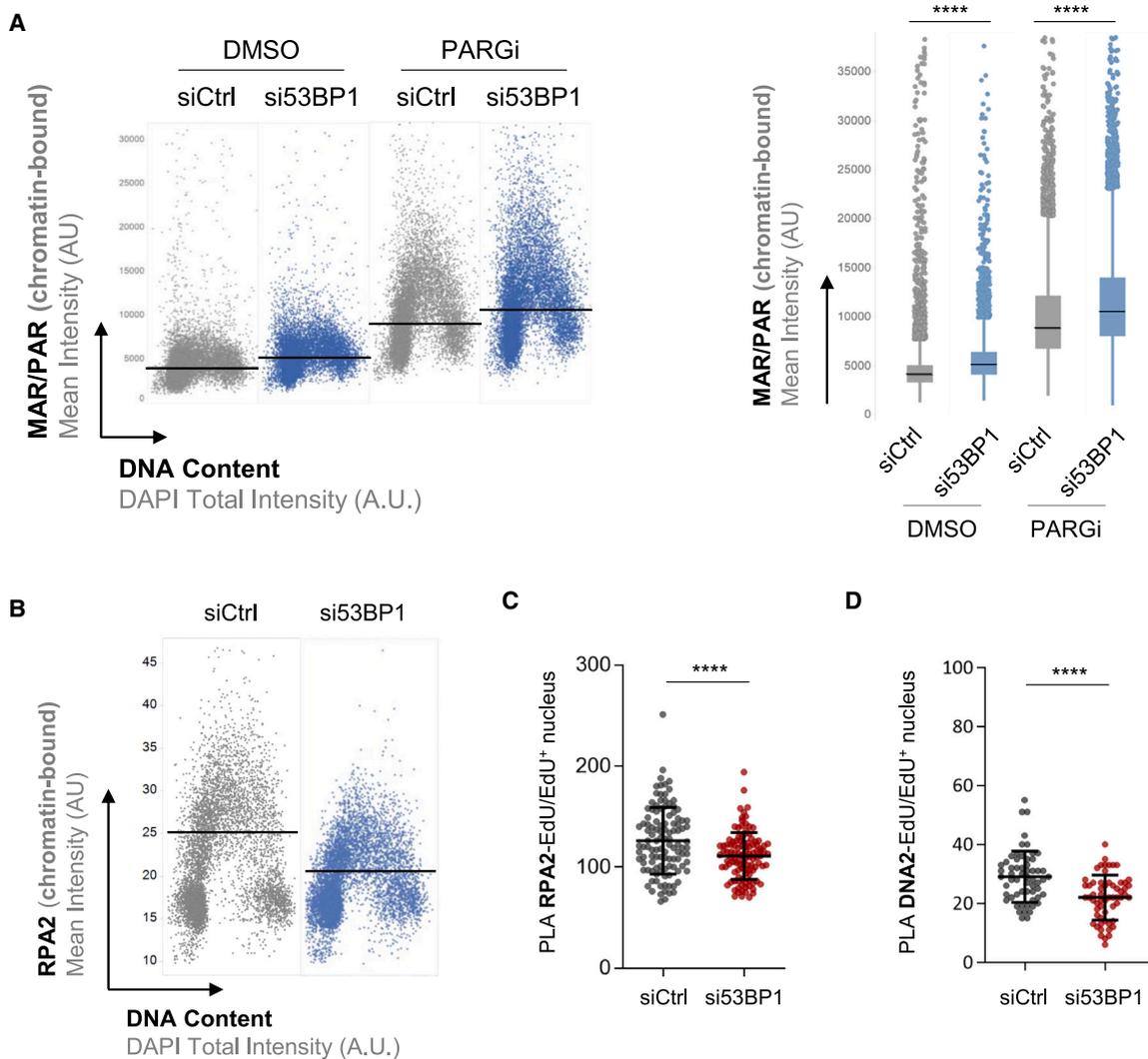


Figure 4. Depletion of 53BP1 perturbs lagging strand processing

(A) QIBC of chromatin-loaded protein mono-ADP-ribosylation (MARylation) or poly-ADP-ribosylation (PARylation). U2OS cells were treated with either control or 53BP1 siRNAs for 72 h and incubated with DMSO or PARGi (10 μ M) for the last 30 min before pre-extraction and cell fixation. Nuclear DNA was counterstained by DAPI. The horizontal lines are medians. Right: quantification of MAR/PAR signals from the experiment on the left. In boxplots, center lines are medians, the boxes indicate the 25th and 75th centiles, and the whiskers indicate Tukey values. p values were determined by one-way ANOVA with Tukey's test. ****p < 0.0001 (n > 10,000 cells from 2 biological replicates).

(B) QIBC of chromatin-loaded RPA2 mean intensity. U2OS cells were treated with either control or 53BP1 siRNAs for 72 h. The horizontal lines are medians (n > 5,000 cells from 2 biological replicates).

(C and D) Representative scatterplot of SIRF experiments of RPA (C) or DNA2 (D) in U2OS cells depleted of 53BP1 (si53BP1) or left untreated (siCtrl) for 48 h. The significance was derived from the Mann-Whitney statistical test. Bars represent the mean \pm SD. ****p < 0.0001 (n = 3 biological replicates).

fragments, a process that is modulated by 53BP1 loss.^{40–42} The Okazaki fragment binding activity of 53BP1 may specifically be involved in the sensitivity of BRCA1-deficient cells to PARPi and may be linked to DNA replication gaps.

Limitations of the study

Our findings show that while the GAR-Tudor domain of 53BP1 is sufficient to bind RNA, it is not able to bind RNA-DNA chimeras in living cells. This indicates that binding of 53BP1 to RNA-DNA chimeras requires an additional RNA-binding domain or an additional

53BP1 domain that recruits the protein in the vicinity of Okazaki fragments. The GAR-Tudor domain alone could also adopt a conformation that affects its binding to RNA-DNA chimeras. Ideally, structural biology approaches will facilitate the defining of the minimal 53BP1 domain able to interact with RNA-DNA chimeras and an associated mutant. Moreover, even if our data indicate that 53BP1 interacts with Okazaki fragments on the lagging strand, the binding of 53BP1 to any RNA-DNA primers including the one present on the leading strand cannot be completely ruled out. Furthermore, our study does not provide visual insight with

electron microscopy of the effect of 53BP1 depletion on Okazaki fragments.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.celrep.2023.113412>.

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AUTHOR CONTRIBUTIONS

M.L., C.B., and P.U. performed the CLIP, SIRF, and 2C experiments. J.J. performed the gel shift and SPR experiments. G.C. and K.S. performed the QIBC experiments. P.B., D.B., and X.V. performed recombinant protein production and associated molecular cloning. S. Mennour and S. Martineau performed all other molecular cloning and laser micro-irradiation experiments. S.V., P.U., K.S., M.L., and C.B. conceived the experiments and analyzed data. S.V. coordinated studies and wrote the manuscript with the help of P.U.,

C.B., K.S., and S.L. and with input from all other authors. S.V., K.S., and P.B. acquired funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-53BP1	Bethyl	Cat# A300-272A; RRID: AB_185520
Mouse monoclonal anti-GFP	Roche	Cat# 11814460001; RRID: AB_390913
Mouse monoclonal anti- α Tubulin	GeneTex	Cat# GTX628802; RRID: AB_2716636
Mouse monoclonal anti-HuR	Santa Cruz Biotechnology	Cat# sc-5261; RRID: AB_627770
Rabbit polyclonal anti-Histone 3	Abcam	Cat# ab1791; RRID: AB_302613
Mouse monoclonal anti-53BP1	Millipore	Cat# MAB3802; RRID: AB_11212586
Mouse monoclonal anti-PCNA	Santa Cruz Biotechnology	Cat# sc-56; RRID: AB_628110
Rabbit polyclonal anti-Biotin	Bethyl	Cat# A150-109A; RRID: AB_67327
Rabbit monoclonal anti-Poly/Mono-ADP Ribose	Cell Signaling Technology	Cat# 83732; RRID: AB_2749858
Human anti-PCNA	Immuno Concept	Cat#2037; RRID: AB_3073928
Mouse monoclonal anti-RPA32/RPA2	Abcam	Cat# ab2175; RRID: AB_302873
Rabbit polyclonal anti-DNA2	Abcam	Cat# ab220883; RRID: AB_3073918
Mouse polyclonal anti-Biotin	Jackson ImmunoResearch Labs	Cat# 200-002-211; RRID: AB_2339006
Mouse monoclonal anti- γ H2A.X	Abcam	Cat# ab26350; RRID: AB_470861
Rat monoclonal anti-BrdU	Abcam	Cat# ab6326; RRID: AB_305426
Chemicals, peptides, and recombinant proteins		
PDD 00017273 (PARGi)	Tocris	Cat# 5952
Critical commercial assays		
Duolink <i>In Situ</i> Detection Reagents FarRed	Sigma-Aldrich	DUO92013
Quick-RNA MidiPrep kit	Zymo Research	R1056
Experimental models: Cell lines		
Human: U2OS cells	ATCC	RRID:CVCL_0042
Human: RPE1 cells	ATCC	RRID:CVCL_4388
Human: HeLa cells	ATCC	RRID:CVCL_030
Human: HEK293T cells	ATCC	RRID:CVCL_0063
Human: A2058 cells	ATCC	RRID:CVCL_1059
Oligonucleotides		
siRNA targeting 53BP1	Dharmacon	SMART pool
siRNA targeting 53BP1	Ambion	s14314
siRNA-1 targeting PRIM1	Ambion	s11050
siRNA-2 targeting PRIM1	Ambion	s11052
siRNA targeting FEN1	Dharmacon	SMART pool
DNA sequence: G1_forward: GGGGACAAGTTT GTACAAAAAAGCAGGCTTCGAAAACCTGTAT TTTCAGGGC	This paper	N/A
DNA sequence: Hs53BP1_F4: GAAAACCTGTA TTTTTCAGGGCGCCCCGGGATCCCCTTCACA GACTGG	This paper	N/A
DNA sequence: Hs53BP1_R4: GGGGACCACT TTGTACAAGAAAGCTGGGTCCTCGAGTCAGG CACGAGGACTTTCT	This paper	N/A
DNA sequence: Hs53BP1-MutGAR_F: AGGGC CGCCACC	This paper	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
DNA sequence: Hs53BP1_R4: GGGCGGCCCTT TCGCCCTTTCCCTTAGGCGTGA CTGGAGCC	This paper	N/A
SC1 RNA sequence: 5'-GCU GCGGUGUGG AAGGAGUGGUCGGGUUGCGCAGCG-3'	Vasilyev et al. ⁴³	N/A
Software and algorithms		
Macro on Fiji software	PICT-IBiSA Imaging Facility (Orsay, FR)	ImageJ: RRID: SCR_003070
Prism	GraphPad	RRID: SCR_002798
Spotfire	Tibco	RRID: SCR_008858

RESOURCE AVAILABILITY

Lead contact

Further information and requests for reagents should be directed to Lead Contact Stéphan Vagner (stephan.vagner@curie.fr).

Materials availability

All unique/stable reagents generated in this study are available from the [lead contact](#) without restriction.

Data and code availability

- Data have been deposited to Mendeley data and are publicly available as the date of publication: <https://doi.org/10.17632/bkmyyt2h4y.1>
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cells were grown in 5% CO₂ humidified incubator at 37°C. U2OS, RPE1, HeLa and HEK293T cells were grown in DMEM supplemented with 10% FBS and 2 mM L-glutamine. A2058 cells were grown in MEM supplemented with 10% FBS and 2 mM L-glutamine.

METHOD DETAILS

siRNA and plasmid transfections

siRNA and plasmid transfections were performed, respectively, using Lipofectamine RNAiMAX (Life Technologies, #13778150) or JetPEI (Polyplus Transfection, #101000053) according to manufacturer's instructions.

CrossLinking and immunoprecipitation (CLIP)

Cells were irradiated with UV-C light at 254nm, lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% Sodium deoxycholate), supplemented with RNase OUT (1 μL/1mL, Invitrogen, #10777019) and protease inhibitor cocktail, and immunoprecipitated with 50 μL of Dynabead Protein G (ThermoFisher, #10009D) using 5 μg of rabbit anti-53BP1 or 5 μg of mouse anti-GFP antibodies for each condition. Immunocomplexes were washed twice with RIPA-S buffer (50 mM Tris-HCl pH 7.4, 1 M NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 0.5% Sodium deoxycholate) and once with PNK buffer (20 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 0.2% Tween 20). Then, crosslinked nucleic acids were radiolabeled using ATP, [³²P] (PerkinElmer, #BLU502A250UC) and T4 polynucleotide kinase (PNK, ThermoFisher, #EK0032), washed twice with RIPA-S buffer and once with PNK buffer. Protein-RNA complexes are eluted and separated by SDS-PAGE. Then the gel was dried and complexes were visualized by exposure to a phosphorimager screen. In the case of nucleic acids extraction, RNA-proteins complexes were transferred onto nitrocellulose membrane 0.45 μm and complexes were visualized by exposure to a phosphorimager screen. The nitrocellulose membrane was cut out at the suspected molecular weight of the radiolabeled protein of interest. Nitrocellulose pieces were treated with proteinase K (1 mg/mL, ThermoScientific, #EO0491) in 200 μL of PK buffer (100 mM Tris-HCl pH 7.4, 50 mM NaCl, 10 mM EDTA), once without urea and then once with urea 7 M. Nucleic acids were purified with phenol/chloroform/isoamyl alcohol 25:24:1 (Sigma) and then precipitated overnight by adding Glycoblue (ThermoFischer, #AM9515), sodium acetate and 100% ethanol. After centrifugation and washes (80% ethanol), nucleic acids pellet was resuspended in water and divided into three conditions. One condition without treatment, one with RNase A treatment (1 μL at 10 mg/mL, ThermoScientific, #EN0531) and one with DNase treatment (1 μL at 2U/μL, Invitrogen,

#AM2238). Nucleic acids were separated using denaturing polyacrylamide gel (TBE-Urea gel, 6%). The gel was dried and nucleic acids were visualized by exposure to a phosphorimager screen.

Complex capture (2C)

Cells were irradiated with UV-C light at 254 nm and lysed in HMGN150 buffer (20 mM HEPES pH 7.5; 150 mM NaCl; 2 mM MgCl₂; 0.5% Nonidet P-40; 10% Glycerol). The lysate is then treated or not with RNase A (15 μL for 1 mg of proteins). A fraction (2%) of the input is kept to load on an SDS-PAGE to control the amount of proteins. The quick-RNA Midiprep kit is used for RNA extraction for the 2C Method. The RNA concentration of the eluate is measured using NanoDrop (ThermoScientific). 50 μg RNA is treated with RNase I (500U, Invitrogen, #AM2295) for 40 min at 30°C. A mix of LDS 4X (ThermoFischer, #NP0007) + Reducing reagent 10X (ThermoFischer, #NP0009) are added to the samples and a Western blot analysis is performed.

Quantitative image-based cytometry (QIBC)

QIBC was performed as previously described.⁴⁴ Briefly, images were acquired with a ScanR inverted microscope high-content screening station (Olympus, IX81) equipped with wide-field optics, air objective, fast excitation and emission filter-wheel devices for DAPI, FITC, Cy3, and Cy5 wavelengths, an MT20 illumination system, and a digital monochrome Hamamatsu ORCA-Flash 4.0LT CCD camera. Images were acquired in an automated fashion with the ScanR acquisition software (Olympus, 3.2.1). 81–100 images were acquired containing at least 5,000 cells per condition. Acquisition times for the different channels were adjusted for non-saturated conditions in a 12-bit dynamic range, and identical settings were applied to all the samples within one experiment. Images were processed and analyzed with ScanR analysis software. First, a dynamic background correction was applied to all images. The DAPI (Sigma-Aldrich) signal was then used to generate an intensity-threshold-based mask to identify individual nuclei as the main objects. This mask was then applied to analyze pixel intensities in different channels for each individual nucleus. For analysis of 53BP1 foci, additional masks were generated by segmentation of individual 53BP1 spots with spot-detector modules included in the software. Each focus was defined as a subobject, and this mask was used to quantify pixel intensities in foci. After this segmentation of objects and subobjects, the desired parameters for the different nuclei or foci were quantified with single parameters (mean and total intensities, area, foci count, and foci intensities). These values were then exported and analyzed with TIBCO Spotfire Software, version 11.1, to quantify absolute, median, and average values in cell populations and to generate all color-coded scatterplots. Within one experiment, similar cell numbers were compared for the different conditions (at least 4,000–5,000 cells), and for visualization, low x axis jittering was applied (random displacement of objects along the x axis) to make overlapping markers visible.

In situ protein interaction with nascent DNA replication forks (SIRF)

U2OS were seeded on Millicell EZ SLIDE 8-well glass (Millipore, #PEZGS0816) 48h before experiment and then incubated with 25 μM EdU for 10 min. After treatment, cells were washed with cold PBS, pre-extracted with cold CSK (10 mM PIPES, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, protease inhibitor cocktail), permeabilized with cold CSK-T buffer (CSK buffer supplemented with 2% Triton X-100) during 5 min at 4°C, treated or not with 1 mg/mL of RNase A for 5 min at 37°C and then fixed with 4% PFA in PBS for 20 min at room temperature. After fixation, slides were placed in a humid chamber, and incubated with a click-it reaction (10 μM biotin azide, 100 mM sodium ascorbate, 2 mM CuSO₄, 1 μM Alexa Fluor 488 Azide, in PBS in this order) at room temperature for 1 h. After the click reaction, slides were washed with PBS and blocked with blocking buffer (10% goat serum and 0.1% Triton X-100 in PBS) for 1 h at room temperature. Primary antibodies (anti-53BP1, anti-biotin, anti-PCNA) were diluted in blocking buffer and incubated at 4°C overnight. Then a PLA procedure is performed using Duolink *In situ* Detection Reagents FarRed. Slides were imaged using Leica 3D upright deconvolution microscope with a CoolSNAP HQ camera, at the PICT-IBiSA Imaging Facility in Orsay, and analyzed using a semi-automatic macro on Fiji software.

Cloning

GFP-RBMX and GFP-53BP1 plasmids correspond to RBMX or 53BP1 sequence inserted in pcDNA 6.2 (Vivid Colors pcDNA6.2/N-EmGFP-GW/TOPO Mammalian Expression Vector, ThermoFisher). The GFP-53BP1-Cter corresponds to the 1235 to 1972 encoding sequence of 53BP1. The Hs53BP1-1288-1659 (HA-NLSx3-GAR-Tudor) encoding sequence was cloned by site-directed recombination using the Gateway technology (Life Technologies). The ORF was amplified by PCR using specific primers: G1_forward; Hs53BP1_F4; Hs53BP1_R4. The resulting PCR products were recombined during BP reaction with pDONO207 to generate Entry clones used for further LR reactions with pGGWA.⁴⁵ The triple mutation (Arg1396Lys, Arg1398Lys, Arg1401Lys) was generated by “rolling-circle” PCR using specific primers as previously described⁴⁶: Hs53BP1-MutGAR_F and Hs53BP1-MutGAR_R.

53BP1 fragments purification

GST-Hs53BP1-1288-1659 fragments (GAR-Tudor or GAR(3K)-Tudor) purifications were performed as described.⁴⁷ Fractions eluted from Superdex 75 10/300 GL (Cytiva) were directly subjected to TEV cleavage at the ratio of 1 TEV for 10 GST-Hs53BP1 fragment (w/w). After 2 h incubation at 20°C, the mixture was incubated with Glutathione Sepharose 4B (Cytiva) and Ni-NTA Superflow (Qiagen). The Hs53BP1 fragment without its GST tag was recovered in the flow through and concentrated using Vivaspinn concentrator (Sartorius).

Electrophoretic mobility shift assay (EMSA)

Appropriate concentrations of each protein and trace amount (25 nM) of ATTO700-labeled oligonucleotides were incubated with binding buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM KCl, 0.1 mM DTT, 5% glycerol, 0.01 mg/mL BSA, pH 7.5) in a 20 μ L final reaction volume at room temperature for 45 min. The incubated samples were loaded on an 8% polyacrylamide (37.5:1 acrylamide/bis-acrylamide) non-denaturing gel. Gels were imaged using an Odyssey scanner.

Surface plasmon resonance (SPR)

The interaction between nucleic acids (ligands) and the proteins (analytes) were measured by the surface plasmon resonance (SPR) technique using a ProteOn XPR36TM (Bio-Rad Laboratories). NLC Sensor chips precoated with NeutrAvidin (Bio-Rad Laboratories) were conditioned with 30- μ L injections of 50 mM NaOH and 1.0 M NaCl at a flow rate of 30 μ L/min in both ligand and analyte flow directions. Immobilization was performed in the running buffer (10 mM Tris-HCl, 200 mM KCl at pH 7.5, Tris-HCl, 0.01% v/v Tween). The temperature of the chip surface was maintained at 25°C. The all biotinylated oligonucleotides were immobilized one by one onto a single channel of the six channel NLC sensor chip. Each oligonucleotide construct was immobilized onto an individual ligand channel by flowing the constructs at a concentration of 10–40 nM for 60 to 240 s injection. Binding to the chip surface resulted in approximately 60–100 response units (RU). The chip was rotated to the analyte flow direction and stabilized by flowing 60 μ L analyte buffer three or more times, as required. Analyte (purified protein or buffer) was applied at various concentrations through the analyte channels at 100 μ L/min for an association phase of 150 s, followed by a dissociation phase (running buffer only) of 600 s. The surface was regenerated with 18 μ L of 0.5% SDS, at a flow rate of 30 μ L/min followed by flowing 60 μ L running buffer in the analyte direction at 100 μ L/min. The resultant sensorgrams for each analyte interaction were analyzed with ProteOn software (Bio-Rad Laboratories). Kinetic parameters for first the dissociation constant (k_d) and then the association constant (k_a) were estimated by fitting the kinetic data according to the 1:1 Langmuir model and values of KD were then calculated (using the ratio k_d/k_a).

DNA damage induction before IF

Micro-irradiation: the confocal microscope TCS SP5 Leica allowed DNA damage induction in cultured living U2OS cells using laser at 405 nm.

QIBC of native BrdU

To detect ssDNA in parental strands, the cells were labeled with 10 mM BrdU for 24 h and then released into a fresh growth medium for 1h prior to fixing cells. Cells were fixed, permeabilized, and processed for immunofluorescence staining as described in the IF and QIBC protocol. To detect BrdU in the native state, fixed cells were incubated with anti-BrdU antibody for 90 min followed by Alexa 594 secondary antibody at room temperature. To assess bulk BrdU incorporation levels as well as expose further BrdU epitopes, fixed cells were treated with DNase prior to immunostaining (data not shown). Briefly, individual coverslips were incubated with 10 units of RNase-Free DNase (M6101, Promega) in 1X Reaction Buffer (400 mM Tris-HCl [pH 8.0 at 25°C], 100 mM MgSO₄, 10 mM CaCl₂) for 40 min at room temperature. After that, coverslips were washed once with Stop Buffer (20 mM EGTA [pH 8.0 at 25°C]) in PBS and further washed two-three times with PBS. Coverslips were then equilibrated in Antibody diluent media for 15 min at room temperature before processing for immunofluorescence staining as described earlier.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed on GraphPad Prism (Version 8.0) and were carried out using either a one-way ANOVA with Tukey's test or a Mann-Whitney U test, as indicated in the figure legends.

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Supplemental information

**53BP1 interacts with the RNA primer from Okazaki
fragments to support their processing
during unperturbed DNA replication**

Melissa Leriche, Clara Bonnet, Jagannath Jana, Gita Chhetri, Sabrina Mennour, Sylvain Martineau, Vincent Pennaneach, Didier Busso, Xavier Veaute, Pascale Bertrand, Sarah Lambert, Kumar Somyajit, Patricia Uguen, and Stéphan Vagner

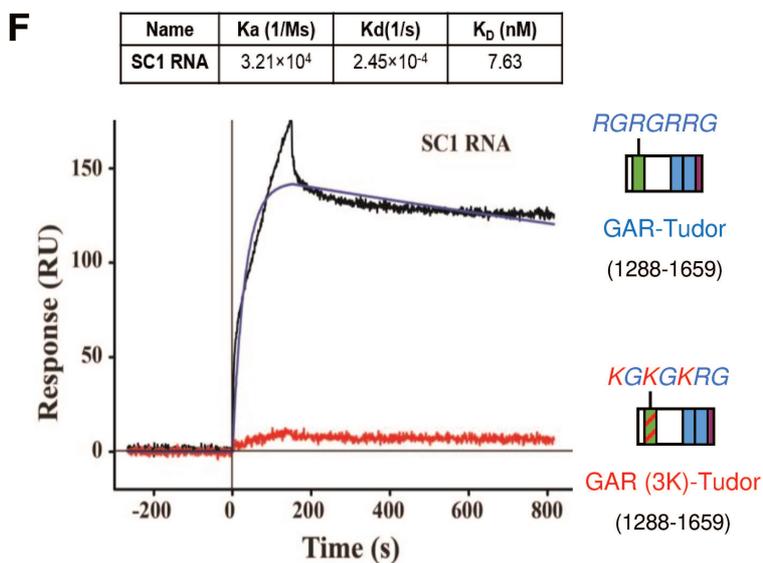
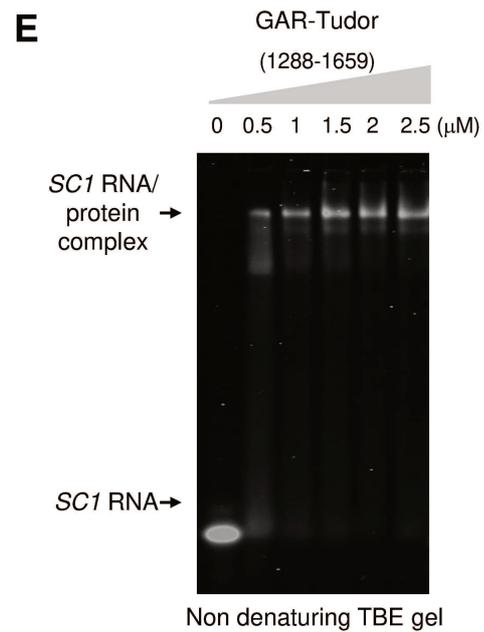
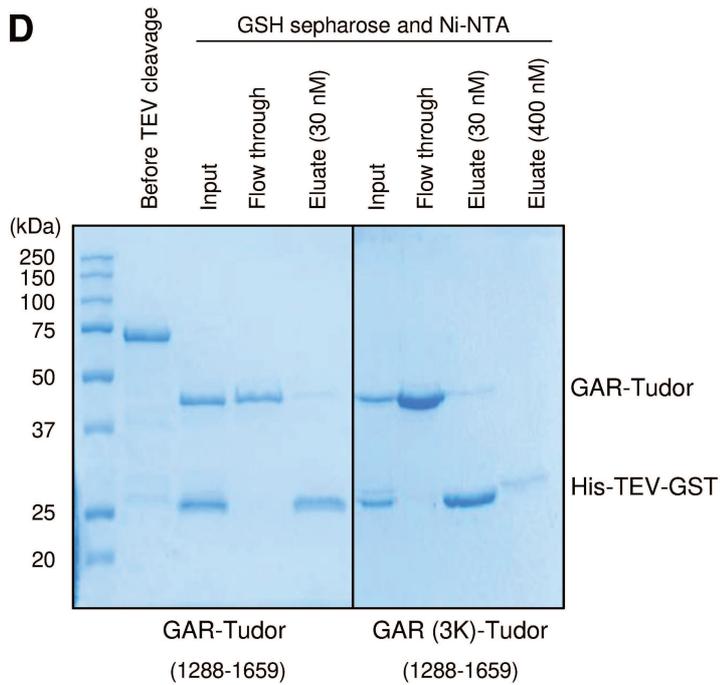
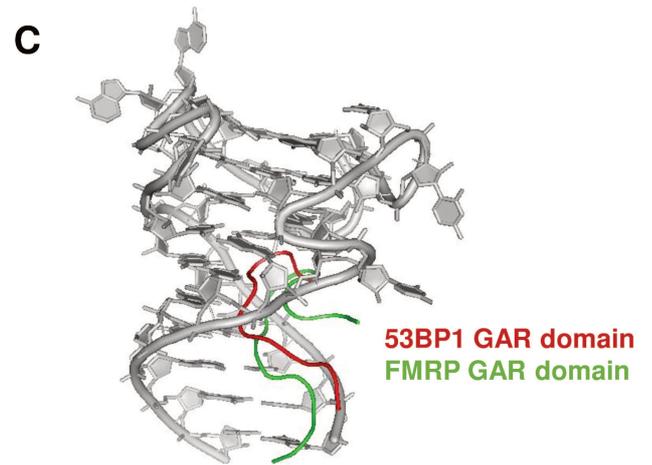
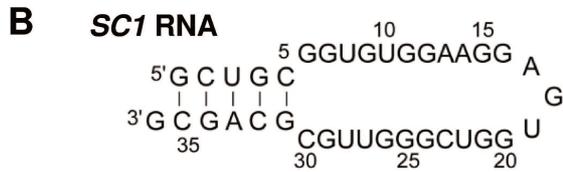
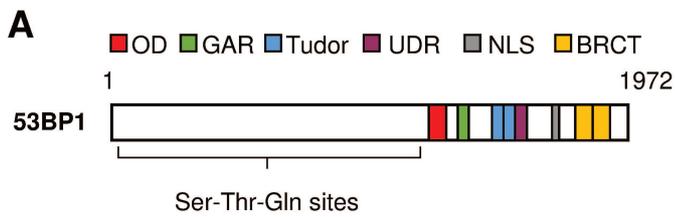


Figure S1. 53BP1 binds RNA *in vitro*

(A) 53BP1 domains map.

(B) Structure of *SC1* RNA.

(C) The predictive docked structure of *SC1* RNA (grey in colour) with GAR-TUDOR RGG motif (PRGRGRRGRP, red in colour) superimposed with X-ray crystal structure of the FMRP RGG peptide (GDGRRRGGGGRGQG, green in colour)–*SC1* RNA complex. The RGG motif of GAR-TUDOR and FMRP protein showed comparable binding mode with *SC1* RNA in the duplex–quadruplex junction.

(D) Gel purification of GAR-Tudor and GAR (3K)-Tudor recombinant proteins.

(E) Electrophoretic Mobility Shift Assay (EMSA) performed using purified recombinant 53BP1 containing the Tudor and the GAR domains incubated with a 5'-end fluorescently labelled synthetic 36 nt-long *SC1* RNA.

(F) Surface plasmon resonance (SPR) experiments with the indicated recombinant proteins and a 5'-end biotinylated *SC1* RNA.

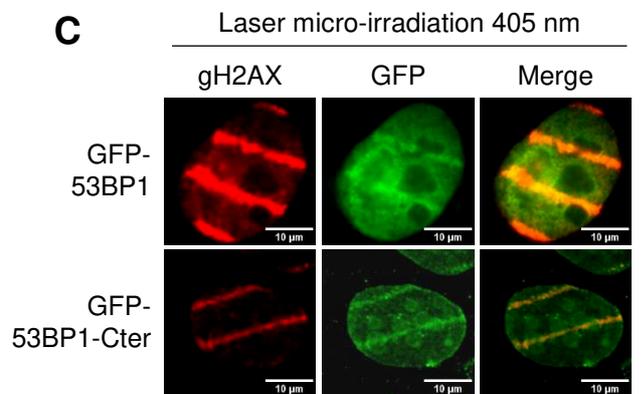
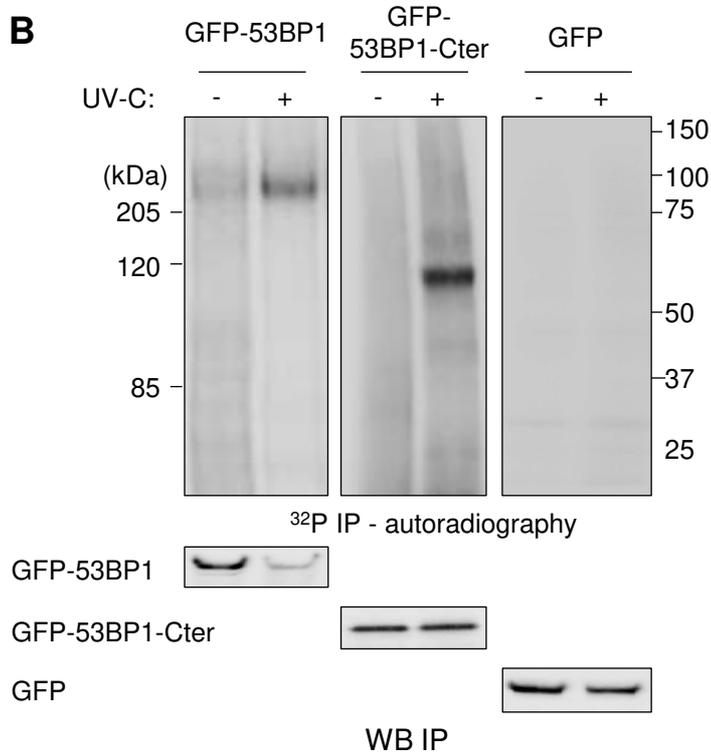
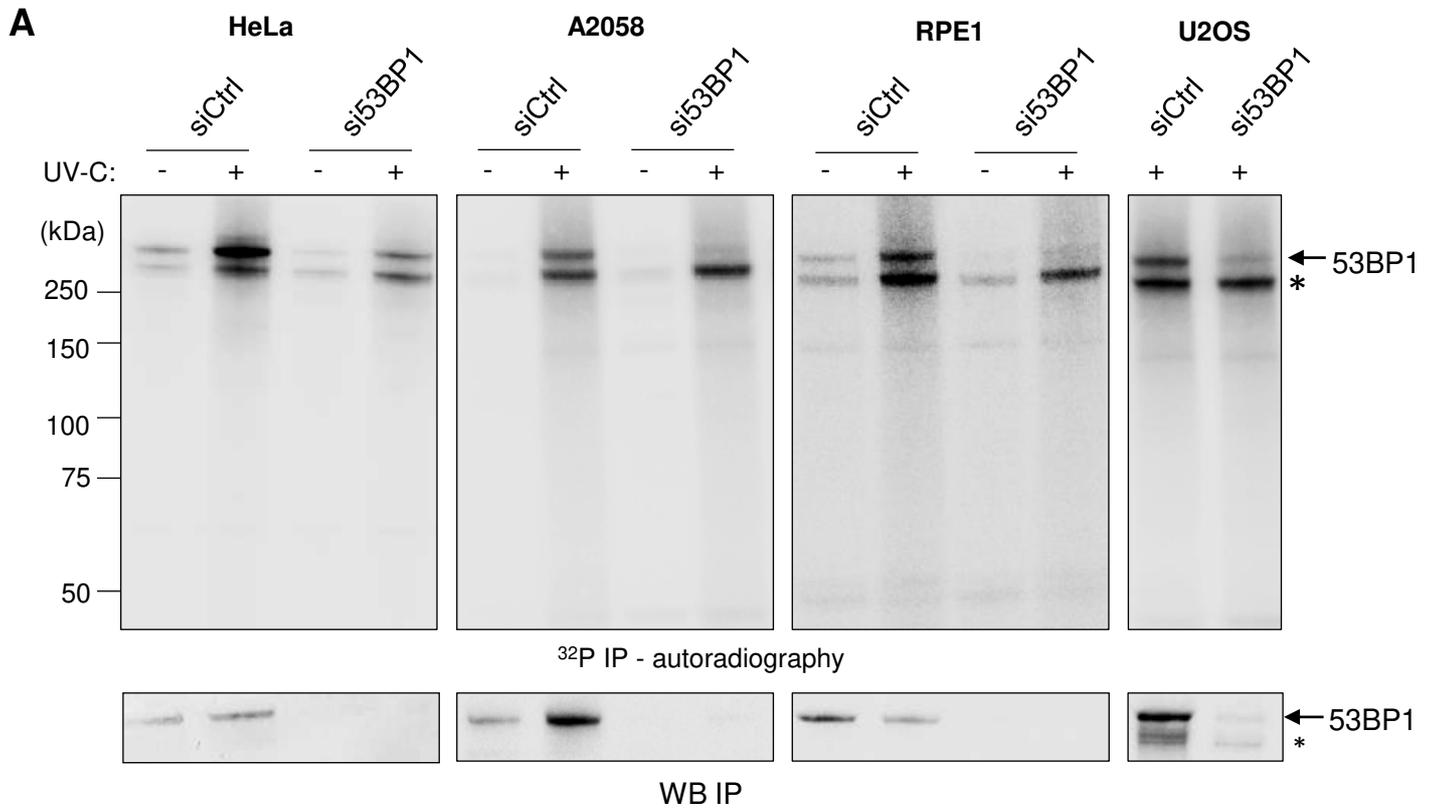


Figure S2. Related to Figure 1. 53BP1 directly interacts with RNA

(A) Autoradiography of 53BP1-nucleic acid complexes in UV-C treated (+) or untreated (-) cervix cancer cells (HeLa), melanoma cells (A2058), osteosarcoma cells (U2OS) or epithelial cells (RPE1) depleted of 53BP1 (si53BP1) or left untreated (siCtrl). The asterisk indicates a non-specific band (*), the arrow indicates the position of 53BP1-nucleic acid complexes.

(B) CLIP experiments performed in HEK29T cells transfected with the plasmids expressing the GFP-53BP1 proteins or the C-terminal part of 53BP1 (1235 – 1972 amino acids) or GFP alone. 10% of the IP was analysed by western blot to confirm immunoprecipitation (lower panel).

(C) Immunofluorescence was performed in U2OS cells expressing the GFP-53BP1 or GFP-53BP1-Cter (1235 – 1972 amino acids) proteins. The cells were irradiated with laser micro irradiation at 405 nm (stripes) and stained with anti- γ H2AX (red) or GFP (green) antibodies.

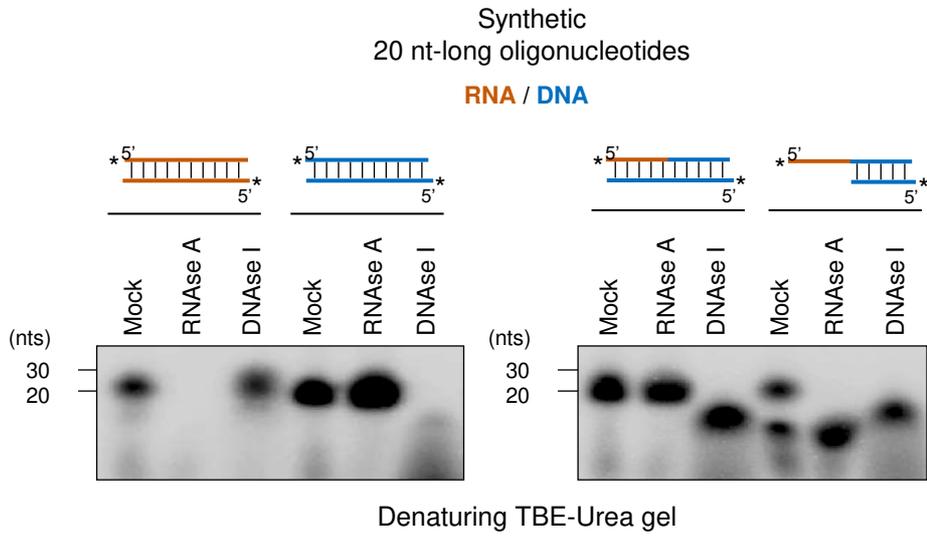


Figure S3. Related to Figure 2. Enzymatic activities of RNase A and DNase I

In vitro assays of RNase A and DNase I activities on synthetic 20 nt-long oligonucleotides. The synthetic oligonucleotides were ^{32}P -5' end labelled on both strands.

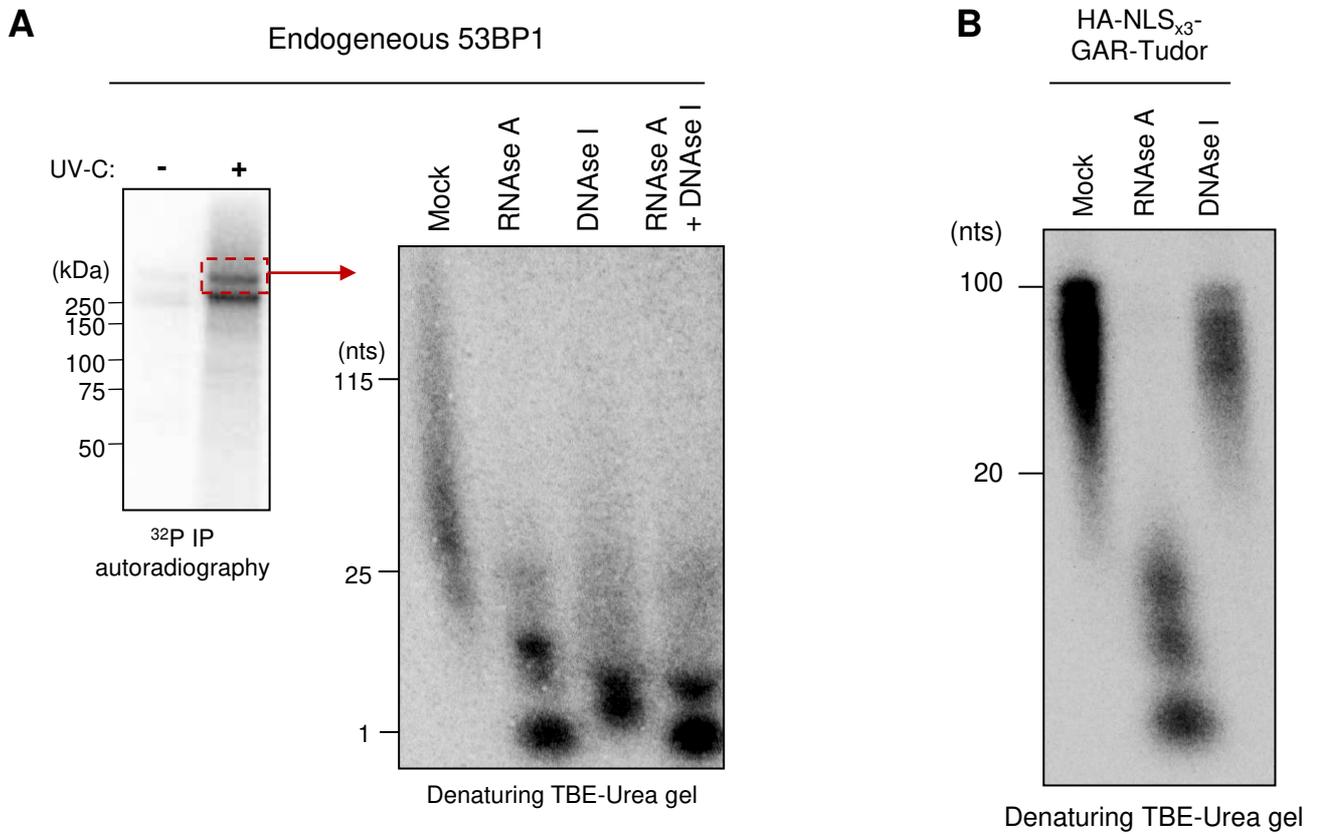


Figure S4. Related to Figure 2. Endogenous 53BP1 binds RNA-DNA chimeras while the GAR-Tudor fragment does not

(A) Nucleic acid extraction after CLIP procedure of endogenous 53BP1 in A2058 cells.

(B) Nucleic acid extraction after CLIP procedure of HA-NLS_{x3}-GAR-Tudor fragment in transfected HEK293T cells.

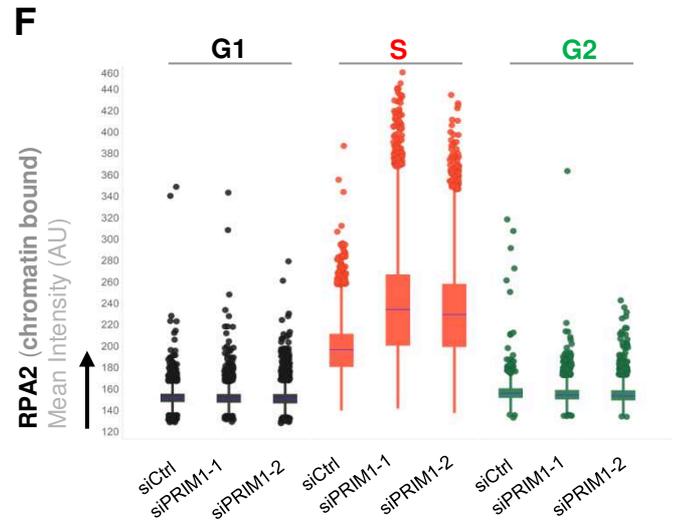
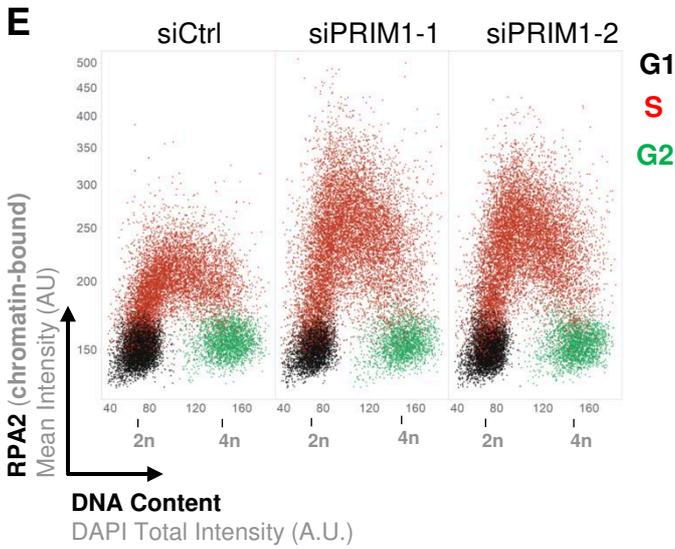
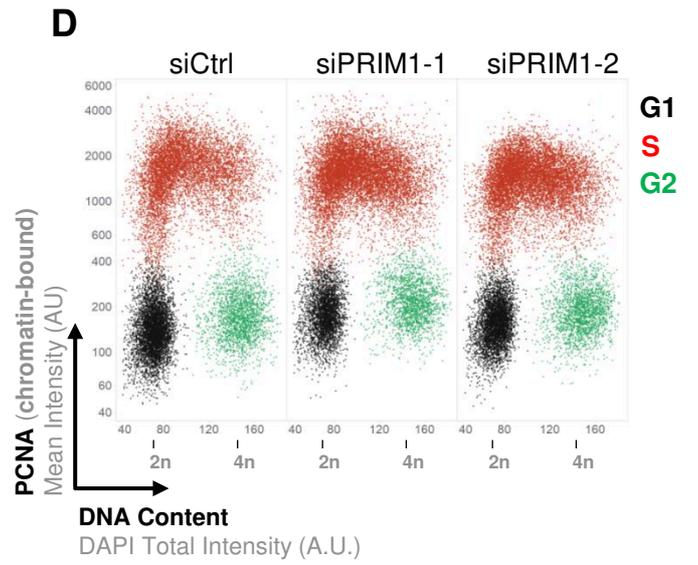
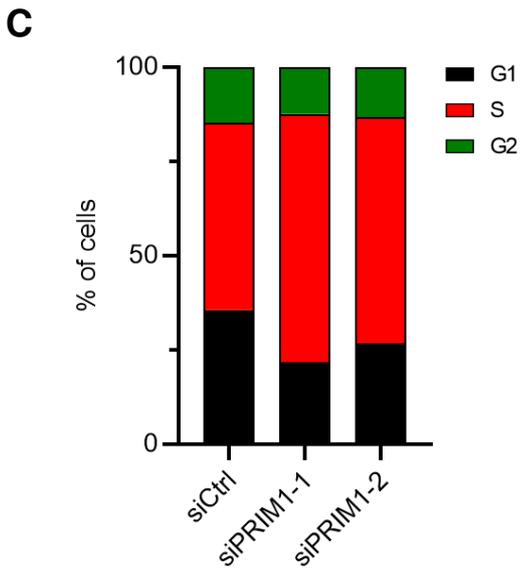
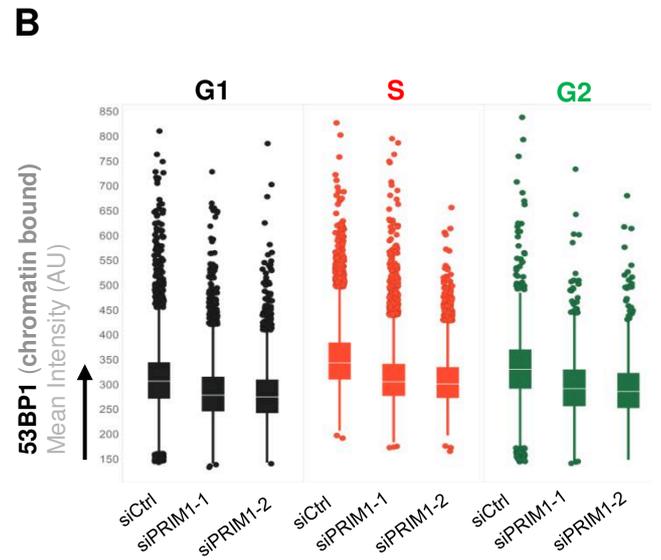
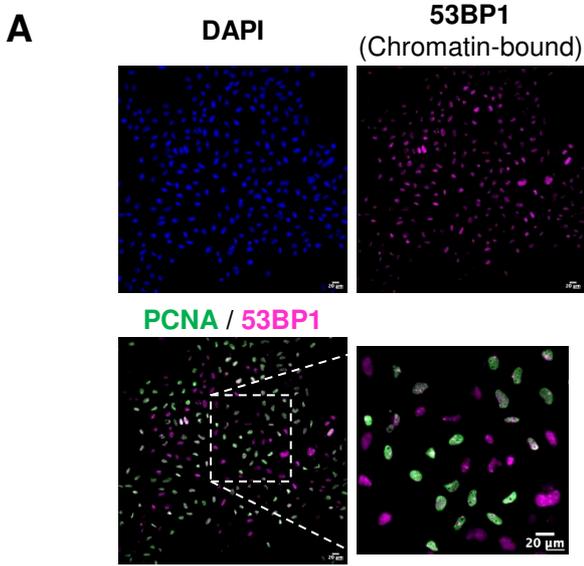


Figure S5. Related to Figure 3. Impact of PRIM1 depletion on the association of 53BP1, PCNA and RPA2 to chromatin

(A) Representative image of pan-chromatin and focus forming 53BP1 quantified in QIBC. Scale bar, 20 μm .

(B) QIBC-based quantification of chromatin loaded 53BP1 mean intensity in different phases of cell cycle (from experiment in Figure 3A). U2OS cells were treated with indicated siRNAs for 48 hours. In box plots, centre lines are medians, the boxes indicate the 25th and 75th centiles, the whiskers indicate Tukey values. P values were determined by one-way ANOVA with Tukey's test, **** $p < 0.0001$ ($n > 10,000$ cells from combined all cell cycle stages per condition from 3 biological replicates).

(C) Percentages of cells from different cell cycle of the QIBC in (B).

(D) Cell cycle stages were gated based on PCNA mean intensity versus DAPI signals.

(E-F) QIBC of RPA2 chromatin loading in cells treated with indicated siRNAs for 48 h. Nuclear DNA was counterstained by DAPI. $n > 10,000$ cells per condition from 2 biological replicates. In box plots, centre lines are medians, the boxes indicate the 25th and 75th centiles, the whiskers indicate Tukey values.

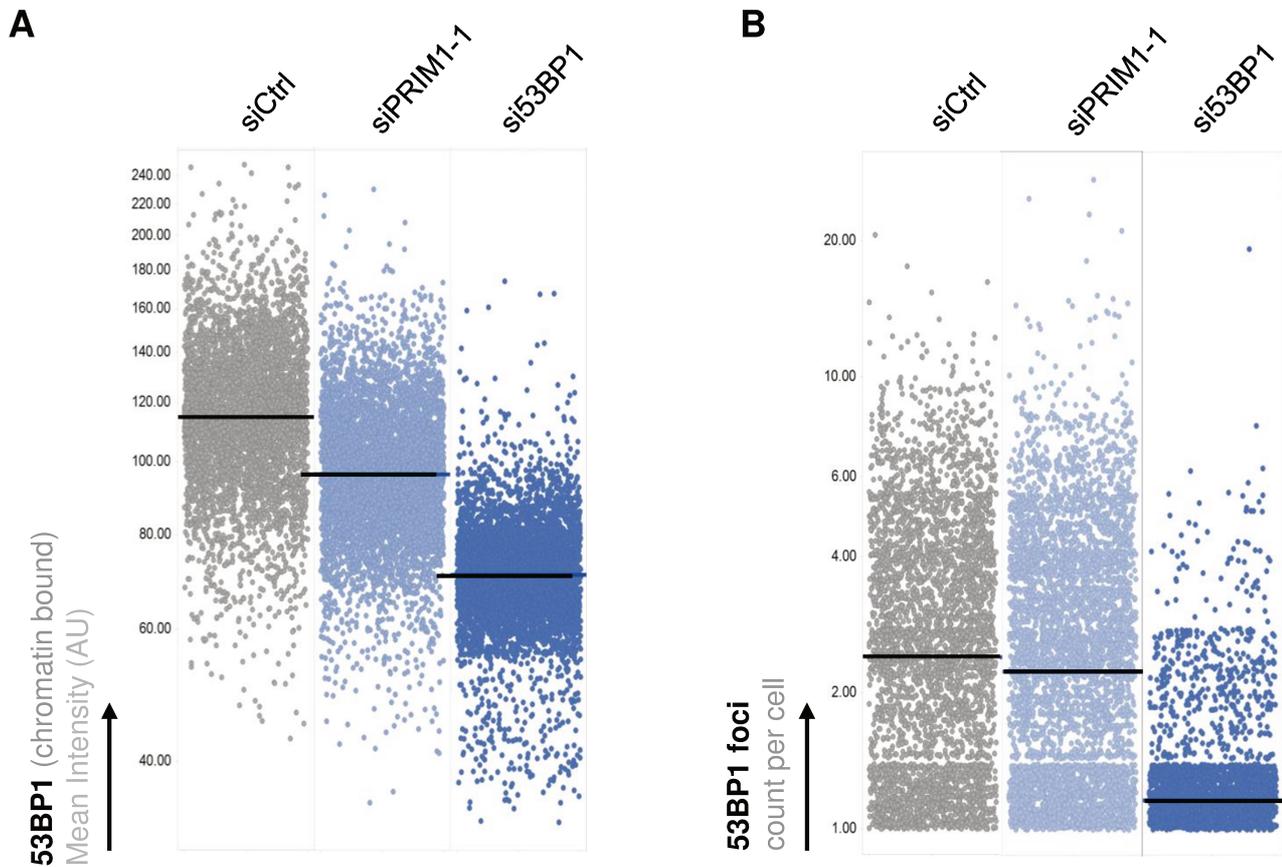


Figure S6. Related to Figure 3. Loss of PRIM1 decreases 53BP1 at the chromatin without impacting the 53BP1 foci assembly

(A) QIBC of chromatin loaded 53BP1 mean intensity. Exponentially growing U2OS cells were treated with PRIM1 siRNAs for 48 h and 53BP1 siRNAs for 72 h. The horizontal lines are median values. $n > 5,000$ cells per condition from minimum of 2 biological replicates.

(B) Quantification of the 53BP1 foci count derived from the QIBC analysis in (A). Average 53BP1 foci/cell are indicated with horizontal lines in black.

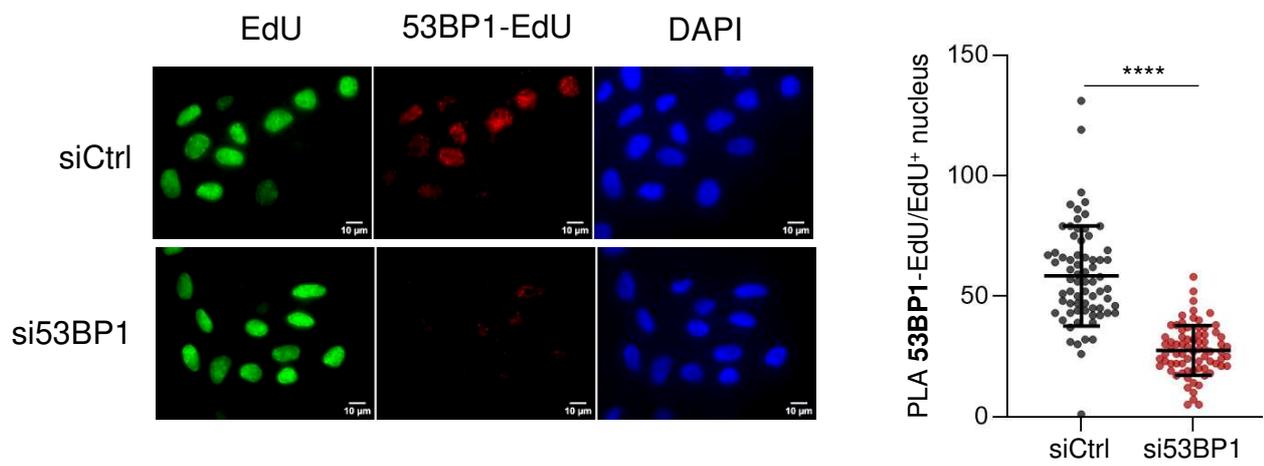


Figure S7. Related to Figure 3. The PLA 53BP1-EdU signal in SIRF experiments is specific to 53BP1

Representative images of 53BP1-EdU SIRF experiments in U2OS cells transfected with indicated siCtrl or si53BP1.

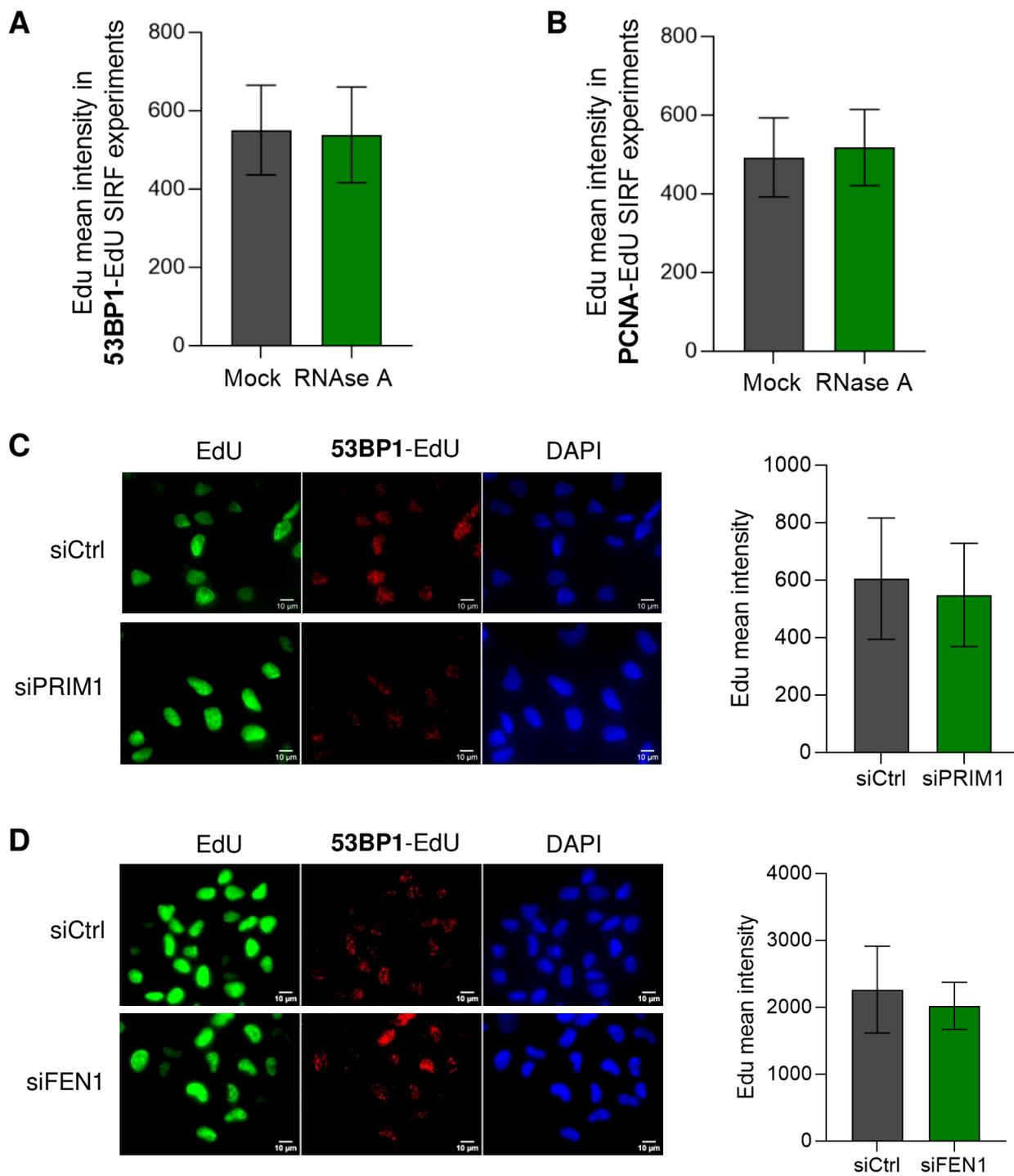


Figure S8. Related to Figure 3. Intensity of EdU incorporation in SIRF experiments

(A-B) EdU mean intensity in 53BP1-Edu (A) or PCNA-Edu (B) SIRF experiments in U2OS cells treated or not with RNase A.

(C-D) Representatives images of 53BP1-Edu SIRF experiments and EdU mean intensity in U2OS cells transfected with indicated siRNAs.

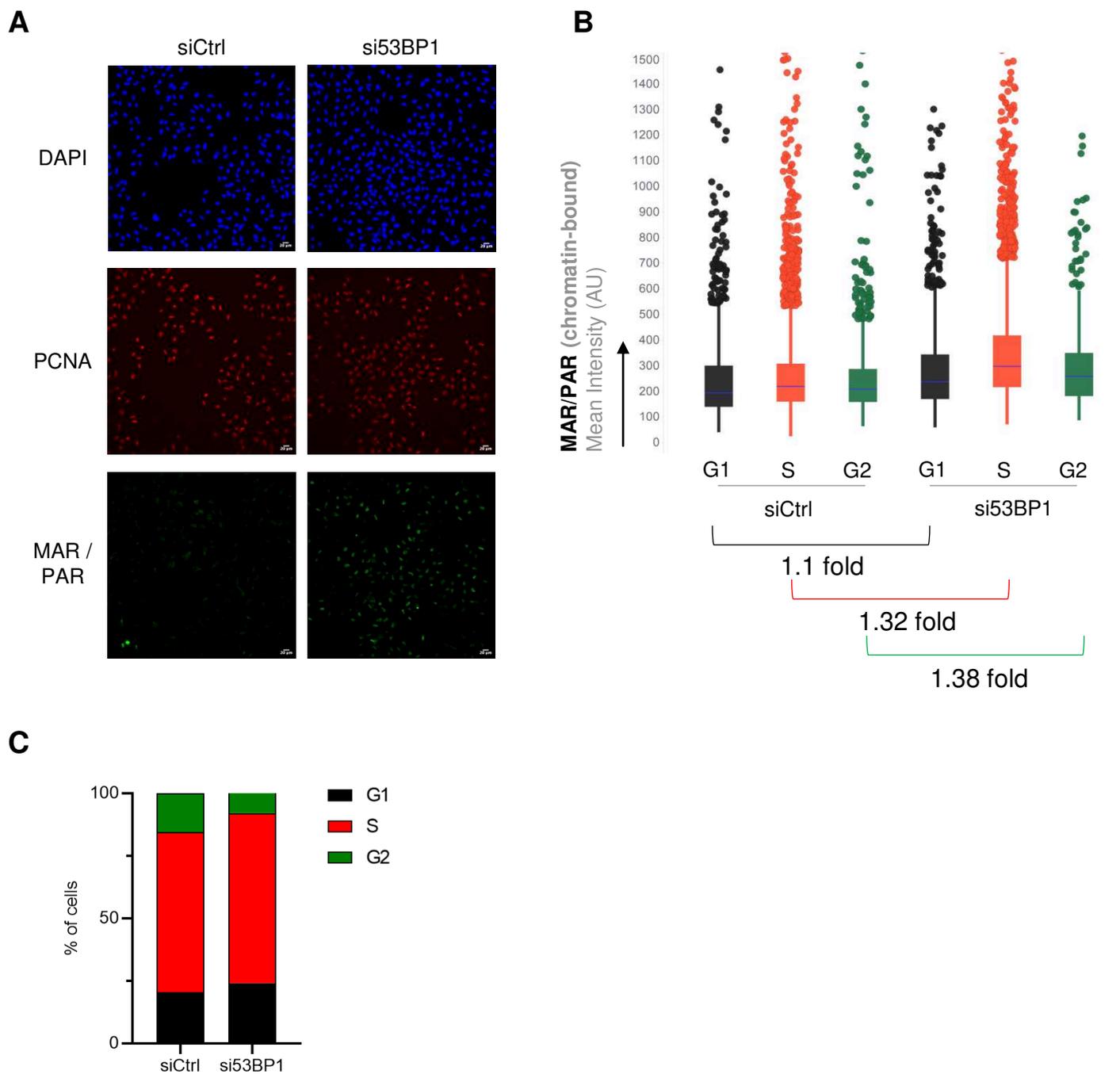


Figure S9. Related to Figure 4. Images and quantifications of MAR/PAR bound to the chromatin

(A) Representative images of PCNA and MAR/PAR bound to the chromatin in QIBC experiment under si53BP1.

(B) QIBC-based quantifications of MAR/PAR signals from the experiment in A. In box plots, centre lines are medians, the boxes indicate the 25th and 75th centiles, the whiskers indicate Tukey values. P values were determined by one-way ANOVA with Tukey's test, **** $p < 0.0001$ ($n > 10,000$ cells from combined all cell cycle stages per condition from 2 biological replicates).

(C) Percentages of cells from different cell cycle from the experiment in A and B.

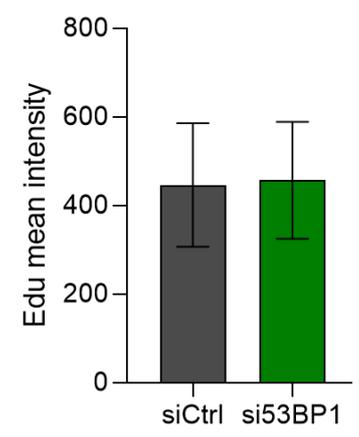
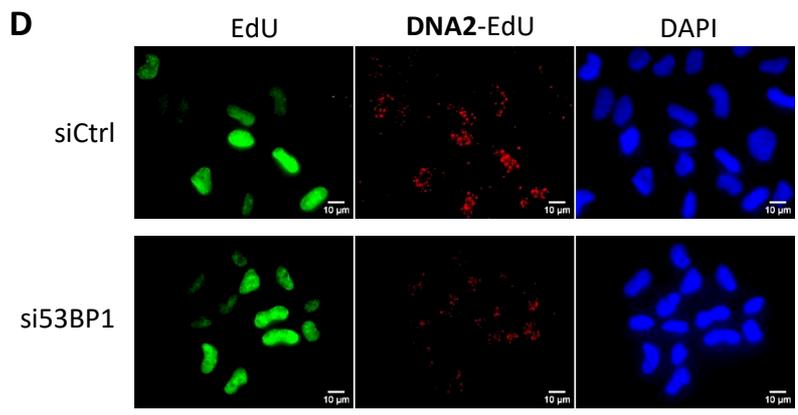
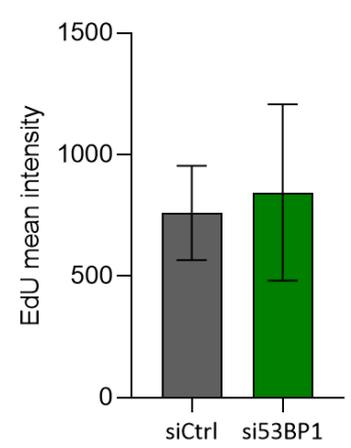
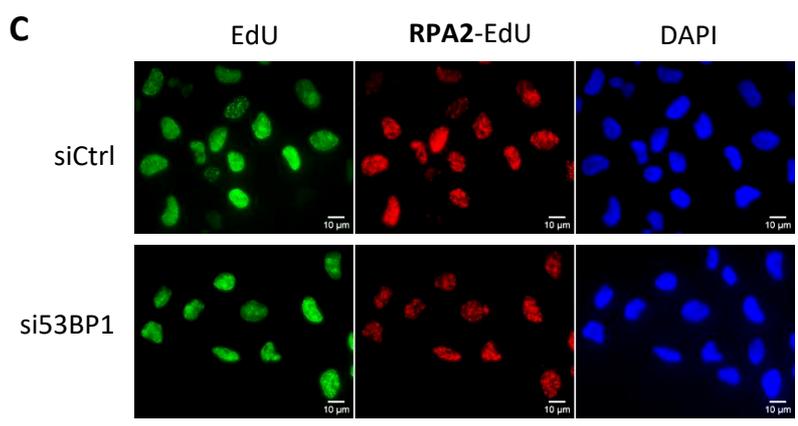
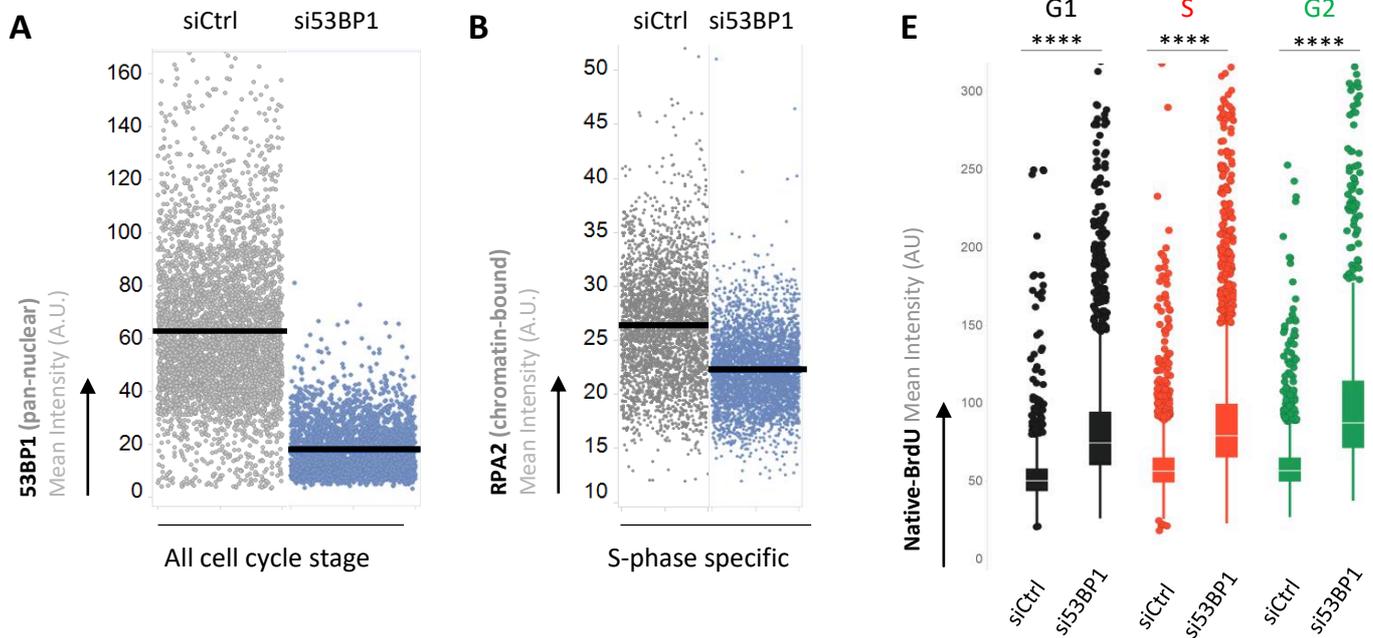


Figure S10. Related to Figure 4. Loss of 53BP1 decreases chromatin-associated RPA2 and increases ssDNA at nascent DNA

(A) QIBC of pan-nuclear 53BP1 mean intensity. Exponentially growing U2OS cells were treated with si53BP1 for 72h. The horizontal lines are median values. $n > 5,000$ cells per condition from minimum of 2 biological replicates.

(B) QIBC of chromatin loaded RPA2 mean intensity. Exponentially growing U2OS cells were treated with si53BP1 for 72h. The horizontal lines are median values.

(C-D) Representative images of RPA2-EdU (C) or DNA2-EdU (D) SIF experiments and EdU mean intensity in U2OS cells transfected with indicated siRNAs for 48 h.

(E) QIBC of ssDNA exposure upon depletion of 53BP1. Parental DNA in replicating U2OS cells was labeled by the addition of 10 μ M BrdU for 24 h followed by a chase into normal medium for 1 h before cell fixing. Cells were fixed and stained with antibodies against BrdU without DNA denaturation to detect parental-strand ssDNA. Box plots of mean BrdU intensity per nucleus is shown. In box plots, centre lines are medians, the boxes indicate the 25th and 75th centiles, the whiskers indicate Tukey values. P values were determined by one-way ANOVA with Tukey's test, **** $p < 0.0001$ ($n > 5,000$ cells from combined all cell cycle stages per condition from 2 biological replicates).

II – Additional results

1. Investigation of the role of 53BP1-Okazaki fragments interaction

53BP1 is involved in the response to replication stress to protect nascent DNA from degradation by nucleases such as MRE11 (Dungrawala *et al.*, 2015; Her *et al.*, 2018; Liu *et al.*, 2020). CLIP and 2C experiments performed in part I (Leriche, Bonnet *et al.*, in revision) were carried out without induced stress, suggesting a new role for 53BP1 during unperturbed DNA replication, in addition to its known one at stalled replication forks.

We first aimed to determine the nature of the RNA that 53BP1 was binding to. We demonstrated that 53BP1 binds to RNA-DNA primers sensitive to RNase A treatment, indicating that the RNA was single-strand. We next wanted to ascertain whether 53BP1 could bind RNA:DNA hybrids, present at the beginning of the elongation of Okazaki fragments. Of note those RNA:DNA hybrids can also be found at sites of transcription-replication conflicts and form R-loops. We used RNase H treatment, which cleaves RNA in RNA:DNA hybrids, and we observed that the nucleic acids bound to 53BP1 is also sensitive to RNase H, indicating that 53BP1 binds RNA:DNA hybrids (**Fig. 1A**). *In vitro* assays further revealed that the GAR-Tudor region of 53BP1 had the ability to bind the RNase H-substrate RNA:DNA hybrids and that the 3K mutation of the GAR domain impaired the binding (**Fig. 1B**). Next, we wanted to ascertain whether the recruitment of 53BP1 to the fork was dependent on RNA. For this purpose, we used the SIRF technique that allows studying protein-protein interactions, close to less than 40nm, at nascent DNA (Roy *et al.*, 2018) (**Fig. 1C**). The thymidine analogue EdU is incorporated into nascent DNA in U2OS cells and then bound by biotin using a click-it reaction. A proximity ligation assay (PLA) is performed to analyse the association between 53BP1 and nascent DNA. The recruitment of 53BP1 to the fork decreased after treatment with RNase A (Leriche, Bonnet *et al.*, in revision) and also with RNase H, without impacting the replisome factor PCNA (**Fig. 1D**).

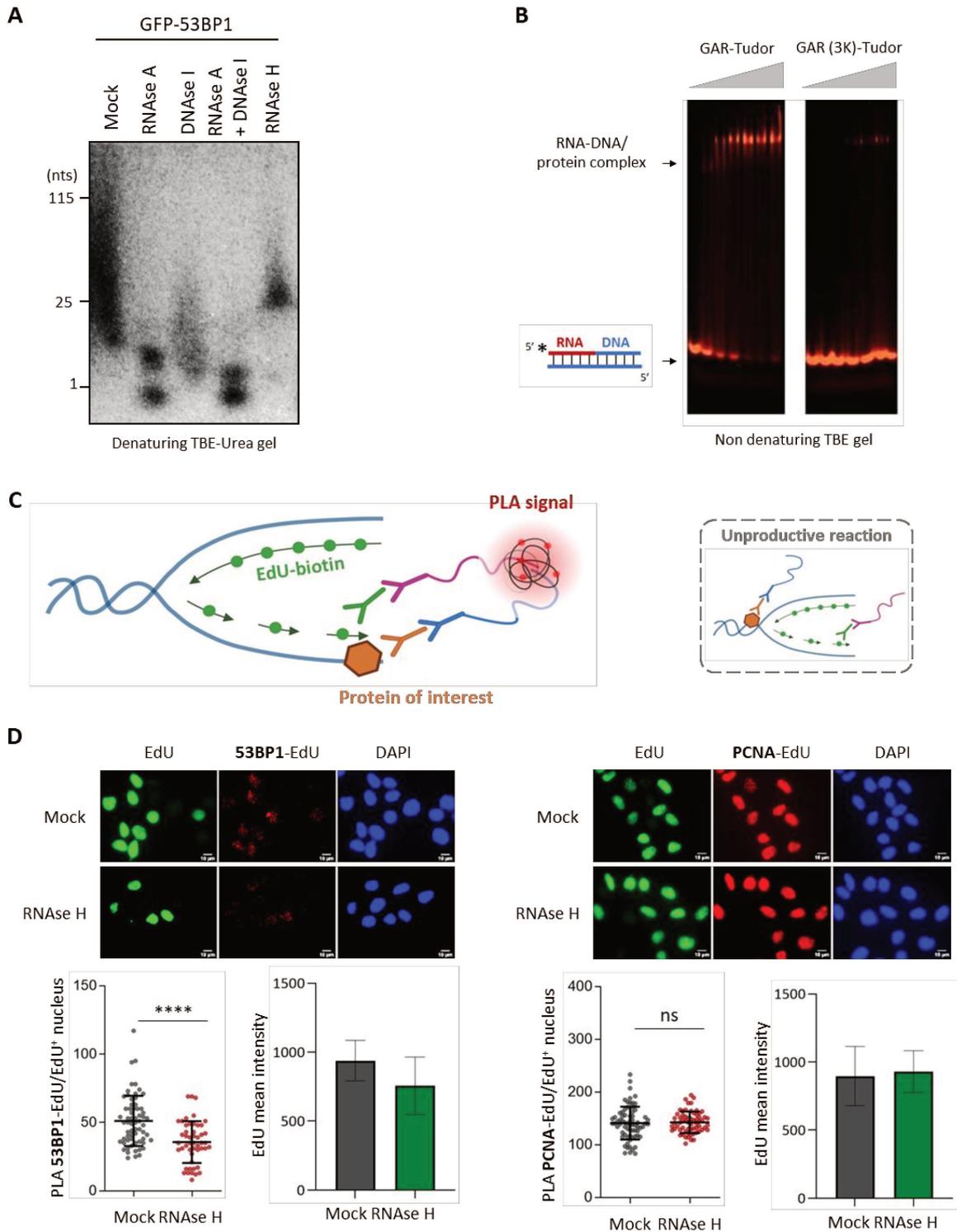


Figure 1. 53BP1 interacts with RNA:DNA hybrids *in vitro* and *in cellulo*

(A) Nucleic acid extraction after CLIP procedure of GFP-53BP1 fragment in transfected HEK293T cells (n=3).

(B) Electrophoretic mobility shift assay (EMSA) performed using purified recombinant 53BP1 containing the Tudor and the GAR domains (0,25mM-1,5mM) incubated with a 5'-end fluorescently labelled synthetic RNA-DNA molecules (25nM).

(C) Schematic representation of the quantitative *in situ* analysis of protein interactions at DNA replication forks (SIRF). U2OS are pulsed with EdU for 10min. Biotin is bound to the EdU using click-it chemistry. A PLA EdU-53BP1 or PCNA is performed.

(D) Representative images and/or analysis of 53BP1-EdU and PCNA-EdU SIRF in U2OS cells permeabilized and treated with RNase H (60U) or left untreated (Mock); PLA signal (red), Alexa Fluor 488-EdU staining (green), and DAPI staining (blue). The EdU mean intensity is measured (right graph). The significance for 53BP1-EdU and PCNA-EdU PLA values (shown as a scatter plot, left graph) was derived from the Mann-Whitney statistical test. Bars represent the mean \pm s.d. **** p<0.0001 (n=3).

This observation could indicate that 53BP1 interacts with the RNA primer of Okazaki fragments early in elongation when the nascent RNA primer is still hybridised to the lagging strand, before being displaced by Pol δ for processing. This suggests that 53BP1 monitors both synthesis and maturation of Okazaki fragments. However, we can't exclude that, even if the experiment is performed without induced stress, 53BP1 interacts with RNA:DNA hybrids present at the replication fork which are induced by the eventual basal level of stress present in cultured cancer cell lines.

In addition, the depletion of 53BP1 leads to the accumulation of PAR chains at the replication fork (Leriche, Bonnet *et al.*, in revision), which constitutes a sensor for immature Okazaki fragments (Hanzlikova *et al.*, 2018). This shows that 53BP1 plays a role in the processing of Okazaki fragments. As a major function of 53BP1 is its anti-resection activity, we can assume that 53BP1 directly interacts with the RNA-DNA primers of the Okazaki fragments under unstressed conditions to protect them and therefore ensure the proper progression of elongation and maturation of the lagging strand.

We next wanted to determine whether the known role of 53BP1 in protecting stalled and reversed forks was actually mediated by its interaction with the RNA primer. First, I used DNA fiber spreading analyses to visualise the impact of 53BP1 depletion on the stability of the replication fork under replication stress (HU treatment). This assay is based on the double incorporation of two thymidine analogues (IdU and CldU) during the same incubation period. The lengths of the DNA having incorporated each

analogue are then measured and the IdU/CldU ratio is calculated (Quinet *et al.*, 2017a) (**Fig. 2A**). If the ratio is less than 1, the fork is degraded while if it is equal to or greater than 1 then the fork is protected. As expected (Liu *et al.*, 2020), the depletion of 53BP1 decreases the IdU/CldU ratio indicating that 53BP1 is involved in the protection of the fork under replication stress (**Fig. 2B**).

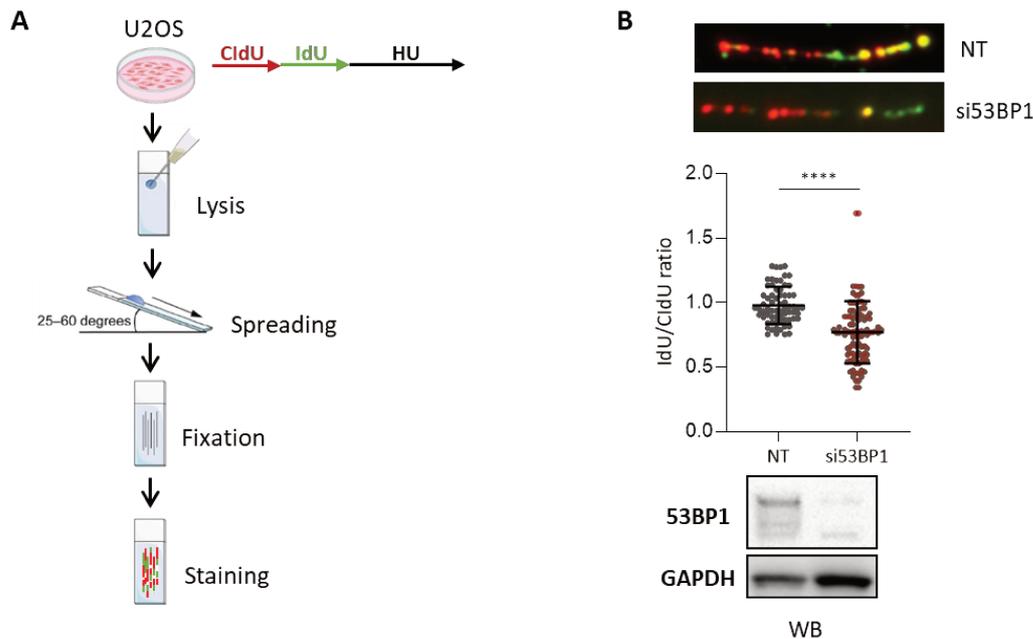


Figure 2. The depletion of 53BP1 induces a degradation of the fork under replication stress

(A) Procedure of the DNA spreading assay. Cells are first incubated with CldU (30 μ M) for 30min, then IdU (250 μ M) for 30min and then treated with HU (4mM, 3h). A drop of U2OS cells is transferred to a microscope slide and lysed. Slide is then tilted at a 25-60 degrees angle to allow DNA spreading down the slide. DNA is then fixed, immunostained and visualized through a fluorescent microscope. Adapted from Quinet *et al.*, 2017a.

(B) The lengths of CldU- and IdU-labeled DNA fibers were measured. The significance is derived from the Mann-Whitney statistical test. **** $p < 0.0001$ (n=2).

In unstressed conditions, the depletion of 53BP1 leads to an accumulation of single-strand DNA (ssDNA) in different phases of the cell cycle, as observed by the QIBC technique (Leriche, Bonnet *et al.*, in revision). We therefore wondered whether the depletion of 53BP1 also resulted in an accumulation of ssDNA at the fork. To investigate this, I developed a novel single-cell approach derived from SIRF, which we named “Repli-Gap”. In U2OS cells, the parental DNA is first labelled with IdU and the nascent DNA is then labelled with EdU. The EdU is biotinylated and a PLA is performed using an anti-biotin antibody and an anti-IdU antibody that specifically

recognises single-strand DNA (**Fig. 3A**). If a signal is observed, it indicates the presence of single-strand DNA in the vicinity of the nascent DNA, which signifies the existence of ssDNA gaps at the replication fork. The presence of ssDNA gaps can be verified through the action of the S1 nuclease, which cleaves single-stranded nucleic acids. We observed that S1 nuclease has a stronger effect in the 53BP1-depleted condition than in the control condition (**Fig. 3B**). This suggests that the loss of 53BP1 induces more single-strand nucleic acids recognized and cleaved by the S1 nuclease. This accumulation of ssDNA could be correlated with the accumulation of PAR chains that we observed when 53BP1 is depleted (Leriche, Bonnet *et al.*, in revision), indicating a defect in Okazaki fragments maturation.

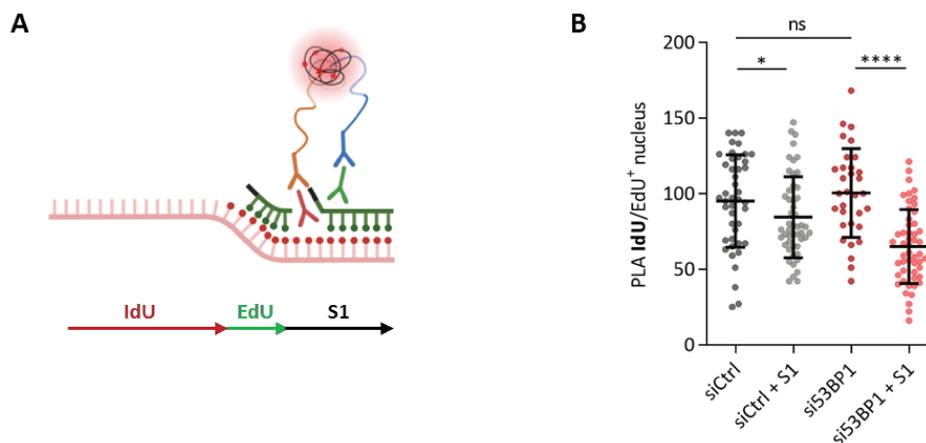


Figure 3. Loss of 53BP1 seems to induce more ssDNA gaps at nascent DNA

(A) Procedure of the Repli-gap. Cells are first incubated with IdU (30 μ M) for 18h to label the parental DNA and next with EdU (100 μ M) for 10min to label nascent DNA. A PLA IdU-EdU is then performed.

(B) Quantification of the PLA IdU-labelled parental ssDNA and EdU-labelled nascent DNA in U2OS cells transfected with an si53BP1 or siCtrl. Cells were treated or not with Nuclease S1 (30U) for 30min. The significance is derived from the Mann-Whitney statistical test. * $p < 0.05$; **** $p < 0.0001$; ns = non significant (n=2).

Taken together, the results suggest that 53BP1 interacts with the RNA-DNA primer of Okazaki fragments at an early stage, which may serve to protect the lagging strand in the event of replicative stress. This is supported by the observation that the loss of 53BP1 leads to fork degradation and appears to result in the accumulation of ssDNA gap at the replication fork.

2. Characterisation of a 53BP1 mutant with altered function in replication

To ensure that the role of the interaction between 53BP1 and Okazaki fragments that we have described is indeed due to the RNA-binding activity of 53BP1, we need to identify a mutant of 53BP1 that loses the binding to the RNA primer. 53BP1 contains a RNA-binding GAR domain composed of a repeated motif of Arginines and Glycines, with the Arginines being methylated by PRMT1 and 5 (Boisvert *et al.*, 2005c; Hwang *et al.*, 2020).

Depletion of the PRMT1 or PRMT5 enzymes reduces the association of 53BP1 with the nascent DNA, and co-depletion further decreases its recruitment (**Fig. 4**). We cannot draw a direct link between the methylation of the 53BP1 GAR domain and its recruitment, nor can we exclude the possibility of PRMT-mediated methylation of intermediate proteins required for 53BP1 recruitment to the replication fork. However, the PRMT- and Arginine methylation-mediated signalling is essential for 53BP1 recruitment to the fork. These observations encourage further in-depth study on the involvement of the GAR domain of 53BP1 in its DNA replication functions.

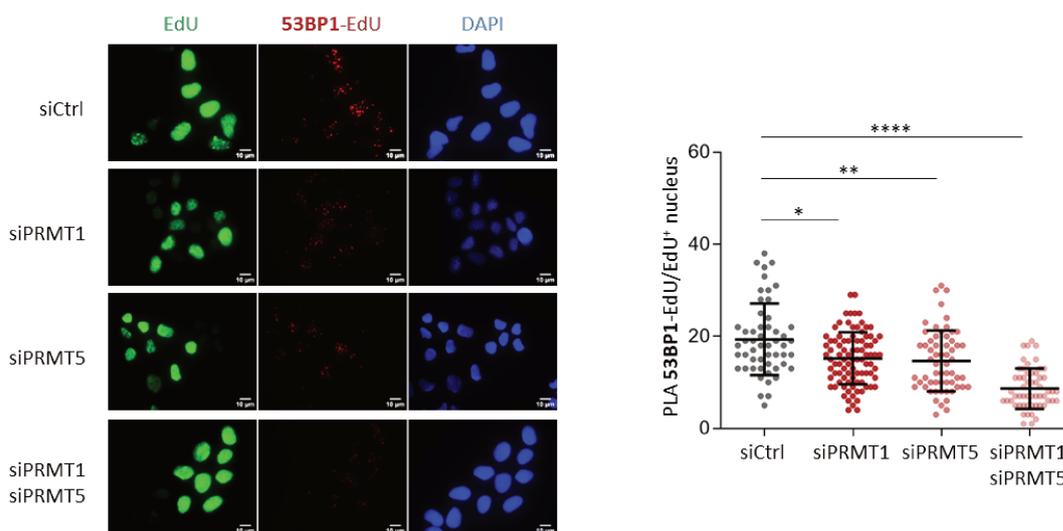


Figure 4. The recruitment of 53BP1 at DNA replication forks is PRMT-dependent

Representative images and analysis of 53BP1-EdU SIRF in U2OS cells transfected with indicated siRNAs. The significance for 53BP1-EdU values (shown as a scatter plot) was derived from the Mann-Whitney statistical test. Bars represent the mean \pm s.d. **** $p < 0.0001$ ($n=3$).

Next, our findings show that while the GAR-Tudor domain of 53BP1 is sufficient to bind RNA, it is not able to bind RNA-DNA primers in living cells (Leriche, Bonnet *et al.*, in revision). This contrasts with our *in vitro* data, where we found that the GAR-Tudor domain alone was sufficient to bind RNA-DNA primers (**Fig. 1C**). This indicates that the GAR-Tudor domain alone could adopt a structural conformation that affects its binding to RNA-DNA primers, since Tudor domain is known to interact with methylated Arginines (Charier *et al.*, 2004; Côté and Richard, 2005). Another hypothesis is that the binding of 53BP1 to RNA-DNA primer could require an additional domain that recruits the protein near the Okazaki fragments. Indeed, using the CLIP technique, I found that the C-terminal region of 53BP1 is the minimal binding region for Okazaki fragments, suggesting that other domains are involved in the interaction with RNA-DNA primers and/or in 53BP1 recruitment to the fork (**Fig. 5A**).

I then deleted the GAR domain and Tudor domain on the 53BP1 protein and performed CLIP experiments to visualize the nucleic acids bound to these constructs. The same profile was obtained for the full length and the GAR-Tudor deleted versions, indicating that the GAR-Tudor region does not appear to be essential for binding to Okazaki fragments (**Fig. 5B**).

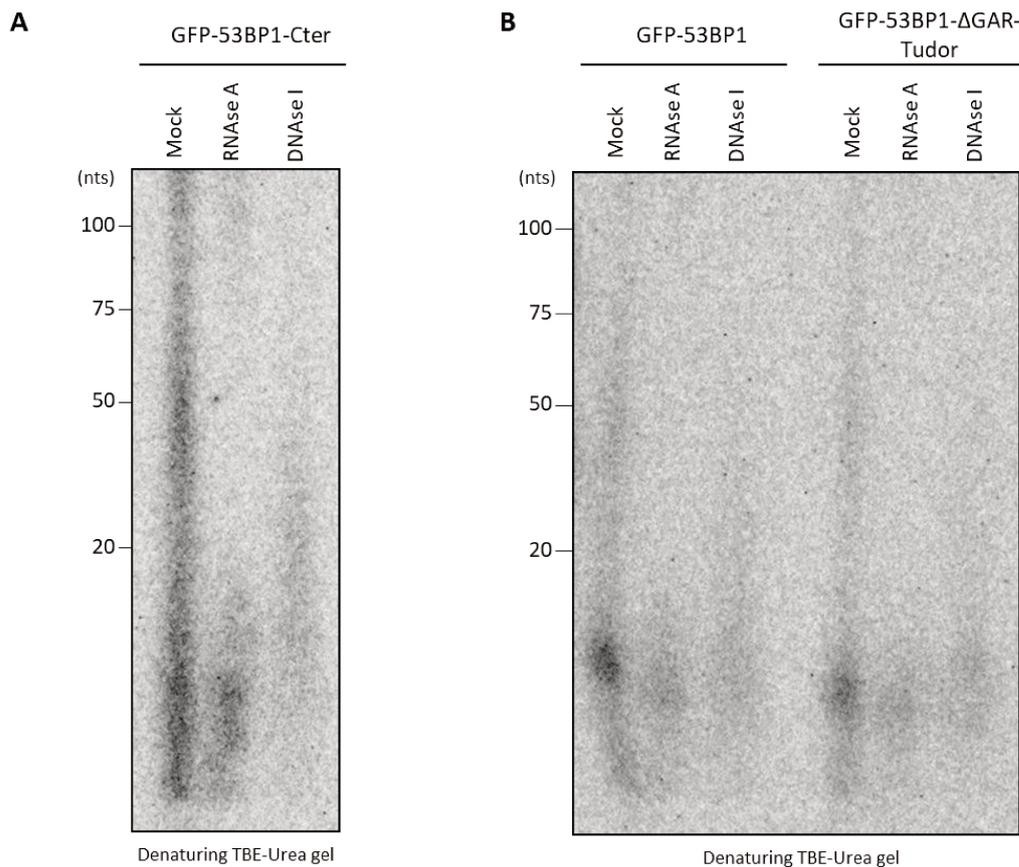


Figure 5. Cter is the minimal region to bind Okazaki fragments and deletion of GAR-Tudor did not impact the binding

(A) Nucleic acid extraction after CLIP procedure of GFP-53BP1-Cter fragment in transfected HEK293T cells (n=3).

(B) Nucleic acid extraction after CLIP procedure of GFP-53BP1 or GFP-53BP1-ΔGAR-Tudor fragment in transfected HEK293T cells (n=2).

However, these observations are not consistent with data obtained with GFP-53BP1-ΔGAR in quantitative microscopy experiments. I performed QIBC experiments in a stable auxin-inducible degron (AID) U2OS cell line depleted for 53BP1 and rescued with different 53BP1 constructs that are transiently transfected. First, in cells expressing OstTIR1, whose promoter is doxycycline inducible, 53BP1 is tagged with a miniAID tag and the addition of auxin in the cell culture media induces the degradation of 53BP1 by the proteasome. The auxin acts as a bridge between the mAID tag and OstTIR1 forming an E3 ligase complex in cells, that ubiquitinates the mAID tag (Natsume *et al.*, 2016) (**Fig. 6A**). Both doxycycline and auxin are required to degrade 53BP1 (**Fig. 6B**).

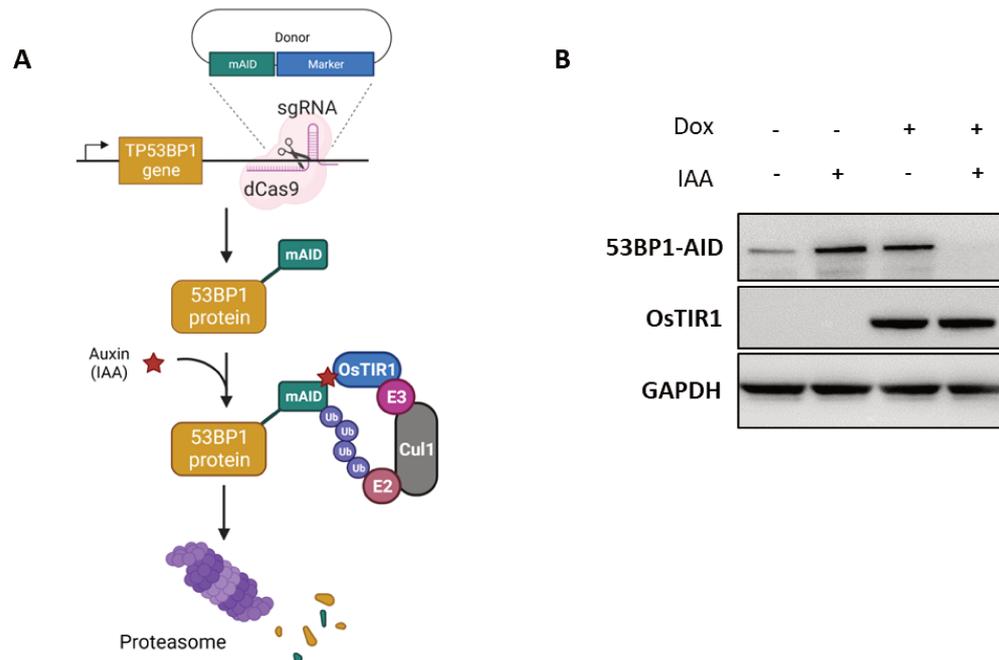


Figure 6. Design of U2OS 53BP1-AID degron cell line

(A) Generation of a U2OS degron cell line. In cells expressing OsTIR1, doxycycline (Dox) inducible, 53BP1 is tagged with a mAID tag. When auxin (IAA) is added, it will act as a bridge between the mAID tag and OsTIR1 and then an E3 ligase complex is formed in cells. This complex ubiquitinates the AID tag, which induces the degradation of the protein by the proteasome. Adapted from Natsume et al., 2016.

(B) Western blot to validate the degron cell line. Both doxycycline, to express OsTIR1, and auxin are required to degrade 53BP1.

The 53BP1 protein in these constructs is tagged with GFP but the presence of copper in the click-it reaction, required for QIBC to detect replicating cells, degrades fluorescent proteins (Garcia *et al.*, 2012). I therefore optimized the QIBC to conduct a copper-safe QIBC, using the Click-iT Plus EdU Cell Proliferation kit (Invitrogen), which relies on the application of a modified Alexa dye and a copper protectant. I observed that deletion of the GAR domain appears to decrease the association of 53BP1 with chromatin, to the same level as the N-terminal control, which is not recruited to chromatin as it lacks a NLS (**Fig. 7A**). Moreover, the deletion of the GAR domain seems to induce the degradation of the replication fork, as visualized by the DNA spreading technique (**Fig. 7B**).

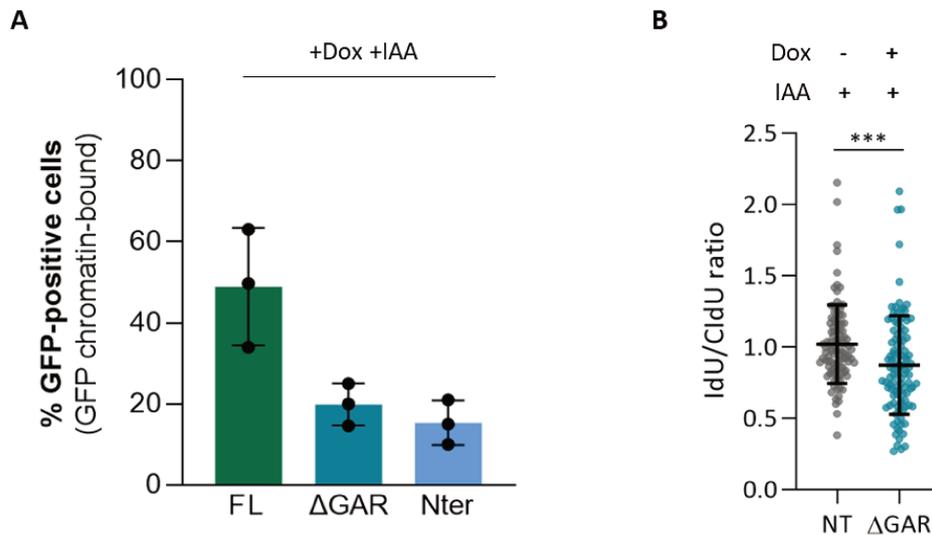


Figure 7. The deletion of the GAR domain reduces the association of 53BP1 to the chromatin and induces the degradation of the replication fork

(A) QIBC-based quantification of chromatin-bound GFP. U2OS 53BP1-AID cells were treated with doxycycline and auxin to deplete endogenous 53BP1 and then transiently transfected with a full length (FL) version, a GAR-deleted version or Nter without NLS version (n=3).

(B) DNA Spreading assay is performed and the length of CldU- and IdU-labeled DNA fibers were measured. U2OS 53BP1-AID were treated with auxin and treated or not with doxycycline. Then the cells were transfected with a GAR-deleted version of 53BP1 or non transfected (NT). The significance is derived from the Mann-Whitney statistical test. *** p<0.001 (n=1).

Of note, unlike the GAR-deleted version, the mutant 3R>3K of the GAR domain does not exhibit the same phenotype in QIBC and DNA spreading (data not shown), indicating that this mutation does not sufficiently impact the structure of 53BP1 (Lysines and Arginines are both positively charged amino acids), even though the 3K mutation is commonly used to mutate GAR domains of RBPs.

Altogether, the GAR domain seems to play a role in the functions of 53BP1 at the replication fork. However, the domains involved in the interaction with RNA are still unclear.

DISCUSSION AND PERSPECTIVES

During my thesis, we characterised 53BP1 as a novel RNA-binding protein that interacts with a specific substrate composed of a short 5' RNA fragment directly followed by a longer 3' DNA fragment. Taken with additional experiments to study the association of proteins with nascent DNA during the S-phase, we concluded that 53BP1 directly interacts with the RNA primer of Okazaki fragments, where the RNA hybridises with the template DNA at the beginning of elongation, or as a flap structure during maturation of the lagging strand. However, the residues/domains of 53BP1 involved in this RNA-binding activity are not yet clearly defined. We particularly focused on the GAR domain, a known RNA-binding domain. The deletion or mutation of the GAR domain does not impact various function of 53BP1 such as foci formation, class switch recombination and anti-resection activity (Lottersberger *et al.*, 2013; Pryde *et al.*, 2005), while it appears to affect the replication functions of 53BP1, suggesting a dissociation of 53BP1 roles mediated by its domains. We thus assume that the GAR domain is important for 53BP1 to bind RNA-DNA primers at the replication fork, however our *in vitro* and *in cellulo* data show that the GAR domain is likely not the only player and other regions are directly or indirectly involved in the RNA-binding activity. Interestingly, a region located in the N-terminal part of 53BP1 may interact with RNA (Queiroz *et al.*, 2019) thus the impact of mutations in the N-terminal part on the RNA-binding activity could also be explored. These observations demonstrate the structural complexity of 53BP1 with domains possessing different functions that may or may not be synergistic. Additional validations are required, and a more in-depth structural biology study would help to define the RNA-binding residues and therefore to identify a mutant of 53BP1 that loses the interaction with RNA.

The factors involved in the signalling and regulation of this interaction remain unknown. We found that 53BP1 is recruited to the fork in a PRMTs-dependent manner. It is therefore possible that methylation of the GAR domain by PRMTs is involved in the signalling. Additionally, a negative regulator of 53BP1 is the RNA-binding protein TIRR. TIRR interacts with the Tudor domains of 53BP1 to prevent its recruitment to chromatin and also to regulate the 53BP1-p53 interaction. It would be interesting to determine whether TIRR also regulates the 53BP1-RNA interaction, for example by masking RNA-binding residues near the Tudor domains.

By identifying this 53BP1 interaction, we have also discovered a new role in DNA replication. Under normal conditions, 53BP1 interacts with the RNA-DNA primers of Okazaki fragments throughout their synthesis to assist the maturation. Okazaki fragments are mainly processed by the short flap pathway through the action of FEN1-LIG1. However in some case, when FEN1-cleavage is delayed, Okazaki fragments are matured through the long flap pathway mediated by RPA/DNA2. We found that 53BP1 acts upstream of RPA/DNA2. In addition, a genetic study in 2011 proposed that Rad9, the 53BP1 homolog in *s. cerevisiae*, was recruited to long flaps in the absence of DNA2, associating with histone marks to induce cell cycle arrest (Budd *et al.*, 2011). Our data show that 53BP1 binds long flaps even in the presence of DNA2 and appears to promote the long flap pathway, consistent with the hypothesis that 53BP1 only binds a subset of Okazaki fragments. Through this role, 53BP1 could help to correct possible errors made during DNA replication by the error-prone Pol α that produces the DNA primer following the RNA primer generated by the primases, and thus maintain genetic stability. Indeed, maturation through the long flap pathway would limit mutations due to transcriptional activity (Balakrishnan and Bambara, 2011; Balakrishnan *et al.*, 2010). Therefore, it would be interesting to measure the mutation rate in the presence and absence of WT/mutated 53BP1. Additionally, sequencing the Okazaki fragments bound to 53BP1 could provide valuable information about the genomic localisation of this interaction, and thus answer to the question of whether 53BP1 binds to flap predominantly in transcriptionally active regions. Moreover, since we have observed that 53BP1 binds RNA:DNA hybrids at the fork, we could suppose that 53BP1 is also involved in the resolution of RNA:DNA hybrids due to transcription-replication conflicts. Indeed, 53BP1 could be recruited to active chromatin and thus be available to play two distinct roles: inducing the long flap on the lagging strand and preventing the formation of R-loops.

Furthermore, sequencing data would also help to better understand the 53BP1-RNA interaction, especially to determine whether this association is mediated by a specific RNA sequence, since the GAR domain is known to recognise G-rich regions. Several sequencing techniques for Okazaki fragments are possible (Ok-seq (Petryk *et al.*, 2016); Gloe-seq (Sriramachandran *et al.*, 2020); TrAEL-seq (Kara *et al.*, 2021)), but they are mostly developed in yeast models or they require a large quantity of cells

making them challenging to perform. Attempts from the lab to perform Ok-seq on nucleic acids covalently bound to 53BP1 (CLIP) were so far unsuccessful.

It is important to highlight that these discoveries were made in unstressed conditions, a context in which 53BP1 is less studied. However, it would be interesting to extend our findings to stressed contexts where 53BP1 is active and thereby determine whether the RNA-binding regulates other functions of 53BP1. As the function of 53BP1 in DNA repair in G1-phase is mediated by RNA, it would be insightful to define whether this mediation is direct or indirect. To assess this, CLIP could be performed on G1-phase cells to elucidate whether 53BP1 exhibit an RNA-binding activity throughout the cell cycle or whether it is specific to the S-phase.

Moreover, the study of 53BP1 in BRCA1-deficient contexts could provide a better understanding of the sensitivity/resistance of certain cancers to clinically used drugs, such as PARP inhibitors. In the context of BRCA1 deficiency, there is a defect in HR and also a defect in processing the lagging strand, which promotes the development of cancers. These BRCA1-deficient cancers are sensitive to DNA-damaging agents, such as PARP1 inhibitors. In such scenarios, the loss of 53BP1 results in resistance to these drugs, and additionally rescues the defect in maturation of Okazaki fragments (Chaudhuri *et al.*, 2016; Cong *et al.*, 2021; Paes Dias *et al.*, 2021; Panzarino *et al.*, 2021). Furthermore, it has been shown that aberrant expression or loss of function of 53BP1 contributes to the onset and development of tumors (Mirza-Aghazadeh-Attari *et al.*, 2019; Rafnar *et al.*, 2011; Timofeeva *et al.*, 2012). By using published databases on 53BP1 mutations, it would therefore be interesting to establish a potential correlation between its RNA-binding activity and predisposition to certain pathologies. Indeed, we can assume that this correlation between 53BP1 defects and cancer risk is not only associated with the DNA repair function of 53BP1 but also with its function in DNA replication, through its RNA interaction. Understanding the role of this binding could therefore be crucial in understanding the mechanisms of tumour progression and potentially lead to better therapeutic treatments, particularly in the context of cancers resistant to PARP inhibitors.

Beyond the 53BP1-RNA association on the lagging strand, our study provides a range of experiments to identify new RBPs at the replication fork and thus better understand the DNA replication process. Indeed, other proteins have been suggested as candidate RBPs, such as Ku and MRE11, and by using the described techniques in our study, it would be possible to validate whether or not they can interact with the Okazaki fragments. For instance, MRE11 also possesses a GAR domain that is methylated by PRMT1, and Arginines mutations disrupt its exonuclease activity, which is necessary for MRE11 functions in response to replication stress or DNA damage (Boisvert *et al.*, 2005a, 2005b). Ku is an identified RBP (Baltz *et al.*, 2012; Queiroz *et al.*, 2019) and is known to play a role in the fork reversal mechanism. A recent study conducted in yeast also determined that the functions of Ku during replication stress are linked to RNA:DNA hybrids originating from Okazaki fragments. In a fork reversal context, the last Okazaki fragment on the lagging strand hybridises with the nascent DNA of the leading strand, forming the regressed arm. The RNA primer contributes to establish the Ku barrier to protect against fork degradation (Audouyraud *et al.*, 2023). Large-scale techniques such as iPOND (Dungrawala *et al.*, 2015), coupled with RNA-binding activity techniques such as OOPS (Queiroz *et al.*, 2019), would potentially allow the identification and characterisation of new RNA-protein interactions at the replication forks.

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RÉSUMÉ

Au cours de cette décennie, des centaines de protéines de liaison à l'ARN (RBP) ont été identifiées. Les interactions ARN-protéines sont essentielles pour de nombreux processus cellulaires, de la régulation post-transcriptionnelle de l'expression des gènes au maintien de l'intégrité du génome. La réplication de l'ADN est un mécanisme critique pour la stabilité du génome et des défauts peuvent entraîner des pathologies. Par ailleurs, des ribonucléotides sont produits pendant la réplication de l'ADN et plusieurs RBP sont présentes aux fourches de réplication. Cependant, leur interaction avec l'amorce ARN, présente dans la matrice d'ADN, reste inexplorée. En utilisant des techniques *in vitro* et des méthodes de couplage induites par les UV-C dans des cellules humaines, le laboratoire a identifié 53BP1 (p53-binding protein 1), impliquée dans la réponse aux dommages de l'ADN et au stress réplcatif, comme une RBP qui interagit directement avec des molécules d'ARN-ADN, semblables aux amorces ARN-ADN synthétisées sur le brin retard pour former les fragments d'Okazaki (OF). L'objectif de ma thèse était alors de mieux caractériser l'interaction entre 53BP1 et les OF et de comprendre

son rôle durant la réplication de l'ADN. Nous avons montré que la partie C-terminal de 53BP1 est la région minimale de liaison à l'ARN. De plus, en utilisant une variété d'approches pour étudier l'association des protéines avec l'ADN naissant pendant la phase S, nous avons montré que le recrutement de 53BP1 aux fourches de réplication était diminué lors du traitement avec la ribonucléase A ou lors de la déplétion de PRIM1, la sous-unité catalytique de la primase. En revanche, la déplétion de l'endonucléase FEN1, qui entraîne l'accumulation d'amorces d'ARN non clivées, a conduit à une présence accrue de 53BP1 à la fourche. En outre, nous avons constaté que la déplétion de 53BP1 induisait une accumulation de poly(ADP-ribose) en phase S, qui constitue un senseur des OF non ligaturés, et à une diminution du recrutement de RPA/DNA2 à l'ADN naissant, qui sont des facteurs de la maturation des OF.

Dans l'ensemble, ces résultats montrent que 53BP1 interagit avec les OF pour aider à la maturation du brin retard, soulignant le rôle d'une nouvelle interaction ARN-protéine au niveau des fourches de réplication de l'ADN.

MOTS-CLÉS

53BP1 ; Fragments d'Okazaki ; Réplication de l'ADN ; Protéine de liaison à l'ARN

ABSTRACT

Over the last decade, hundreds of RNA-binding proteins (RBPs) have been identified. The RNA-protein interactions are essential for many cellular processes, from post-transcriptional regulation of gene expression to maintenance of genome integrity. DNA replication is a critical mechanism in genome stability and defects can lead to diseases. Furthermore, ribonucleotides are produced during DNA replication and several RBPs are found at replication forks. However, their interaction with the RNA primer, present in the DNA template, remains unexplored. Using *in vitro* assays and UV-C-induced crosslink methods in living human cells, the laboratory identified 53BP1 (p53-binding protein 1), involved in the response to DNA damage and replication stress, as an RBP that directly interacts with RNA-DNA species, similar to the RNA-DNA primers synthesized on the lagging strand to form the Okazaki fragments (OF). The aim of my thesis was to better characterise the interaction between 53BP1 and OF and to understand its role during DNA replication.

We found that the C-terminal part of 53BP1 is the minimum RNA-binding region. Furthermore, using a variety of approaches to study the association of proteins with nascent DNA during S-phase, we showed that the recruitment of 53BP1 to replication forks was decreased upon treatment with ribonuclease A or upon depletion of PRIM1, the catalytic subunit of primase. In contrast, depleting the endonuclease FEN1, which results in the accumulation of uncleaved RNA primers, led to an increased presence of 53BP1 at the fork. In addition, we found that 53BP1 depletion induced an accumulation of S-phase poly(ADP-ribose), which constitutes a sensor of unligated OF, and led to reduced DNA2/RPA loading at nascent DNA, which are factors of OF maturation.

Altogether, these results show that 53BP1 interacts with OF to help in the processing of lagging strand, highlighting the role of a novel RNA-protein interaction at DNA replication forks.

KEYWORDS

53BP1; Okazaki Fragments; DNA Replication; RNA-binding protein