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Jo-Ann Nettersheim

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ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTE - ED414
Laboratoire Biotechnologie et signalisation cellulaire, UMR 7242 du CNRS, Strasbourg

THÈSE

présentée par :

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Discipline/ Spécialité : Aspects Moléculaires et Cellulaires de la Biologie

**Interplay between the translesion DNA polymerase η and
the calpain system**

**Mise en évidence d'un lien entre l'ADN polymérase
translésionnelle pol η et le système calpaïne**

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The first principle is that you must not fool yourself - and you are the easiest person to fool

Richard Feynman



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Résumé en Français

Introduction et objectifs de la thèse

L'ADN est constamment altéré du fait du métabolisme oxydatif de la cellule ou de l'exposition à des agents génotoxiques environnementaux. Malgré l'existence de systèmes de réparation de l'ADN efficaces, certaines lésions sont encore présentes lors de la phase S du cycle cellulaire et bloquent la progression des fourches de réplication. Néanmoins plusieurs mécanismes « de tolérance » permettent à la cellule de poursuivre la synthèse d'ADN. L'un de ces mécanismes, appelé synthèse translésionnelle (TLS), fait intervenir des ADN polymérases spécialisées, dites « translésionnelles », qui remplacent les ADN polymérases répliquatives bloquées au niveau des lésions de l'ADN, au risque d'introduire des mutations dans le génome.

L'importance du mécanisme TLS est illustrée par la maladie *Xeroderma pigmentosum variant* (XPV) caractérisée par une forte incidence de cancers cutanés. Les patients atteints sont déficients dans l'ADN polymérase η (pol η), capable de franchir très efficacement et sans erreur un dimère de pyrimidine T-T de type cyclobutane (lésion CPD majoritairement formée après irradiation des cellules aux rayonnements ultraviolets). En son absence, d'autres ADN polymérases TLS interviennent dans la réplication d'ADN endommagé, avec moins de fidélité et d'efficacité. Le maintien de l'intégrité génétique des cellules dépend donc d'une régulation fine de l'activité des ADN polymérases TLS. Ceci concerne non seulement leur recrutement au niveau des lésions de l'ADN, mais aussi leur démobilisation une fois la TLS réalisée, pour permettre la reprise de la réplication « classique », non mutagène.

L'étude de la régulation de la TLS est l'objectif de ce travail de thèse. En particulier nous avons cherché à déterminer le rôle de l'interaction que nous avons mis en évidence entre pol η et CAPNS1, la sous-unité régulatrice des calpaïnes 1 et 2. Ces protéases ubiquitaires dépendantes du calcium régulent de nombreux processus cellulaires fondamentaux en effectuant une digestion contrôlée de leur protéine cible. Il a été décrit qu'un stress répliquatif induit une élévation de la concentration intracellulaire de Ca_{2+} dans le noyau (Li et al., 2019) et que les calpaïnes sont activées après irradiation des cellules aux rayons ultra-violet (UV) (Gulati et al., 2004). Nos résultats permettent d'établir que les calpaïnes participent à la régulation de la TLS en impactant positivement la localisation de pol η dans les foyers de réplication dans les cellules irradiées aux UV.

Résultat acquis

L'ADN polymérase translésionnelle pol η interagit avec CAPNS1

En utilisant le système double hybride de levure nous avons montré que pol η interagit avec CAPNS1. Nous avons ainsi déterminé que la partie C-terminale de pol η , incluant les acides aminés 595 à 662, est suffisante, mais que la structure en doigt de zinc contenue dans cette région n'est pas impliquée. Après irradiation aux UV des cellules surexprimant la protéine de fusion eYFP-pol η , pol η est relocalisée dans les foyers de réplication, visibles et dénombrables sous un microscope à fluorescence. Nous avons observé que CAPNS1 s'accumule avec eYFP-pol η dans ces foyers de réplication suggérant que les deux protéines puissent y être associées.

Clivage *in vitro* de pol η par la calpaïne

Pol η , produite dans un lysat de réticulocytes de lapin, est coupée spécifiquement autour du résidu 465 par la calpaïne 1 purifiée. En outre, l'incubation d'extraits protéiques en présence de calcium induit le clivage de pol η par une calpaïne endogène, comme le montre l'effet de la calpeptine, un inhibiteur des calpaïnes 1 et 2. Des analyses par spectrométrie de masse de la forme clivée de pol η confirment que le clivage s'effectue à la position 465 (Plateforme Protéomique Strasbourg IBMC) dans nos conditions expérimentales, ce qui est compatible avec une analyse informatique de prédiction des sites de coupure de la calpaïne (*CaMPDB.org*).

Clivage de pol η dans les cellules après activation de la calpaïne

L'activation de la calpaïne par le traitement des cellules avec un ionophore transporteur de calcium induit le clivage de pol η à la même position que lors de la coupure *in vitro*. Ce clivage peu efficace est augmenté lorsque l'on réduit l'expression de la calpastatine, protéine inhibitrice des calpaïnes, à l'aide de siRNA. Enfin, dans ces conditions d'inhibition de la calpastatine, le clivage est également observé après irradiation aux UV.

La version clivée de pol η (1-465) est fonctionnelle pour la TLS

Le produit de clivage de pol η par la calpaïne contient le domaine catalytique de pol η intact (1-435) et est dépourvu des domaines d'interaction avec l'ubiquitine et PCNA nécessaires au recrutement de pol η dans les foyers de réplication après irradiation des cellules aux UV. Cette version tronquée de pol η contient néanmoins une séquence alternative d'interaction avec PCNA dont le rôle est controversé (Acharya et al., 2008). Par mutagenèse dirigée nous avons établi que ce motif est fonctionnel car il confère à la version tronquée de pol η (1-465) une activité de TLS *in vitro* non négligeable par rapport à la protéine complète.

Les calpaïnes sont impliquées dans la localisation de pol η dans les foyers de réplication

Curieusement, le nombre de foyers de pol η formés après irradiation aux UV est diminué lorsque les cellules sont traitées avec un inhibiteur des calpaïnes, alors que le cycle cellulaire et la monoubiquitination de PCNA ne sont pas modifiés. Cette réduction, qui atteint 50% après 6h et 9h d'irradiation montre que l'activité des calpaïnes est impliquée dans la mobilisation de pol η dans les foyers de réplication. La région où s'effectue le clivage de pol η est le siège de plusieurs autres modifications post-traductionnelles (GlcNAcylation à la position 457 et polyubiquitination à la position 462) qui sont nécessaires à l'élimination de pol η après la TLS, limitant ainsi la mutagenèse (Ma et al., 2017). Le clivage de pol η à la position 465 pourrait empêcher ces modifications et contrecarrer ainsi son départ de la chromatine.

Conclusion

Nous avons établi que pol η interagit avec CAPNS1, et que les deux protéines s'accumulent conjointement dans les foyers de réplication après irradiation des cellules aux UV. De plus, pol η est clivée *in vitro* et dans les cellules irradiées aux UV par la calpaïne. Le produit clivé comporte le domaine catalytique de pol η et un motif d'interaction avec PCNA fonctionnel. Enfin, le résultat majeur de notre étude réside dans le fait que l'activité des calpaïnes impacte de façon positive la localisation de pol η dans les foyers de réplication. Une perspective intéressante de ce travail est de rechercher si ce mécanisme de régulation pourrait avoir lieu lors de processus spécifiques au cours desquels l'activité mutagène de pol η est observée. Ceci pourrait être le cas dans les cellules B activées dans lesquelles la concentration de calcium intracellulaire est augmentée de façon significative et où pol η participe à l'hypermutation somatique en synthétisant de larges portions d'ADN de façon mutagène. L'activité mutagène de pol η est également responsable de l'activation d'oncogène dans les cellules tumorales subissant un stress réplicatif (Tsao and Eckert, 2018). La dérégulation du système calpaïne et de l'homéostasie du calcium de ces cellules pourrait être à l'origine de l'activité soutenue de pol η dans ces conditions.

La compréhension de la régulation de pol η est essentielle pour le développement d'inhibiteurs des mécanismes de tolérance des dommages à l'ADN. De telles molécules pourraient sensibiliser les cellules tumorales aux agents génotoxiques (par exemple le cisplatine) et pourrait réduire les effets secondaires mutagènes de ces agents.

Tables of Contents and Abbreviations

Table of Contents

Acknowledgements	3
Résumé en Français	4
Tables of Contents and Abbreviations	7
Table of Figures	12
List of symbols and abbreviations	13
Introduction	16
Introduction orientation	17
Replication	17
Origin of replication firing	17
The DNA replication fork	20
DNA damage	21
DNA repair.....	22
Single-strand DNA lesions repair mechanisms.....	22
Double-strand break repair mechanisms.....	25
Importance of DNA repair and tolerance mechanisms for the cell cycle progression.....	27
DNA damage tolerance mechanisms.....	28
Template switching.....	29
Translesion Synthesis – TLS	30
TLS polymerases.....	31
Polymerase switch	33
TLS and cancer	35
Translesion DNA Polymerase η	38
Pol η implication in Xeroderma pigmentosum variant	38
Domain structure of pol η	38
Functions of pol η	39

Translesional synthesis by pol η	39
Tolerance and resistance against cisplatin by pol η	43
Somatic hypermutation	44
Implications of pol η in non-canonical mismatch repair	48
Other functions of pol η	49
Regulation of pol η by protein interactions and posttranslational modifications	50
Proteins interacting with pol η	50
Posttranslational modification of pol η	51
<i>Calpains</i>	54
The calpain family.....	54
Regulation of calpain by calcium and calpastatin	56
Activation of calpain.....	56
Inhibition of calpain by the endogenous inhibitor calpastatin	58
Cleavage site prediction of calpain 1/2	59
Calpains in disease	61
Unregulated calpain in human pathologies	61
Calpain inhibitors	61
Calpain in infectious disease	63
Calpain mutations causing diseases.....	64
Calpain as prognostic marker in cancer	64
Functions of calpain	65
The role of calpain in inflammation	65
Autophagy and calpain.....	66
Calpain activity during apoptosis	67
Calpain activity on proteins involved in the same pathways as pol η	68
<i>Thesis objectives</i>.....	70
<i>Preliminary Article</i>.....	71
Calpain is involved in the pol η-dependent UV response	72
Abstract.....	72
Introduction	72
Material and Methods	75
Cell lines and plasmids	75

Two-hybrid analysis (Y2H).....	75
<i>In vitro</i> calpain cleavage assay	75
Translesion synthesis assay.....	76
RNA interference transfection	76
Whole cell extracts and Immunoblotting.....	76
Clonogenic assay cell survival	77
Fluorescence Microscopy.....	77
Statistical Analyses.....	78
Results.....	79
Pol η interacts with CAPNS1	79
Pol η is a calpain substrate <i>in vitro</i>	80
Pol η is a substrate of calpain <i>in vivo</i>	81
Translesion synthesis efficiency of truncated pol η across a TT-CPD lesion	82
Calpain is involved in pol η foci formation.....	83
Discussion.....	84
References.....	87
Figure legends.....	72
Figure 1. Pol η interacts with CAPNS1, the small subunit of calpain	93
Figure 2. Association of CAPNS1 and eYFP-pol η within the nucleus.....	93
Figure 3. pol η is a substrate of calpain 1 <i>in vitro</i>	94
Figure 4. Ionophore induces eYFP-pol η cleavage	95
Figure 5. Catalytic and TLS activity of wild type and truncated mutant pol η	95
Figure 6. Calpain is involved in pol η foci formation and UV survival	96
Figure 7. Hypothesis of the regulation of pol η by calpain	96
Supplementary figure 1. Cell cycle and PCNA ubiquitination after calpeptin treatment	96
Supplementary figure 2. Foci formation after UV irradiation in calpastatin depleted cells	97
<i>Additional results and discussion</i>	107
Ionophore induces eYFP-pol η and POLD1 cleavage	108
Cleavage of pol η by calpain after UV irradiation.....	111
<i>Discussion</i>	113
Discussion.....	114
Perspectives	114
Conclusion	115

References..... 117

Appendix..... 159

 Image analysis of foci formation script 160

Abstract 166

Résumé 167

Table of Figures

Figure 1: Origin of replication	19
Figure 2: Replication fork in human cells.....	21
Figure 3: DNA damage and the cellular response	22
Figure 4: DNA repair pathways of single strand DNA damages	24
Figure 5: Double strand break repair mechanisms.....	26
Figure 6: Interstrand crosslink repair pathway	27
Figure 7: Template switching	30
Figure 8: Dogma of TLS	31
Figure 9: Domains of translesional polymerases.....	33
Figure 10: Models for TLS activation	34
Figure 11: Models for polymerase switching during TLS.....	35
Figure 12: Polymerases error rates.....	36
Figure 13: Correlation between polymerase activity and genome instability and mutations	37
Figure 14: Pol η domains and some interaction partners.	39
Figure 15: UV induced DNA damage	40
Figure 16: Translesion synthesis	42
Figure 17: Somatic hypermutation - DNA Deamination Model	47
Figure 18: Somatic hypermutation - RNA based model or reverse transcriptase model	48
Figure 19: Proteins interacting with pol η from BioGrid3.5 software	51
Figure 20: Posttranslational modifications of pol η	53
Figure 21: Calpain family classification	55
Figure 22: Calcium dependent activation of calpain	58
Figure 23: Calpastatin domains	59
Figure 24: Frequency of amino acids in proximity to CAPN1/2 cleavage site	60
Figure 25: Inflammatory mechanisms and calpain.....	66
Figure 26: Calpain targets in the autophagic machinery.....	67
Figure 27: Calpain activities during apoptosis	68
Figure 28: Ionophore induced cleavage of pol η and POLD1..	110
Figure 29: Cleavage of pol η by calpain after UV irradiation.....	112

List of symbols and abbreviations

1-MeA	N1-Methyl Adenine
8-oxoG	8-oxoguanine
AID	Activation-induced deaminases
ALT	Alternative Lengthening of Telomeres
AraC	Cytarabine
ATP	Adenosine TriPhosphate
ATR	Ataxia Telangiectasia and Rad3-related protein
BER	Base Excision Repair
Bpa	4-benzoyl-L-phenylalanine
BPDE-N ² -dG	10S-trans-anti-Benzo[a]Pyrene-7,8-Dihydrodiol-9,10-Epoxyde
BRCA 1/2	BRCA1 Carboxyl Terminus
BRCT	BRCA1 Carboxyl Terminus
CAPN1	Calpain 1 (μ -calpain)
CAPN2	Calpain 2 (m-calpain)
CAPNS1	Calpain Subunit 1
CBSW	Calpain-type Beta-SandWich domain
CDC6	Cell Division Control protein 6 homolog
CDK2	Cyclin Dependent Kinase 2
CPD	Cyclobutane Pyrimidine Dimers
CTD	C-terminal domain
CysPC	Calpain-like CYSteine Protease domain
DNA	Deoxyribonucleic Acid
DNA-PK	DNA-dependent Protein Kinase
dNTP	Deoxyribonucleotide phosphate
DSB	Double Strand Break
EGF	Epidermal Growth Factor
EJ	End Joining
ER	Endoplasmic Reticulum
FA	Fanconi Anaemia
FEN1	Structure-Specific Endonuclease 1

GFP	Green Fluorescent Protein
GG-NER	Global Genomic NER
GST	Glutathione Sepharose Transferase
HLTF	Helicase-Like Transcription Factor
HR	Homologous Recombination
ICLs	Interstrand Crosslinks
LF	Little Finger
LGMD2A	Limb-Girdle Muscular Dystrophy type 2A
LGMDR1	Limb-Girdle Muscular Dystrophy type 1 or 2A
LigI	DNA Ligase I
m6G	6-O-methyl Guanine
MCM	Mini-Chromosome Maintenance
MIT	Microtubule Interacting and Transport motif
MMR	Mismatch Repair
NCX	Na ⁺ /Ca ²⁺ eXchanger
NER	Nucleotide Excision Repair
NHEJ	Non-Homologous End Joining
NLS	Nuclear Localization Sequence
ORC	Origin Recognition Complex
PAD	Polymerase-Associated Domain
PALB2	Partner And Localizer of BRCA2
PARP	Poly (ADP-ribose) polymerase
PARPBP	PARP1 Binding Protein
pAzF	Amino acids p-azido-L-phenylalanine
PC	protease core domain
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PEF	Penta-EF-hand domain
PIP	PCNA Interacting Peptide
PMCA	Plasma Membrane Ca ²⁺ transport ATPase
Pol	Polymerase
RFC	Replication Factor C

RIR	Rev1 Interacting Region
ROS	Reactive Oxygen Species
RPA	Replication Protein A
SCJ	Sister Chromatid Junction
SHM	Somatic Hypermutation
SHPRH	SNF2 Histone linker PHD RING Helicase
SSB	Single Strand Break
SSB	Single Strand Break
STUbL	SUMO-Targeted Ubiquitin Ligase
TC-NER	Transcription-Coupled NER
TLS	Translesion Synthesis
TM	TransMembrane domain
TS	Template Switching
TT	Thymine-Thymine dimer
UBM	Ubiquitin Binding Motif
UBZ	Ubiquitin Binding Zinc finger
UV	Ultra Violet light
XP-C	Xeroderma Pigmentosum C Protein
XP-F	Xeroderma Pigmentosum F Protein
XP-G	Xeroderma Pigmentosum G Protein
XPV	Xeroderma Pigmentosum Variant
XRCC1	X-ray Repair Cross-Complementing protein 1
Y2H	Yeast two Hybrid
α	alpha
δ	delta
ε	epsilon
ζ	zeta
η	eta
ι	iota
κ	kappa

Introduction

Introduction orientation

This PhD thesis is about the regulation of the translesion DNA polymerase η (pol η). I will set the scene by introducing replication and by giving an overview of DNA damages and repair mechanisms. These paragraphs lead to an introduction of DNA damage tolerance mechanisms, which is followed by a detailed description of translesion synthesis (TLS), and more specifically about pol η . Pol η was found in our laboratory to interact with CAPNS1, the subunit of calpain 1 and 2, which are calcium dependent, modulating proteases. I focus my PhD research on this CAPNS1/ polymerase η interaction to study its biological relevance. Therefore, adherend to the pol η introduction is a chapter about calpains and their multiple functions and implications in human pathologies.

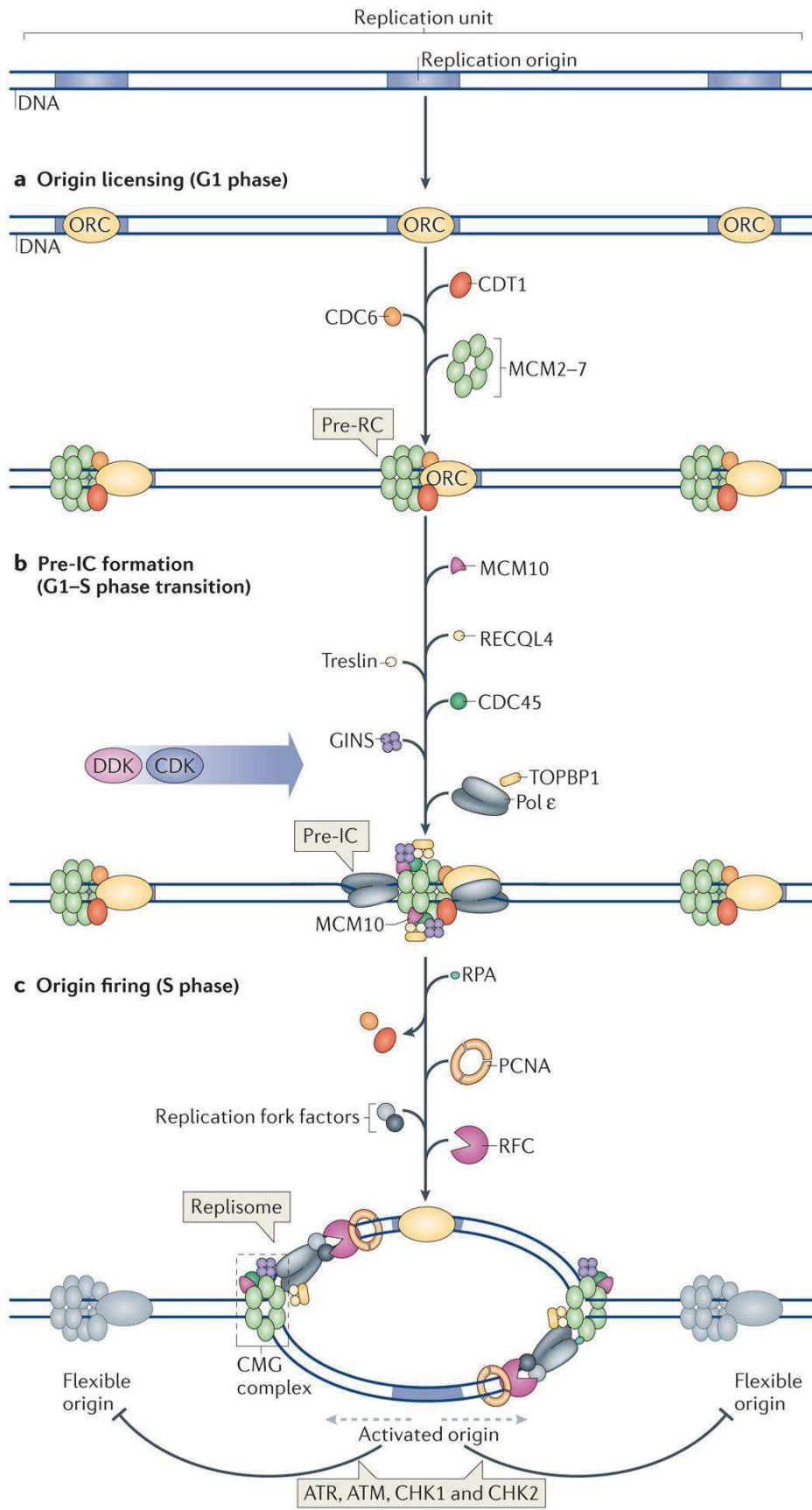
Replication

Origin of replication firing

At each cell division, the genome is duplicated by replication. The replication is initiated at about 30,000 - 50,000 replication origins. Replication origins contain DNA sequences which are recognized by replication initiation proteins (Figure 1). During G1-phase, licensing of replication occurs by formation of the origin recognition complex (ORC), consisting of the hexamer ORC1-6, at the site of replication origin. This is controlled by a “licensing checkpoint” delaying entrance into S-phase until sufficient origins are licensed and therefore insures the replication of the complete genome. Secondly, the pre-replication complex is formed by binding of the DNA replication factor CDT1 and cell division control protein 6 homolog (CDC6), as well as loading of mini-chromosome maintenance (MCM) helicase complex. MCM contains six subunits: MCM2–7 (Fragkos et al., 2015). During G1- to S- phase transition, DBF4-dependent kinase (DDK) and cyclin-dependent kinases (CDKs) phosphorylate numerous replication factors; most importantly MCM10, CDC45, ATP-dependent DNA helicase Q4 (RECQL4), treslin, GINS, DNA topoisomerase 2-binding protein 1 (TOPBP1) and DNA polymerase ϵ (Pol ϵ), leading to the formation of the pre-initiation complex (pre-IC) and the activation of the MCM helicase complex. The MCM helicase double hexamer complex splits into two hexamers, subsequentially, both MCM helicases unwind the DNA and thereby creating two replication forks. Replication protein A (RPA) binds to the unwound single

stranded DNA, protecting it from degradation and hinders the formation of secondary structures. The later process is termed origin firing, which occurs in domains, which have a mean replication domain sizes of 1.4–3.6 Mbp and contain at least six replicons. Replicons are determined as the DNA region replicated from a single origin. The focal sites of DNA synthesis at a single origins are termed replication foci, which can be visualized by either labelling replisome components or by detecting sites of nucleotide incorporation upon pulse labeling (Chagin et al., 2016). When the replication of one domain is completed the origin firing spreads to its neighboring domains. Dormant origins can be activated in case of an absent origin firing in its vicinity or when a DNA damage stalls incoming forks, helping to complete replication. Unfired dormant origins are inactivated to prevent re-replication, if their DNA is replicated by an incoming fork, generated by a neighboring origin. Transcriptionally active open euchromatin replicates in early S-phase and heterochromatin generally replicates late in S-phase, often at the nuclear periphery and around nucleoli. Active replication and transcription take place separately from one another to avoid replication-transcription interference and therefore genome instability (Blow et al., 2011; Fragkos et al., 2015). Late replication is associated with elevated levels of mutations (Gaboriaud and Wu, 2019). It is proposed that during S-phase progression specific biochemical aspects change, such as dNTP levels/balance, activity of TLS DNA polymerases, and mismatch repair activity, causing effects on the introduction or repair of DNA lesions (Koren et al., 2012).

Furthermore, early or late S-phase replication regions are associated with different mutation types. Whereas copy number variations and large-scale rearrangements occur in early S-phase, while deletions and single-nucleotide polymorphisms arise in late S-phase.



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Figure 1: Origin of replication from Fragkos et al., 2015

The DNA replication fork

The DNA replication fork consists of many proteins that form a large complex, the replisome which includes all proteins described in the following paragraph. The replisome facilitates the efficient initiation and elongation of DNA synthesis (Figure 2). Proteins traveling with the replication fork are involved in chromatin-associated events, such as, repair, recombination, chromatin formation, chromatin modification, chromatin remodeling, maintenance of epigenetic information and the prevention of re-replication during replication fork progression (Mueller et al., 2019). DNA synthesis is started by primase, which synthesizes an RNA primer, in complex with the replicative DNA polymerase α , extending the RNA primer with DNA. The DNA sliding clamp proliferating cell nuclear antigen (PCNA) is loaded onto the DNA 3'-OH ends with the help of replication factor C (RFC) in an ATP (Adenosine triphosphate) dependent manner (Shiomi and Nishitani, 2017). RPA directly interacts with RFC to facilitate the specific binding of RFC to the 3' junction, thereby directing PCNA loading. PCNA plays a fundamental role in coordinating multiple events on the DNA. During the next step, PCNA tethers the fidel replicative DNA polymerases δ and ϵ (pol δ and ϵ), stabilizing the interaction of the polymerases with the DNA. The daughter-stand is synthetized in 5' to 3' direction, originating from the RNA-DNA primer. Leading strand (3' to 5') synthesis is done in a continues manner in 5' to 3' direction towards the helicase, mainly by pol ϵ (subunits: PolE1-PolE4), whereas the lagging strand (5' to 3') is synthetized in about 200 nt fragments in 5' to 3' direction (Okazaki fragments) mainly by pol δ (subunits: PolD1-PolD4). The RNA parts of the primers are removed by ribonuclease H and Flap Structure-Specific Endonuclease 1 (FEN1). DNA pol δ fills the gaps with deoxynucleotides and the fragments are coupled by DNA ligase through 5' to 3' phosphodiester bonds (Nick McElhinny et al., 2008; Stillman, 2008). DNA topoisomerases bind ahead of the MCM helicase, nicking one or both of the DNA strands to release torsional tension induced by the unwinding of the DNA (Bjornsti and Kaufmann, 2019; Oakley and Hickson, 2002).

During replication about 120 000 misplaced bases are integrated into the DNA per cell. If those mistakes aren't repaired, they manifest into mutations during the next cell division. Fortunately, Pol δ and ϵ possess the ability to proofread their newly synthetized DNA strand and therefore, demonstrate an increased fidelity of 10^2 - 10^3 (Bębenek and Ziuzia-Graczyk, 2018; Reha-Krantz, 2010). Furthermore, base mismatches can be repaired after the

replication due to a repair pathway called the mismatch repair (described below). Organisms deficient in MMR demonstrate an estimated mutation rate of 1.6×10^{-8} causing hereditary cancers (Meier et al., 2018). Faithful DNA replication, with an error rate at about 10^{-9} - 10^{-10} , is essential to prevent accumulations of mutations and to maintain genome integrity. Accordingly, germline mutations in the catalytic subunits of pol δ and pol ϵ lead to proofreading-associated polyposis (Briggs and Tomlinson, 2013; Palles et al., 2013).

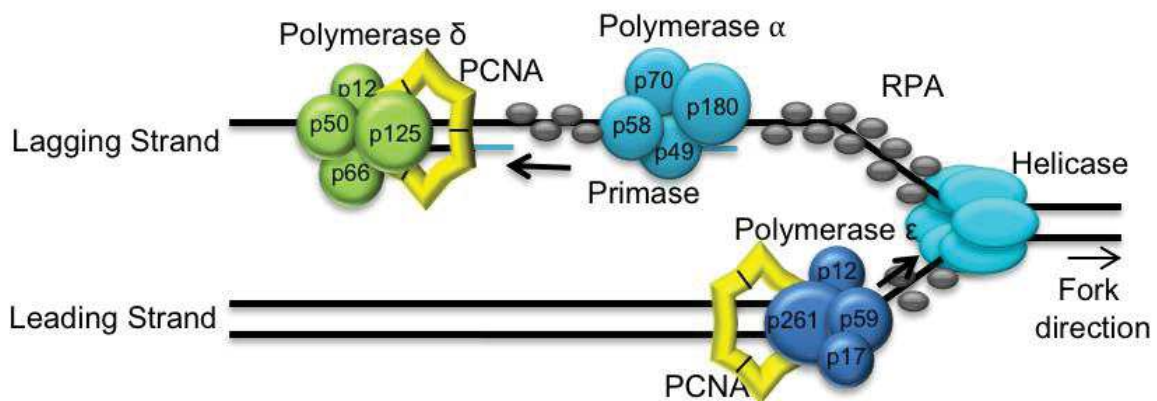


Figure 2: Replication fork in human cells. The replicative DNA polymerase α forms a complex with Primase, initiating the synthesis of the DNA fragment. The replicative DNA polymerases ϵ and δ synthesize the leading and the lagging strand, whereas PCNA serves as anchor for polymerase ϵ and δ .

DNA damage

The human body consists of approximately 10^{13} cells and each cell experiences tens of thousands DNA lesions per day (Lindahl and Barnes, 2000). DNA damage can be categorized into two types, endogenous and exogenous. Endogenous DNA damage is caused by the cell itself through reactive oxygen species (ROS), AP (apurinic or apyrimidic) sites, DNA methylation, active enzymatic DNA processes and other metabolic products (Lindahl and Barnes, 2000; Tubbs and Nussenzweig, 2017). Exogenous damage occurs when cells are exposed to ionizing radiation, ultraviolet radiation or chemical agents (e.g.: DNA damaging chemotherapeutics, carcinogens in tobacco smoke, alcohol) (Brooks, 1997; Ma et al., 2019). The DNA damages that occur can be base damage, sugar damage, single strand breaks (SSBs), or double strand breaks (DSBs) (Figure 3) (Kuo and Yang, 2008; Shiloh, 2003). DNA damage can lead to mutations and genome instabilities, which can result in many different medical conditions, including cancer. Approximately 38.4% of all people worldwide will be diagnosed

with cancer at some point during their lifetimes (based on data from 2013–2015 (National Cancer Institute, 2015)). This incident rate is expected to rise.

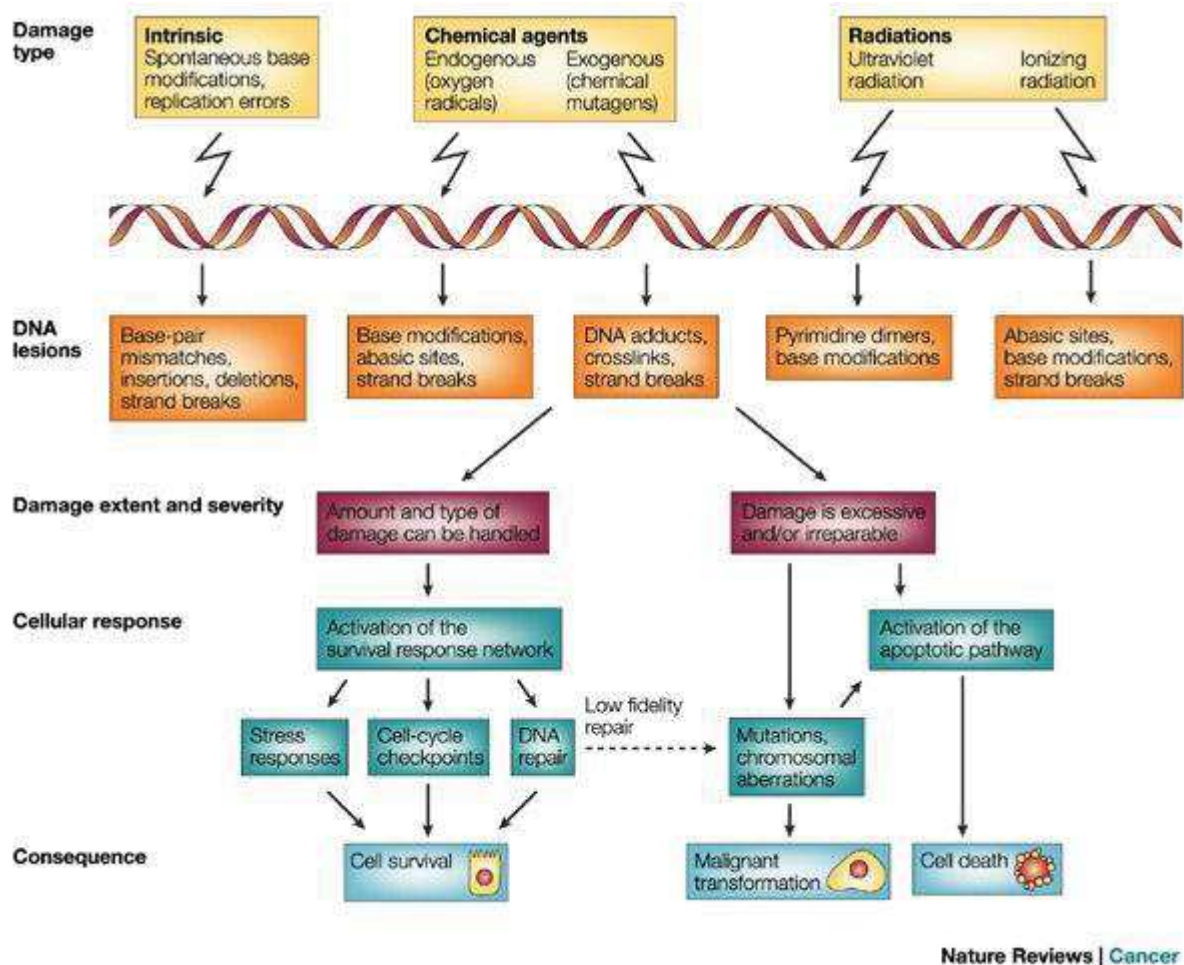


Figure 3: DNA damage and the cellular response as shown in Shiloh, 2003.

DNA repair

DNA repair is crucial to an organism's ability to maintain its genome integrity and thus its function. Depending on the damage type there are different pathways to repair them (see Figure 4 and 5 for main repair pathways). The repair pathways are described in the following in two groups: single-strand DNA lesions and double-strand breaks.

Repair mechanisms of lesions affecting one strand of the DNA

Base excision repair (BER): BER is a mechanism during which a single non-helix distorting DNA alteration is removed and replaced with the correct base. Eleven different DNA glycosylases in humans recognize the different base damages. X-ray repair cross-complementing protein 1

(XRCC1) interacts with several of these DNA glycosylases involved in the repair of both oxidative and alkylated base lesions, and stimulates their activity. DNA glycosylases catalyze the cleavage of the N-glycosidic bond between the substrate base and the 2'-deoxyribose, producing an AP (Apyrimidinic/Apurinic) site. Subsequently, AP endonuclease (APE1) cleaves the DNA backbone. The following steps of gap filling and ligation are carried out either via the short-patch or the long-patch BER sub-pathways. The short-patch BER sub-pathway is dependent on the pol β , which can hydrolyze the 5'dRP and fills the single nucleotide gap. This prepares the strand for ligation by either a complex of DNA Ligase III α (LigIII α) and XRCC1 or LigI. The interaction between the BRCT II domain of XRCC1 with DNA Ligase III α protein is required to prevent the DNA Ligase degradation. For the long-patch sub-pathway a complex is formed, including pol δ or ϵ coupled with PCNA, flap endonuclease (Fen1) and DNA Ligase I (LigI). This complex is synthesizing DNA to fill the gap. Finally, the DNA strand integrity is restored by closing the nick with LigI (Lodish, 2008; Wallace, 2014).

Single-strand break repair (SSBR): Single-strand breaks can be repaired through the SSBR pathway. The discontinuities in one strand of the DNA double helix are usually accompanied by loss of a single nucleotide. The binding of the PARP-1 and PARP-2 proteins to the single strand break activates the synthesis of poly(ADP-ribose) (PAR) polymers, thereby stimulating the recruitment of DNA repair proteins to the DNA damage site including the scaffold protein XRCC1. The nick is then repaired by either the long patch pathway or the short patch pathway as during the BER pathway (Brem and Hall, 2005; Polo et al., 2019).

Mismatch repair (MMR): MMR is a pathway to repair mismatches after replication. The mismatch is identified by MSH2 and 6 and forms a ternary complex with the MLH1/PMS2 in an ATP dependent manner. A DNA helicase unwinds the double-stranded DNA and several hundred bases before and after the miss-matched base are nicked by the DNA endonucleases MLH1/PMS2. This complex interacts with PCNA which helps to identify the newly synthesized DNA strand. Subsequently, EXO1 removes about 800 nt including the mismatched DNA (Jeon et al., 2016). Finally, the gap is filled by the DNA pol δ and the DNA ends are joined by DNA ligase 1 (Hsieh and Zhang, 2017).

Nucleotide excision repair (NER): NER is a mechanism that repairs helix-distorting DNA damage in a multistep 'cut and patch'-type reaction. One of the lesions the NER pathway can repair are thymine-thymine dimers caused by ultraviolet light. There are two types of NER, global genomic NER (GG-NER) and transcription-coupled NER (TC-NER). GG-NER repairs

damages occurring in active and inactive genes throughout the genome. GG-NER require Xeroderma pigmentosum C Protein (XP-C) and Rad23B for the recognition of helix distortion. TC-NER ensues in transcriptionally active regions of the genome and is initiated directly by CSA/CSB complex when RNA polymerase stalls at a lesion in DNA. The following mechanism is the same for both GG-NER and TC-NER. After activation of NER, the transcription factor TFIIH is recruited, activating its helicase subunit. An unwind DNA stretch of about 25 bases is formed with the help of XP-G and RPA proteins. XP-G and XP-F act as endonucleases and cut the unwind DNA segment that contains the distorted DNA backbone. The gap is filled by replicative DNA polymerases and sealed by DNA ligase (Lodish, 2008; Spivak, 2015).

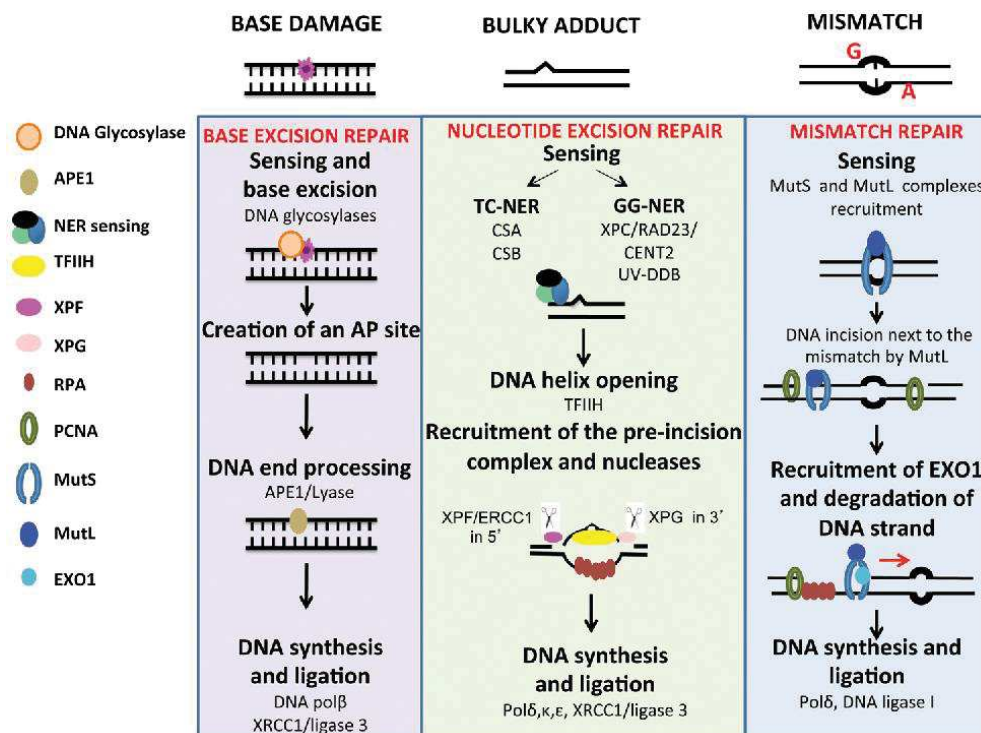


Figure 4: DNA repair pathways of single strand DNA damages as presented in Gourzoune-Dmitriev et al., 2013.

DNA-Protein Crosslink (DPC) repair: The DPC repair pathway removes proteins irreversibly covalently bound to DNA. The trapped protein is cleaved by the DNA-dependent metalloproteases Wss1 or SPRTN. The DNA-bound peptide remnant can be bypassed by the DNA damage tolerance mechanism translesion synthesis during replication. For the repair the peptide remnants can be excised by the tyrosyl-DNA-phosphodiesterases 1 and 2. Alternatively, nucleases can also remove DPCs by cleaving the DNA to which DPCs are attached. Furthermore, the NER pathway can process DPCs (Fielden et al., 2018).

Double-strand break repair mechanisms

Homologous recombination (HR): HR repairs double strand breaks by using the homologous DNA sequence as template and thus is restricted to the S and G2 phases of the cell cycle. For this, overhanging 3' single strands are produced by exonucleases. The two DNA single strands intertwine with the intact double strand via Rad51, forming a holiday junction in order to synthesize the missing DNA fragment. After DNA synthesis and ligation of the fragments, the holiday junction needs to be resolved by cleavage and new ligation of the nicked DNA. This pathway can also repair collapsed replication forks. An unrepaired collapsed replication fork is likely to be lethal to the daughter cell due to the loss of genetic information. Furthermore, HR is used for the cross-over of genetic material between the maternal and the paternal chromosomes (Lodish, 2008; Wright et al., 2018).

Non-homologous end joining (NHEJ): NHEJ is the dominant double-strand repair pathway and occurs in disregard of homology. NHEJ modifies the two broken ends and fuses them. This mechanism is about 3-6 times faster than HR but it is error prone and results in the loss of several base pairs (Mao et al., 2008). The DNA-dependent protein kinase (DNA-PK) and the KU70/KU80 heterodimer bind to the loose ends. Nucleases bind to the DNA ends and remove the overhang, producing blunt ends. These ends are ligated together resulting in a repaired but shortened DNA double strand (Lodish, 2008).

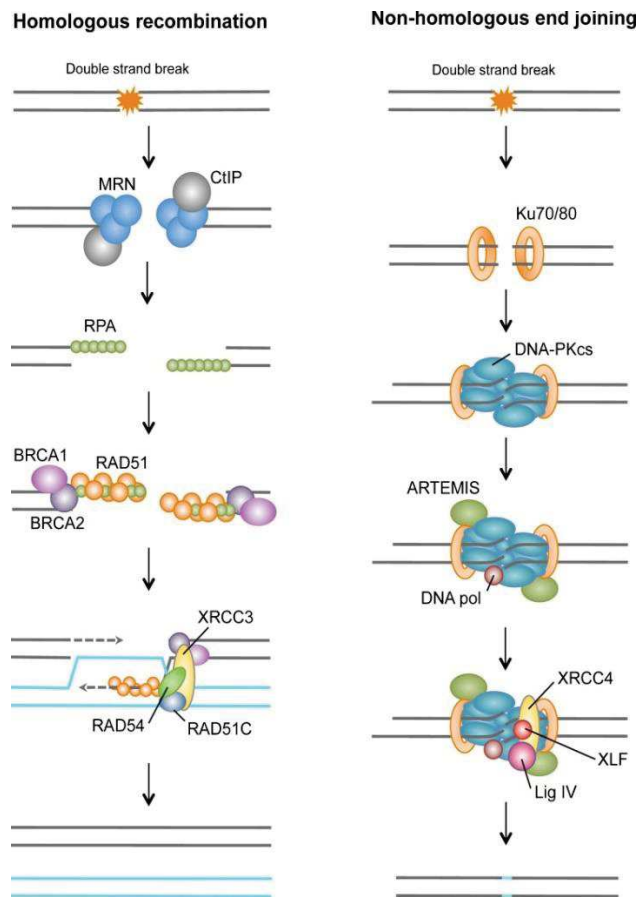
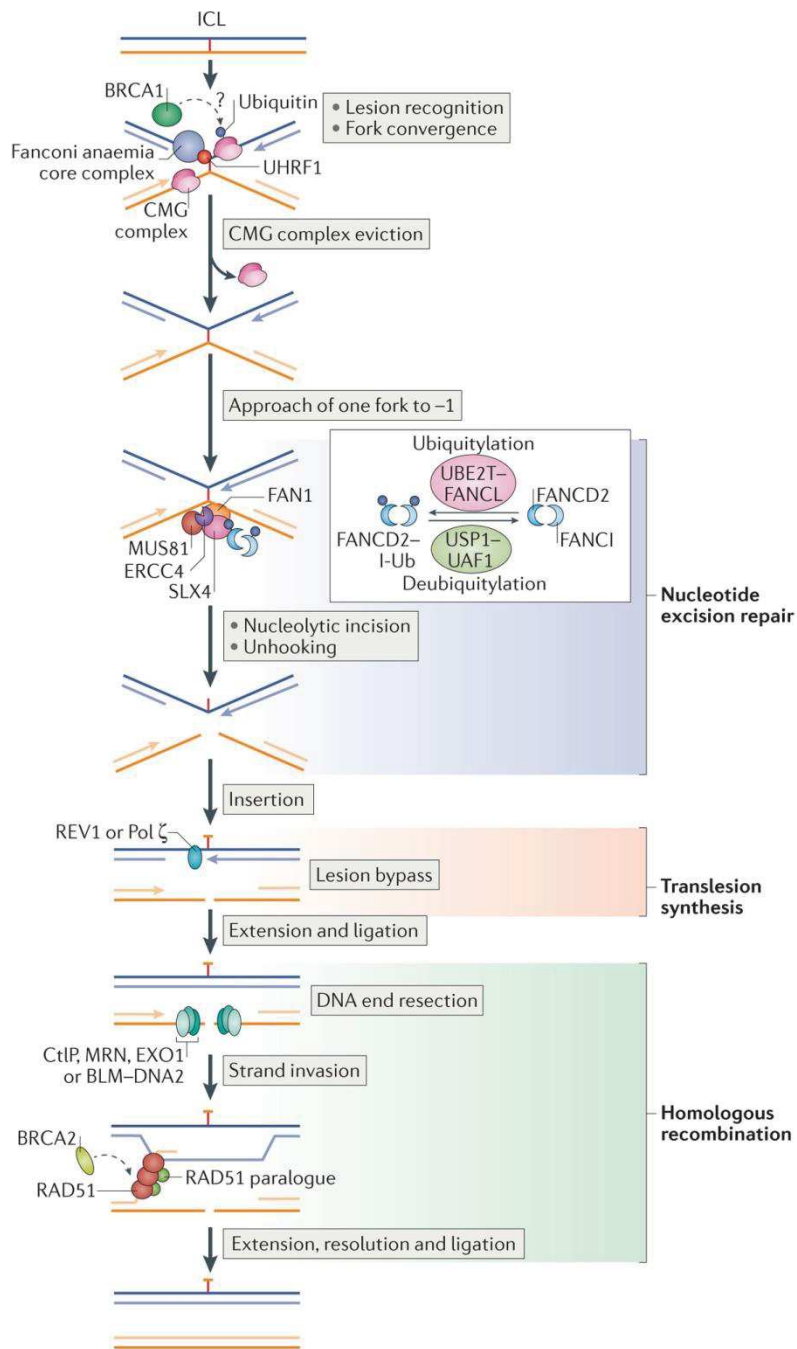


Figure 5: Double strand break repair mechanisms as presented in Brochier and Langley, 2013.

Fanconi anaemia (FA) pathway: The FA pathway (Figure 6) is involved in repairing interstrand crosslinks (ICLs), highly toxic DNA lesions that can block replisome progression as well as transcription. The ICLs are recognized by the FANCM–FAAP24–MHF1–MHF2 complex and subsequently the Fanconi anaemia core complex ubiquitinates two other Fanconi anemia proteins, FANCD2 and FANCI. FANCM activates the ATR checkpoint, which phosphorylates and activates multiple Fanconi anaemia proteins. Ubiquitinated FANCD2 at the ICL region recruits several factors, including SLX4 and Fanconi-associated nuclease 1 (FAN1), and coordinates nucleolytic incisions by ERCC4 or MUS81. Afterwards, the DNA sequences are unhooked, leaving the crosslinked nucleotide attached to the complementary strand. This moiety is bypassed by translesion synthesis polymerases such as REV1 or DNA polymerase ζ . Ligation restores an intact DNA duplex, which functions as a template for homologous recombination-mediated repair of the double-strand break. The USP1–UAF1 complex deubiquitylates the FANCD2–I heterodimer and completes repair (Ceccaldi et al., 2016).



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Figure 6: Interstrand crosslink repair pathway - Cooperation of Fanconi anaemia, nucleotide excision repair, translesion synthesis and homologous recombination proteins as presented in Ceccaldi et al.,2016

Importance of DNA repair and tolerance mechanisms for the cell cycle progression

There are two vulnerable points during cell cycle progression where it is incremental that the DNA contains as little damage as possible to prevent mutations, loss of genetic information or further damage of the DNA. One vulnerable point during the cell cycle is mitosis. Unrepaired DNA damage during mitosis can lead to loss of genetic information due to the absent of homology. End joining (EJ) and homologous recombination operate throughout

interphase but are inhibited during mitosis. (Branzei and Foiani, 2008; Hustedt and Durocher, 2017).

The other vulnerable point during the cell cycle is during DNA replication, because mismatches can be transformed into mutations and DNA damage can lead to replication fork break down and subsequently to the loss of genetic information. To prevent this, firstly, the genome is scanned directly by the replicative polymerase, which incorporated the nucleotides, by its proofreading capacity for mis-incorporations. Secondly, several repair mechanisms take place to ensure the genome integrity, such as MMR. Notably, the S-phase DNA damage checkpoint is slowing replication in response to DNA damage but not halting cell cycle progression. The two S-phase checkpoint kinases, Ataxia Telangiectasia Mutated (ATM) and ATM and Rad3-related (ATR), respond to DNA damage. ATM is mainly activated upon double-strand breaks and ATR is activated in response to a variety of genotoxins such as UV, MMS, hydroxyurea (HU), aphidicolin, and psoralen. ATR also functions in unperturbed S-phase in the regulation of origin firing. DNA damages during the S-phase such as interstrand crosslinks and DNA-protein crosslinks can furthermore hinder transcription process. However, repair throughout S-phase of lesion in the replication fork will lead to double strand-breaks. To avoid this, the cell initiates two DNA damage tolerance pathways upon stalled replication forks: template switching (TS) and translesion DNA synthesis (TLS).

DNA damage tolerance mechanisms

DNA damage tolerance (DDT) mechanism (sometimes referred to as DNA damage bypass or post-replication repair) allows to circumvent DNA lesions and thereby the completion of DNA replication, leaving the damages to be repaired after the replication (Bi, 2015). The two DNA-damage tolerance mechanisms TLS and TS are regulated by PCNA mono- and K63-linked polyubiquitination, respectively.

Upon DNA damage during replication, RPA coated single-stranded DNA recruits the E3-ubiquitin ligase Rad18, whereas pol η can facilitate the Rad18 recruitment by binding to PCNA and Rad18 (Durando et al., 2013). Furthermore, Nijmegen breakage syndrome 1 (NBS1) and breast cancer type 1 (BRCA1) can also help to recruit Rad18. RAD6 binds to RAD18 and the complex monoubiquitinates PCNA at K164, promoting TLS. However, the monoubiquitin can be polyubiquitinated by the E3-ubiquitin ligases helicase-like transcription factor (HLTF) or

the E3 ubiquitin-protein ligase SNF2 histone linker PHD RING helicase (SHPRH) (human orthologs of yeast Rad5) together with the E2-conjugating complex MMS2-UBC13 thus promoting TS. This polyubiquitination depends on the presence of HLTf in the replication fork. HLTf is stimulated by double-stranded DNA via recruitment to stalled primer ends. By contrast, when PCNA is monoubiquitinated in the absence of HLTf, it is not subsequently polyubiquitinated (Masuda et al., 2018).

Template switching

During template switching the damaged strand invades a homologous duplex DNA and uses it as template for replication (Figure 7), in an error free manner albeit at the cost of lost heterozygosity. As described above TS is activated by PCNA poly-ubiquitination, followed by strand invasion and pairing of newly synthesized strands from the two sister chromatids mediated in *saccharomyces cerevisiae* by Rad51, Rad52, Rad54, and Rad55/Rad57. Pol δ synthesizes the missing gap along the homologous template DNA. Subsequently, the sister chromatid junction (SCJ) is resolved by the yeast complex Sgs1/Top3/Rmi1 (Bi, 2015). It was shown that in late S-, G2/M-phase the DNA damage tolerance can be achieved by the salvage HR pathway, which similar to the TS pathway invades the sister chromatin, synthesizing along the homologous DNA, followed by the resolution of the SCJ in a hyper-recombinogenic manner (Branzei et al., 2008; Minca and Kowalski, 2010; Pfander et al., 2005). Salvage HR is independent from PCNA ubiquitination and can be inhibited by recruitment of human PARPB (PARP1 Binding Protein, also termed PARI; *Saccharomyces cerevisiae*: Srs2) to sumoylated PCNA at K164 and K254. The PARPB helicase can disrupt Rad51 presynaptic filaments and thus prevent HR (Burkovics et al., 2016; Moldovan et al., 2012).

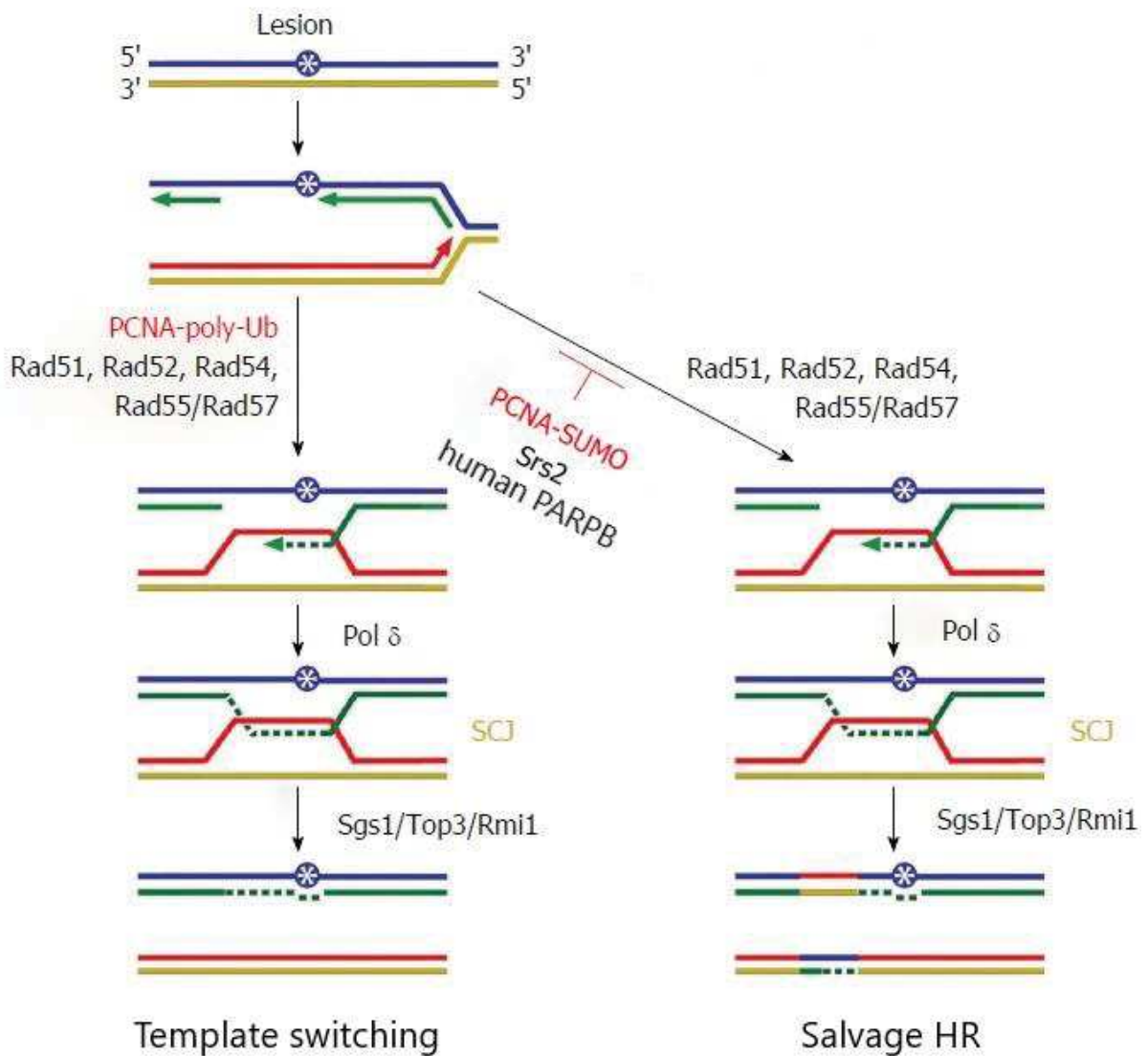


Figure 7: Template switching in *saccharomyces cerevisiae* adopted from Bi, 2015

Translesion Synthesis – TLS

Replication forks can be stalled by unrepaired DNA damage or secondary structures. As described above, once DNA has been unwound at the replication fork, replication must proceed despite lesions to prevent more deleterious and mutagenic consequences. TLS polymerases are capable of synthesizing new DNA strands complementary to the sequence containing a lesion. TLS is active during S-phase replication, interestingly TLS has been described to be active in budding yeast during G2-phase.

It is possible for TLS polymerases to synthesize new DNA strands across lesions because they possess a relaxed geometric selectivity in the nascent base pair binding pocket of their

catalytic domain, which is more open and accessible for bulky lesions than those of replicative polymerases. Without conformational selection or proofreading, accuracy of Y-family polymerases is achieved by chemical selection alone. General TLS includes the following steps: Firstly, the DNA is replicated by replicative DNA polymerases until the lesion. Secondly, the replication fork stalls and recruits TLS polymerases, which insert nucleotides across the lesion. Thirdly, oftentimes another TLS polymerase extends the newly synthesized strand (two-step mechanism). The extension can be achieved by the same TLS polymerase as the insertion in a one-step mechanism. Lastly, replicative polymerases continue the replication process (Figure 8).

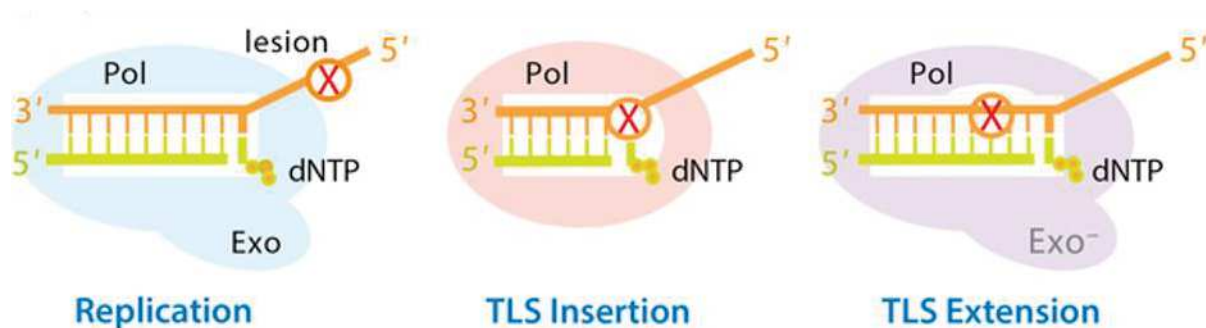


Figure 8: Dogma of TLS as presented by Yang and Gao, 2018.

TLS polymerases

DNA polymerases can be divided into different families: A, B, C, D, X, Y, RT (reverse transcriptase, including telomerase), and PrimPol (primase and polymerase) families, in dependence of their structure (Figure 9)(Garcia-Diaz and Bebenek, 2007; Ito and Braithwaite, 1991; Ma et al., 2020). The main TLS polymerases include polymerase η , ι , κ , ζ and Rev 1.

B family: DNA translesional polymerase ζ (subunits: REV3L, REV7, POLD2 and POLD3) belongs to the same family as the replicative polymerases α , δ , and ϵ . B family polymerases are multisubunit enzymes. Unlike other B family polymerases, pol ζ does not possess a proofreading function. Pol ζ extends the DNA primer, made by other translesion DNA polymerases at site of a lesion, so that the replicative DNA polymerases can continue the replicative process. Pol ζ is also capable of completing TLS without an insertion polymerase. DNA pol ζ is essential for normal embryogenesis, DNA replication and cell proliferation. Pol ζ operates as a tumor suppressor by preserving chromosomal stability at the cost of point mutations in mammals. In yeast, pol ζ is responsible for at least half of spontaneous

mutations, demonstrating its importance (Martin and Wood, 2019). Recently, it was shown that pol ζ is cleaved by *taspase1*, preventing ubiquitination dependent degradation, which appears to be important for DNA lesion response (Wang et al., 2020).

Y family: DNA translesional polymerase η , ι , κ , and Rev 1 belong to the Y family and are encoded by the genes *POLH*, *POLI*, *POLK*, and *REV1* respectively. Y family members have identical right-hand thumb, palm and finger domains. The active site, however, differs between family members in accordance with the different lesions across which they replicate. Y family members are generally recognized as insertion polymerases in two-step mechanisms, meaning that these polymerases are capable to insert nucleotides in front of lesion. The extension of this fragment is done by the B family pol ζ and Y family pol κ in dependence of the lesion. However, pol η is capable of extending the newly synthesized strand after CPD lesions until a replicative polymerase continues the synthesis, in a one-step mechanism (insertion and extension) (Livneh et al., 2010). Pol κ is also capable to bypass BPDE-N²-dG (10S-trans-anti-benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide) lesions in a one-step mechanism (Stern et al., 2019).

A family: The prototype of this family, bacterial pol I, was the first DNA polymerase to be isolated. DNA polymerases θ and ν encoded by the genes *POLQ* and *POLN* belong to the A family. Both polymerases have been implicated in somatic hypermutation and TLS (Masuda et al., 2006; Quinet et al., 2018). Pol θ can bypass 6-4PP in the presence of Pol ι , and is capable to extend past CPD and 6-4PP after insertion by another polymerase (Seki and Wood, 2008). Pol θ is a key enzyme of theta-mediated end joining, which is independent from the Ku heterodimer in comparison with the “classical” NHEJ (Schimmel et al., 2017). Pol ν and pol θ bypass alkylated guanine lesions (Du et al., 2020).

PrimPol family: PrimPol is a member of the archaeo-eukaryotic primase (AEP) superfamily. PrimPol performs TLS across both CPD and 6-4PP lesions as well as it synthesizes a de novo DNA primer at the leading strand downstream a UV induced lesion (Bianchi et al., 2013; Mourón et al., 2013).

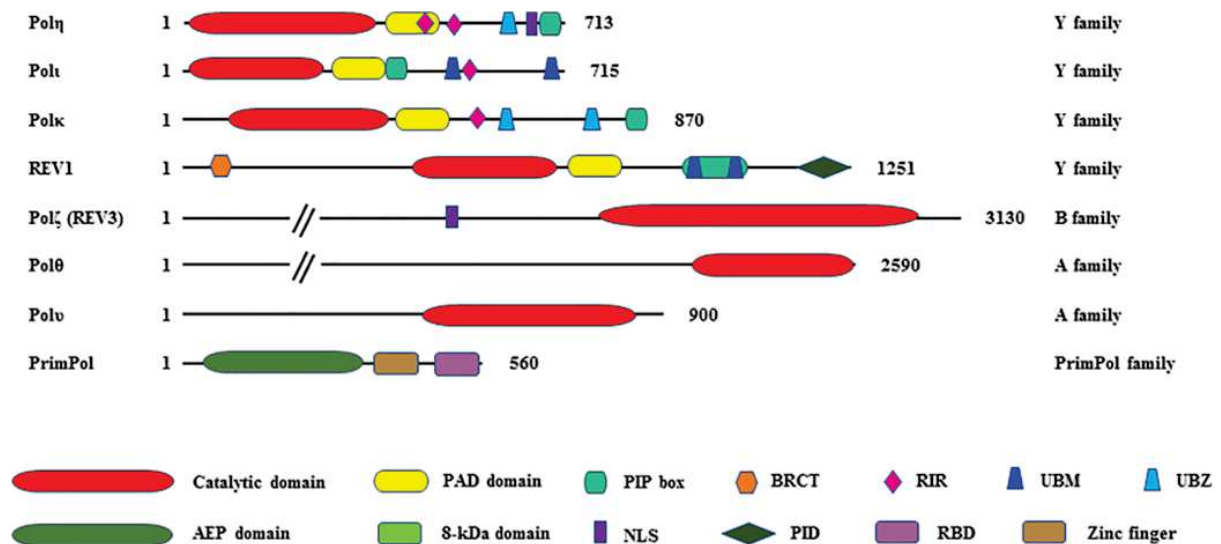


Figure 9: Domains of translesional polymerases adapted from Ma et al. 2020. AEP domain, archaeo-eukaryotic primase domain; BRCT, BRCA1 carboxyl terminus; NLS, nuclear localization signal; PAD, polymerase-associated domain (also known as the little finger); PID, polymerase interacting domain (of REV1); PIP, PCNA-interacting peptide; RBD, RPA binding domain (RBD); RIR, REV1-interacting region (of other Y-family polymerases); UBM and UBZ, ubiquitin-binding domains.

Polymerase switch

TLS is dependent on several polymerase switches, that means that different polymerases are exchanged during the TLS process to accomplish specific tasks. Two general models of TLS activation and polymerase switches are discussed in the literature (Bertolin et al., 2015). Firstly, TLS is activated post-replicative, as shown in Figure 10A: when a replication fork stalls due to a lesion, replication restarts downstream of that lesion, leaving a gap behind. PCNA-ubiquitination marks the gap in front of the DNA lesions, which then can be closed by TLS polymerases at a later time (Quinet et al., 2018). Secondly, TLS is replication-coupled (“on the fly”), Figure 10B: at the stalled replication forks PCNA is ubiquitinated and TLS polymerases are loaded. TLS polymerases elongate DNA across the DNA lesions and afterwards replicative polymerases are re-loaded to continue the replication. Consistently, it was shown in avian cells that both models serve to maintain genome stability. In these cells the C-terminus of REV1 is needed for coordination of TLS at stalled replication forks and PCNA ubiquitination is control TLS postreplicatively, behind the replication forks (Edmunds et al., 2008).

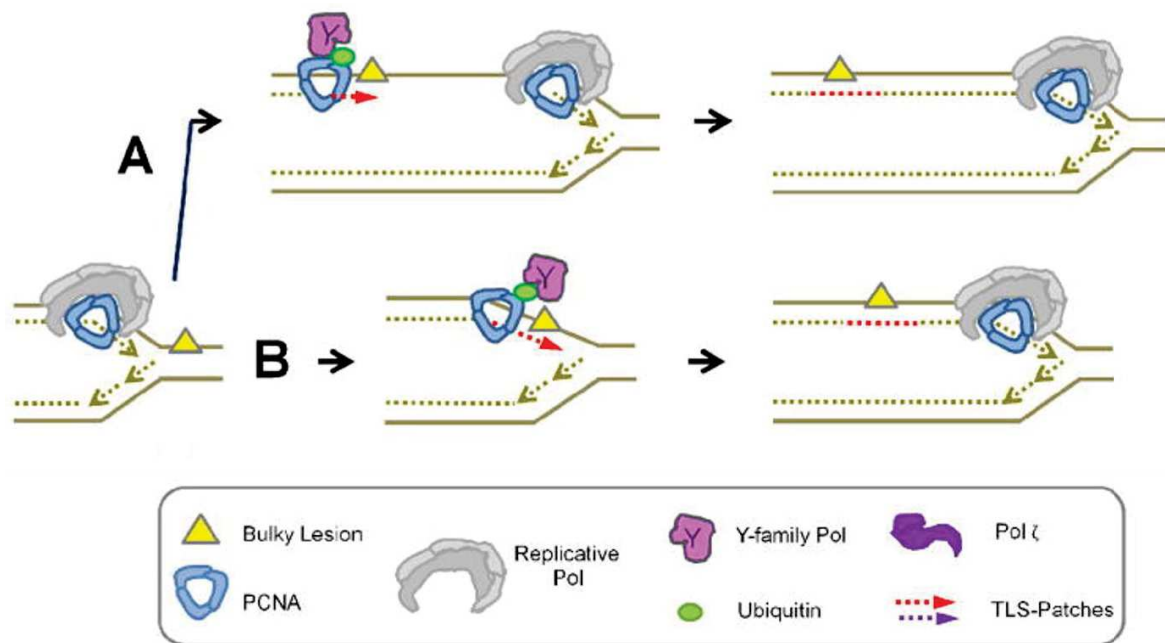


Figure 10: Models for TLS activation adapted Bertolin et al, 2015.

Independently from the model of TLS there are three mechanisms proposed for polymerase switches at stalled replication forks (Ma et al., 2020) (Figure 11). Firstly, PCNA ubiquitination facilitates TLS polymerases interaction at the stalled replication fork, enabling replicative polymerase \rightarrow TLS polymerase switch (discussed further for pol η in the paragraph “TLS by pol η ”) (Kanao et al., 2015; Kannouche et al., 2004). Secondly, REV1 binds to PCNA with its BRCA1 carboxyl terminus (BRCT) and polymerase-associated domain (PAD) domain, and to other polymerases with the C-terminal domain (CTD), building a bridge and facilitating replicative/TLS polymerase \rightarrow TLS polymerase switch (Guo et al., 2003, 2009a; Zhao and Washington, 2017). The REV1 CTD N-terminal interacts with the REV1 interacting region (RIR) motifs of Y family polymerases and PolD3, while the REV1 CTD C-terminal binds REV7 (Boehm et al., 2016; Kikuchi et al., 2012; Pozhidaeva et al., 2012; Pustovalova et al., 2016; Wojtaszek et al., 2012a, 2012b; Xie et al., 2012). Lastly, the switch of polymerases can transpire due to sharing of subunits. It is shown that the pol δ subunit POLD2 interacts with the pol ζ catalytic subunit REV3. It is proposed that at stalled replication forks PolD2 and POLD3 stay at site of replication during TLS and form a complex with REV3-REV7 pol ζ (PolD2/PoID3/REV3/REV7), which may facilitate the polymerase switch between pol $\delta \leftrightarrow$ pol ζ (Baranovskiy et al., 2012).

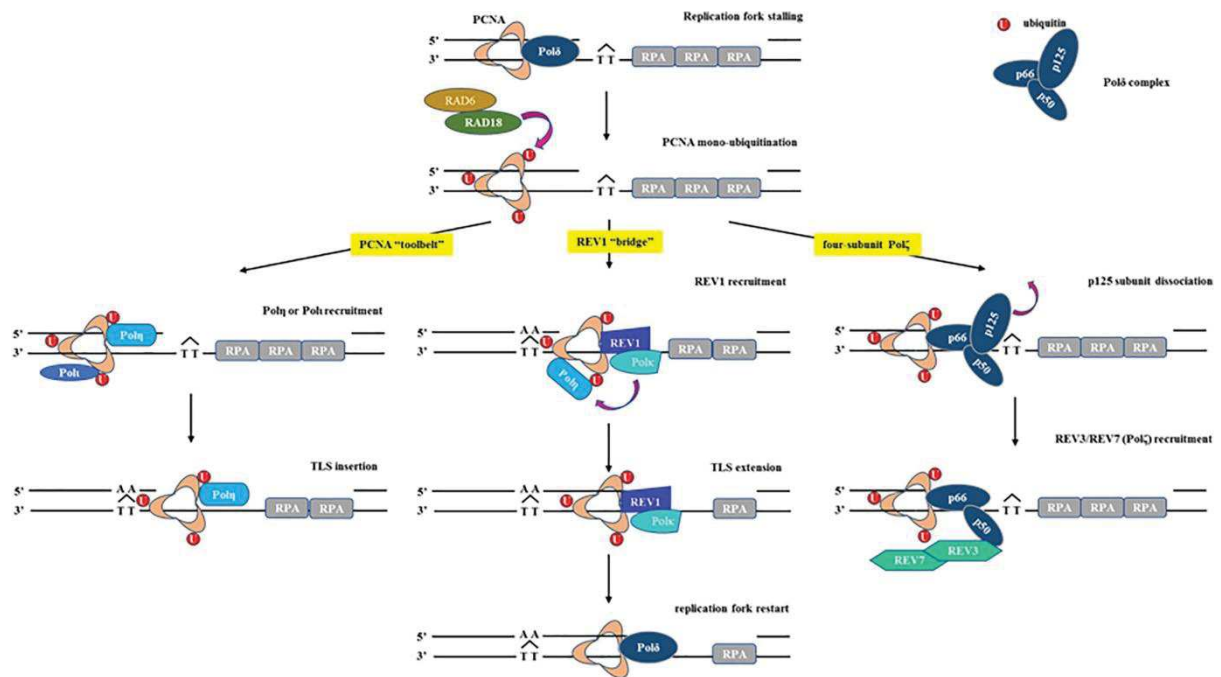


Figure 11: Models for polymerase switching during TLS as shown in Ma et al., 2020.

TLS and cancer

Translesional polymerases have been implicated in the development of cancer by its mutational capacity's and the development of resistance to some cancer treatment. On the other hand, translesional polymerases are important to protect from gross chromosomal instabilities and protection from developing cancer. Replicative DNA polymerases have an error rate of 10^{-6} to 10^{-8} , which is decreased to 10^{-8} to 10^{-10} when those polymerases interact with auxiliary proteins such as PCNA, RPA and MMR (Figure 12). In contrast, TLS polymerases demonstrate low fidelity, with an error rate range of 10^{-1} to 10^{-3} when replicating undamaged DNA (Kunkel, 2004; Nick McElhinny et al., 2008). However, TLS polymerases replicating across their specialized lesions are thought to be error free (Gibbs et al., 2005; Washington et al., 2000).

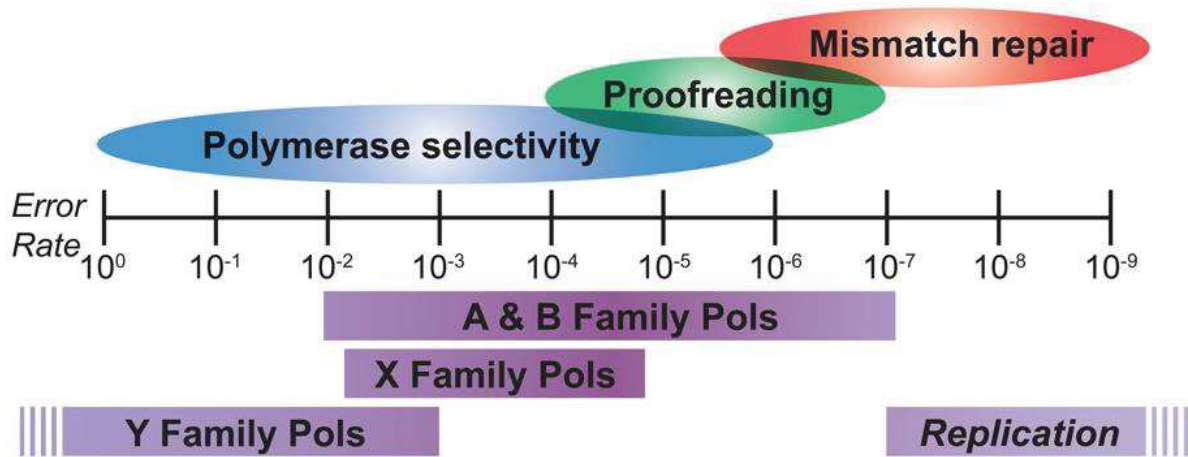


Figure 12: Polymerases error rates from McElhinny et al., 2008. Depiction of error rates of different DNA polymerases when replicating undamaged DNA.

Several TLS polymerases have been reported to be overexpressed in cancers, for example, pol η in ovarian cancer stem cells and HNSCC, pol ι in glioblastoma, pol κ in glioblastomas and lung cancer, which has been associated with resistance against chemotherapeutics and bad prognostics (Albertella et al., 2005; Lemée et al., 2010; O-Wang et al., 2001; Srivastava et al., 2015; Wang et al., 2010, 2004; Zhou et al., 2013). These results show that TLS polymerases can be mutagenic, however, inhibition of TLS polymerases is correlated with an increase in gross chromosomal instability, a hallmark of cancer. Consistently, XPV patients who are deficient for TLS pol η activity, have a very high cancer incidence rate (see chapter on pol η for details). Furthermore, mutations in several TLS polymerases have been associated with cancer, such as, pol ι and REV1 in lung cancer (Sakiyama et al., 2005; Starcevic et al., 2004). Additionally, decreased expression of pol ζ , pol η , pol ι and pol κ have been found in human tumors (Lemée et al., 2007; Pan et al., 2005). Furthermore, the selection of TLS polymerases to specific lesions is crucial to maintain genome stability without the cost of possible mutations. This is demonstrated by TLS across the UV lesion cyclobutane pyrimidine dimers, which can be accomplished by pol η and pol θ . Whereas pol η has a low error rate and pol θ is error prone. Yet, pol θ protects against genome instabilities, as shown by pol θ deficient mice who have a higher tumor rate than WT mice after UV irradiation (Yoon et al., 2019a). Take together it shows on one side the importance of TLS polymerases to maintain genome stability and on the other the mutagenic potential of these polymerases (Figure 13).

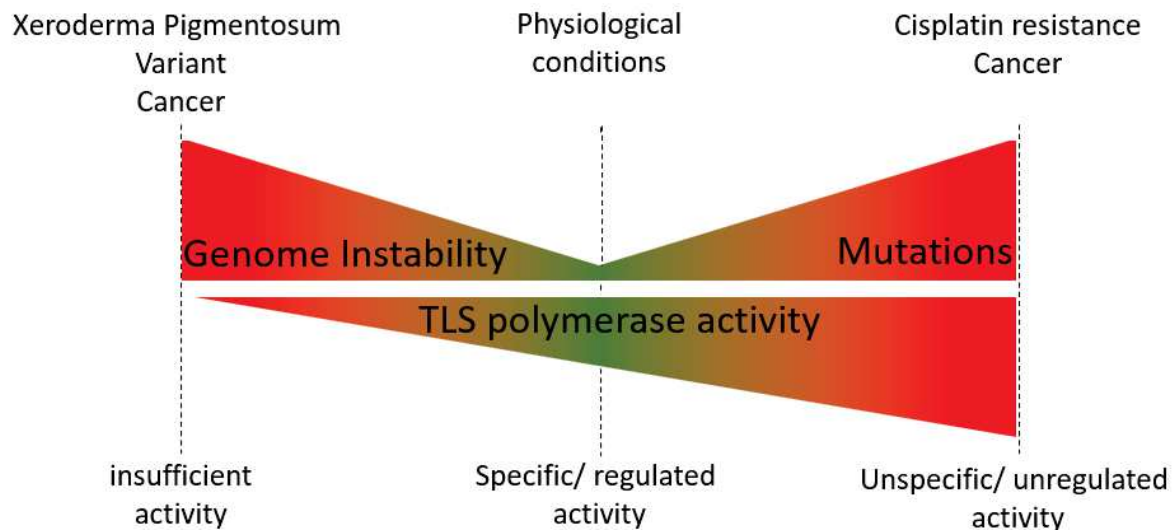


Figure 13: Correlation between polymerase activity and genome instability and mutations

The implication of TLS in cancer can be exploited for cancer treatment in two ways: firstly, sensitizing tumors to genotoxins by inhibition of DNA damage tolerance mechanisms and secondly, using deficiencies in DNA damage tolerance mechanisms to exploit synthetic lethal relationships. Therefore, transient inhibition of TLS activity might be beneficial in the treatment of cancers with specific mutations, triggering synthetic lethality. For example, the ATR replication checkpoint is overactivated in pol η deficient cells leading to cell cycle arrests and these cells rely on ATR to maintain viability during replication stress. Depletion or inhibition of ATR was shown to be synthetically lethal with pol η deficiency, particularly when tumor cells were treated with replication stress-inducing drugs (Barnes et al., 2018; Despras et al., 2010). Another example for the clinical interest of synthetic lethality is the inhibition of poly(adenosine diphosphate [ADP]-ribose) polymerase (PARP) in ovarian- and breast cancer of patients with *BRCA* mutations (Fong et al., 2009). Targeting nonessential TLS pols that serve to facilitate resolution of replication stress and chemoresistance in tumors could be one route to reducing toxic side effects and improving outcomes for patients treated with genotoxic drugs. Inhibition of *REV3* or *REV1* expression by siRNA, sensitized cancers to cisplatin and decreased the formation of cisplatin resistant cells *in vitro*, demonstrating the potential of small molecule inhibitors on TLS mechanisms (Doles et al., 2010; Wu et al., 2004; Xie et al., 2010). Recently, it was shown that the small molecule inhibitor JH-RE-06 targets REV1, inhibiting the dimerization with REV7, disrupting TLS. JH-RE-06 inhibits mutagenic TLS and enhances cisplatin induced toxicity *in cellulo* and suppresses the growth of xenograft human

melanomas in mice (Wojtaszek et al., 2019). Though, inhibition of TLS could result in chromosomal aberrations that promote secondary malignancies or other complications.

Translesion DNA Polymerase η

Pol η belongs to the Y family polymerases and is involved in several mechanisms beyond TLS. It demonstrates mutagenic but also genome stabilising functions, which is why it is important to fully understand its interactome and its regulation.

Pol η implication in Xeroderma pigmentosum variant

The importance of functional pol η is demonstrated by the genetic disease Xeroderma Pigmentosum Variant (XPV). XP-patients display a high sensitivity to ultraviolet light and short exposure to sunlight can lead to severe sunburns. Furthermore, affected people also present with freckling in sun exposed areas, dry skin and changes in skin pigmentation. Moreover, XP-patients have a 1.000 to 10.000-fold increase to develop skin cancers (melanoma and non-melanoma skin cancers) and may present with neurological degeneration (DiGiovanna and Kraemer, 2012). These symptoms are caused by genetic mutations in proteins which manage UV damages (Cleaver, 1968). There are eight different types of Xeroderma pigmentosum, the first seven are caused by mutations in the XPA to XPG genes, which are involved in the NER pathway. The last type is Xeroderma pigmentosum variant (XPV), which makes up about 20 % of all XP patients. These patients have an inactivating bi-allelic mutation in the *POLH* gene coding for pol η . In comparison with the first seven groups of XP patients, people affected with XPV have milder presentations. XPV patients show a delayed onset of cancer with tumors appearing in 20–30 years old individuals and display variable severity of UV sensitivity. Interestingly, XPV patient do not show neurologic abnormalities as it is seen in XPA to XPG patients (Opletalova et al., 2014; Stary and Sarasin, 2002).

Domain structure of pol η

Mammalian pol η consists out of a polymerase catalytic domain in its N-terminus, several PCNA Interacting Protein regions (PIP) and a Ubiquitin-Binding Zinc finger domain (UBZ), two Rev1 interacting Regions (RIR) and a Nuclear Localization Sequence (NLS) in its C-terminus (Kannouche et al., 2001) (Figure 14). In accordance with Y family polymerases, human

catalytic domain of pol η contains four domains: palm, finger, thumb and little finger (LF). The active site resides in the palm domain and DNA is bound between thumb and LF (Biertümpfel et al., 2010).

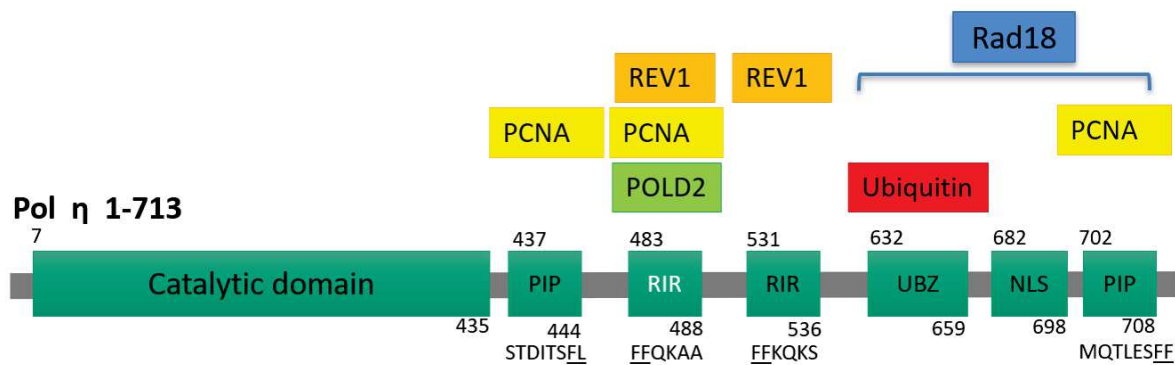


Figure 14: Pol η domains and some interaction partners.

Functions of pol η

Translesion synthesis by pol η plays a key function in genome stability. Furthermore, it is involved in somatic hypermutation, telomere maintenance, DNA repair pathways and replication of difficult to replicate DNA.

Translesional synthesis by pol η

Pol η is specialized on the bypass of Ultraviolet (UV) induced lesions. UV can alter DNA by inducing a variety of DNA lesions (Figure 15). The most energetic part of the solar spectrum that reaches the earth's surface is UVB with a wavelength of 280–320 nm, which accounts a typical proportion of about 0.3% of the whole solar light on the earth surface. UVB irradiation causes the formation of cyclobutane pyrimidine dimers (CPDs) (about 75%) and pyrimidine (6–4) pyrimidone photoproducts (6-4PPs) (about 25%) (Sinha and Häder, 2002). UVA light reaches the earth less energetic with a wave length of 320-400 nm, but 20–times more intense (5.1% of solar light) than UVB light. UVA can induce the formation of CPDs as well as a wide variety of oxidatively generated lesions such as single-strand breaks and oxidized bases, most commonly 8–oxo–7,8–dihydroguanine (8–oxo-Gua) (Figure 15). 8–oxo-Gua can also be produced by other mechanisms causing oxidative stress, such as cellular metabolism and xenobiotics. Lesions, such as CPDs, cause a distortion of the DNA backbone. Replicative polymerases are not capable of synthesizing across such lesions, because they possess a tighter catalytic pocket than pol η . Pol η is capable of synthesizing across the lesion in an error

free manner. p53 plays an important role in preventing cell death after UV light exposure, by favoring translesion DNA synthesis by pol η . UV induces p53 stabilization and transactivation leads to increased expression of pol η (Lerner et al., 2017).

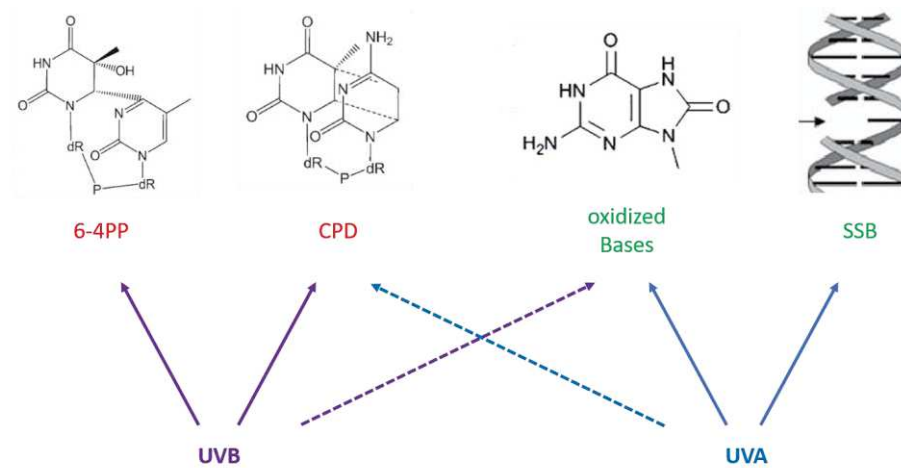


Figure 15: UV induced DNA damage adapted from Cadet et al., 2018

Mechanism of pol η mediated TLS

Upon replication fork blockage, PCNA is monoubiquitinated at K164 by Rad18/6, which facilitates TLS synthesis. Yet, Rad18 lacks a PCNA-binding motif. It has been shown that Rad18 is targeted to PCNA by pol η , promoting PCNA monoubiquitination. This function is unique to pol η among Y-family TLS polymerases (Durando et al., 2013). Interestingly, PCNA K164 is NEDDylated (ubiquitin like) by Rad18 at later time points after oxidative stress or UV damage. This NEDDylation hinders the recruitment of pol η and antagonizes K164 ubiquitination (Guan et al., 2018). Pol η interacts with ub-PCNA via its UBZ and PIP interacting domains. However, these motifs are not required for pol η recruitment but for its retention at stalled replication foci (Despras et al., 2012). Consistently, Sabbioneda et al. state that the pol η residence times increase at replication forks, which is further facilitated by PCNA ubiquitination (Sabbioneda et al., 2008). Furthermore, using purified proteins it was shown that PCNA ubiquitination is not required for the binding of TLS polymerases to PCNA nor for the TLS polymerase switch (Hedglin et al., 2016).

Spartan (also termed C1orf124, contains PIP and UBZ domain) interacts with pol η and (ubiquitinated) PCNA. Spartan interacts also with POLD3 and this interaction is reduced after UV irradiation (Ghosal et al., 2012). It was shown that spartan promotes pol η accumulation to stalled replication forks by its capacity to interact with DNA (Centore et al., 2012; Kim et

al., 2013; Toth et al., 2017). Consistently, spartan deficient cells demonstrated a highly defective pol η foci formation. Remarkably, it was shown that spartan recruits the ubiquitin-selective chaperone p97 (transitional endoplasmic reticulum ATPase) to blocked replication forks. It is proposed that thereby spartan promotes pol η dissociation during DNA repair, preventing excessive TLS (Davis et al., 2012; Mosbech et al., 2012).

Pol η completes insertion and elongation across e.g. CPD lesions in a one-step mechanism and achieves insertion across e.g. 6-4PP in a two-step mechanism, whereas pol ζ does the elongation. After the completed synthesis across the lesion and its elongation by, another polymerase switch occurs and the replicative polymerases continue to replicate along the DNA. PCNA is ISGylated (ubiquitin like) by EFP on K164 and K168, this recruits USP10 which in turn facilitates PCNA deubiquitination and TLS termination (Cipolla et al., 2016; Park et al., 2014). Furthermore, USP1/UAF1 can deubiquitinate PCNA and inhibition leads to pol η dependent UV sensitivity (Liang et al., 2014). Remarkably, UVC irradiation leads to down regulation of USP1 (Huang et al., 2006). Additionally, USP7 can de-ubiquitinate Ubi-PCNA *in vitro* and it suppresses UV- and oxidative-stress-induced PCNA monoubiquitylation *in vivo* (Kashiwaba et al., 2015) (Figure 16). Consistently, USP7 controls the stability of p53 by counteracting the activity of Mdm2 and thereby decreasing the expression of pol η . Yet, USP7 also deubiquitinates pol η directly, stabilizing it (Qian et al., 2015). Furthermore, it was shown that USP7 stabilizes Rad18 and therefore PCNA monoubiquitination (Zlatanou et al., 2016).

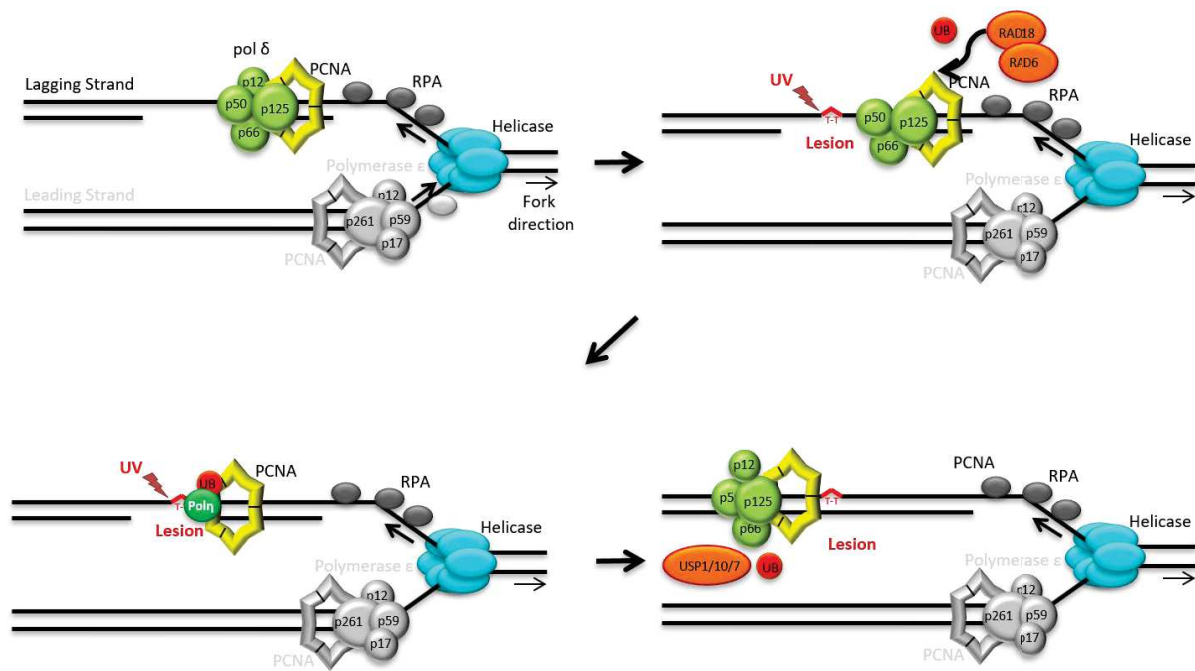


Figure 16: Translesion synthesis

Pol η is capable to replicate through other lesions beside UV induced ones. For instance, N1-methyl adenine (1-MeA) lesions are bypassed by pol η . 1-MeA lesions impair the Watson-Crick base pairing and block replicative DNA polymerases. TLS at 1-MeA lesion site in human cells occurs via three different pathways in which Pol ι and θ function in one pathway and pol η and pol ζ , respectively. TLS opposite this lesion in human cells occurs in a highly error-free fashion (Conde et al., 2015). Additionally, 8-oxo-Gua which can occur after UV irradiation (see above) but is mainly a major lesion that is a consequence of oxidative stress, can be circumvented by pol η . 8-oxoGua is associated with cancer, aging, hepatitis, and infertility (Fraga et al., 1990, 1991; Malins and Haimanot, 1991). Pol η is bypassing 8-oxoGua in a mostly error-free manner, therefore, preventing GC \rightarrow AT transversion mutations (Patra et al., 2014). Moreover, *O*²-[4-(3-Pyridyl)-4-oxobutyl]thymine, a DNA lesion formed from tobacco carcinogens, can be bypassed by a combination of pol η and pol ζ (Gowda and Spratt, 2016). The oxidized form of rNTPs, can be generated in the nucleotide pool by the action of oxygen radicals. These ribonucleotides are used as substrate during DNA replication. Pol η can efficiently and accurately bypass undamaged and damaged ribonucleotides (rG and 8-oxo-rG, respectively) in a more error-free manner compared with deoxynucleoside 8-oxo-dG (Sassa et al., 2016). The capability of pol η to synthesize across ribonucleotides is also used for somatic hypermutation (see “somatic hypermutation”).

Importantly, pol η can replicate along cisplatin induced lesions as described below in “Tolerance and resistance against cisplatin by pol η ”. Similar to the cisplatin resistance by pol η it was recently shown that pol η is mutagenic when replication through cytarabine (AraC) lesions. AraC is a main treatment form against acute myeloid leukemia (Rechkoblit et al., 2019; Yoon et al., 2019b).

Tolerance and resistance against cisplatin by pol η

As described above pol η is involved in the development of cancer, furthermore, it is involved in the development of resistance against cancer treatments. Cisplatin (cisplatinum, or *cis*-diamminedichloroplatinum (II)) is a first line platinum based chemotherapeutic, which is widely used against cancers including bladder-, breast-, cervical-, esophageal-, head and neck-, lung-, ovarian-, and testicular cancers as well as mesotheliomas, brain tumors and neuroblastomas. It was the first FDA-approved platinum based drug against cancer in 1978 (FDA; Kelland, 2007). Cisplatin crosslinks purine bases of the DNA most frequently, about 90%, within one DNA strand (intrastrand) and less often between two strands (interstrand) (Eastman, 1987; Fichtinger-Schepman et al., 1985; Jamieson and Lippard, 1999; Poklar et al., 1996). Furthermore, monoadduct are formed which are likely bypassed by a replicative polymerase with a low error frequency, such as polymerase δ . The crosslinks interfere with DNA repair and replication mechanisms, which cause more DNA damage and subsequently leads to apoptosis of cancerous cells. However, the drug also attacks cell organelles, triggering the production of ROS, the release of lysosomal proteases and the deregulation of calcium storages and the misfolding of proteins (Dasari and Tchounwou, 2014). Unfortunately, this mechanism of action can cause severe side effects, which is one of the main disadvantages. The other main disadvantage of this drug is the development of resistance. The less common cisplatin adducts, the interstrand crosslinks, can be resolved by NER and TLS. Cisplatin induced intrastrand cross-links can be bypassed by pol β , ζ , η , and μ . Although, pol η demonstrated to be the most efficient of these polymerases to insert a base opposite the platinated purines (Havener et al., 2003; Hoffmann et al., 1995; Vaisman et al., 2000). In vitro bypass of cisplatin lesions requires pol η to insert dCTP opposite the 3' guanine and pol ζ , to extend the primers (Lee et al., 2014a). Structural and biochemical analyses revealed that pol η inserts the correct nucleotide opposite the first G of cisplatin-GG, but it is less efficient and promiscuous in the bypass of the 5'dG (Alt et al., 2007; Ummat et al., 2012). This leads to mutations, which can

develop into resistance against cisplatin, through e.g. decreasing drug uptake, increasing drug efflux, and inducing drug detoxification by covalent binding to glutathione or metalloproteins. Consistently, high levels of pol η are associated with the development of cisplatin resistance in bladder, lung and ovarian cancers (Srivastava et al., 2015; Zhang et al., 2019a). It was shown that pol η is strongly induced after cisplatin treatment in a p53-dependent manner (Lerner et al., 2017). Furthermore, pol η is needed to overcome the cisplatin dependent S-phase arrest (Albertella et al., 2005).

Interestingly, when the cells were treated with transplatin, which preferentially forms interstrand crosslinks, pol η -dependence was not detected (Albertella et al., 2005). Furthermore, cisplatin-induced mutations are 2- to 2.5-fold higher in fibroblasts lacking pol η in comparison with normal or high expressing pol η fibroblasts, confirming that pol η is mostly involved in error-free translesion synthesis past cisplatin crosslinks (Bassett et al., 2004; Vaisman et al., 2000). Taken together, pol η is important for intrastrand lesion tolerance and intrinsic resistance to cisplatin. Mutagenic effects of cisplatin in pol η negative cells suggests that other polymerases synthesizing DNA across the adducts in an error prone manner, leading to acquired resistance. Indeed it was shown that pol κ in combination with pol ζ results in error-prone TLS past cisplatin adducts (Shachar et al., 2009). Additionally, pol ζ has been shown to be active in translesion DNA synthesis, complementing pol η during cisplatin adduct bypass in *in vitro* experiments (Lee et al., 2014b).

To overcome the disadvantage of cisplatin treatment combination therapies are highly researched and are applied in the clinic (Dasari and Tchounwou, 2014). Inhibition of TLS polymerases can have a dual anticancer effect, sensitizing the tumors to cisplatin and limiting the emergence of resistance.

Somatic hypermutation

The mutative capacity of pol η is needed during somatic hypermutation, a process by which B-cells mature in germinal centers of secondary lymphoid organs. This process generates cells displaying receptors with the highest affinity and specificity for a given antigen that will differentiate into either plasmocytes or memory B cells (Longo and Lipsky, 2006). During this process mutations accumulate in rearranged variable (V), diversity (D), and joining (J) genes on the heavy (H), kappa (κ) and lambda (λ) Ig loci (Saribasak and Gearhart, 2012). Point mutations, the most common alterations, occur with a frequency of 10^{-2} to 10^{-3} per bp in

these regions, a rate that is about 10^6 -fold higher than the background mutation rates observed in other genes (Lebecque and Gearhart, 1990). Additionally, tandem double base substitutions, deletions, and insertions can occur. With every B-cell division mutations accumulate, however, many of these mutations reduce the affinity of the antibody. B-cells carrying affinity-increasing mutations have a selective advantage when competing for the antigen, provoking clonal selection. Therefore, B-cells can produce a generation of highly affine antibodies. Somatic hypermutation in antigen-activated B-cell is triggered by T-cells, upon which the B-cell expresses activation-induced deaminases (AID). AID deaminates cytosine residues to uracil on both strands (Golding et al., 1987). There are two types of mechanisms discussed in the literature following the DNA deamination to produce point mutations (Steele, 2016). Firstly, the DNA Deamination Model by which mutations are generated by five different modes by DNA repair and TLS proteins on the DNA (Figure 17) (Pilzecker and Jacobs, 2019). Secondly, the RNA/Reverse Transcriptase model which describes the use of RNA as a template to synthesize DNA strands containing mutations (Figure 18) (Steele, 2016). It was shown that pol η is implicated in repeat expansion diseases at RNA polymerase II-transcribed genes due to slipped hairpin-stabilized which can be explained with both the DNA deamination model and the RNA/Reverse transcriptase model (Franklin et al., 2020).

DNA Deamination Model

There are five mutagenic pathways described during SHM, which follow the DNA deamination model. 1. The uracil synthesis pathway, during which adenine is directly inserted opposite the uracil template (Figure 17-1). 2. The UNG2 TLS pathway; the glycosylase UNG2 converts an uracil into an abasic site, which is filled by TLS, generating both transitions and transversions (Figure 17-2). 3. The ncMMR UNG2 hybrid pathway is a mix between the non-canonical mismatch repair (ncMMR) and UNG2 dependent TLS (Figure 17-3). 4. The ncMMR pathway generates the majority of A/T mutagenesis. Therefore, MSH2/MSH6/EXO1 remove part of the uracil containing strand and PCNA-Ub/pol η fill the gap (Figure 17-4). 5. The UNG2 PCNA-Ub pathway; UNG2 converts uracil into an abasic site and PCNA-Ub/pol η fill the abasic site. This non-canonical long-patch BER pathway generates a minor but significant subset of A/T mutations (~8%) (Figure 17-5).

G/C base pair mutations involve several error-prone DNA polymerases (Rev1, pol ι and pol ζ), while A/T base pairs mutations depend exclusively on the activity of pol η (Franklin et al., 2020; Wilson et al., 2005). Coherently, XPV patients present a decreased frequency of mutations at A/T sites in immunoglobulin genes and more G/C template mutations, while the frequency of overall mutations changes just slightly (Delbos et al., 2005, 2007; Reynaud et al., 2009). Furthermore, it has been shown that XPV patients demonstrate an increased frequency of large deletions (Lerner et al., 2020).

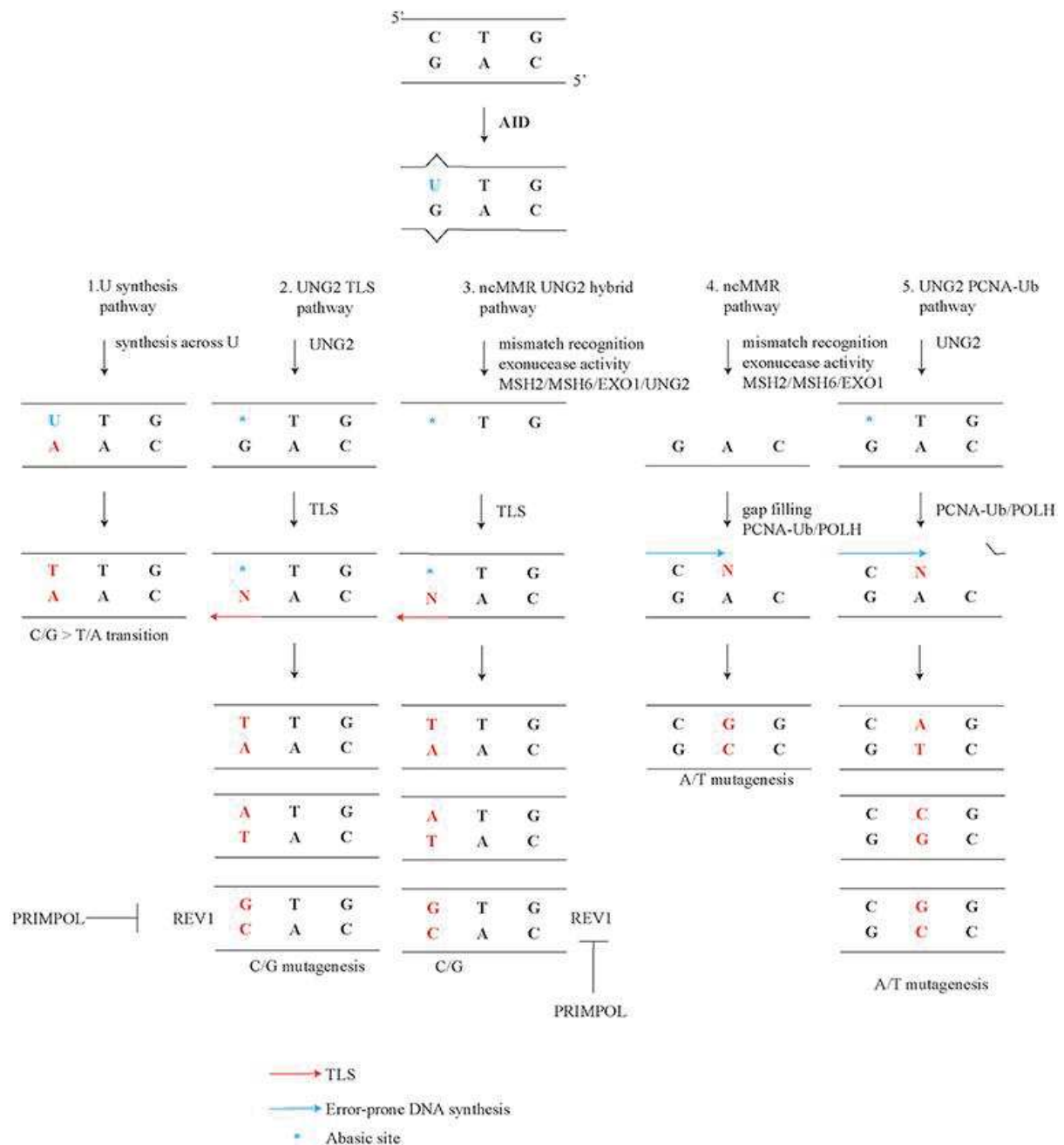


Figure 17: Somatic hypermutation - DNA Deamination Model as shown in Pilzecker and Jacobs, 2019. Depiction of five modes of mutagenic Uracil processing.

RNA/Reverse Transcriptase model

Similar to the DNA based model, the RNA/Reverse transcriptase model starts with the deamination of cytosines. Then, RNA Pol II introduces mutations into the newly synthesized mRNA as it copies deaminated nucleic acids from the transcribed DNA strand (Kuraoka et al., 2003). Followed by ADAR1-mediated (adenosine deaminases that act on RNA) A-to-I RNA editing of WA sites (adenine followed by an adenine or uracil)(Steele et al., 2006). These mRNA is then reverse transcribed to cDNA by pol η . Afterward follows an unknown process

of strand invasion, heteroduplex formation and/or resolution of heteroduplex, integrating the mutated cDNA into the VDJ genes.

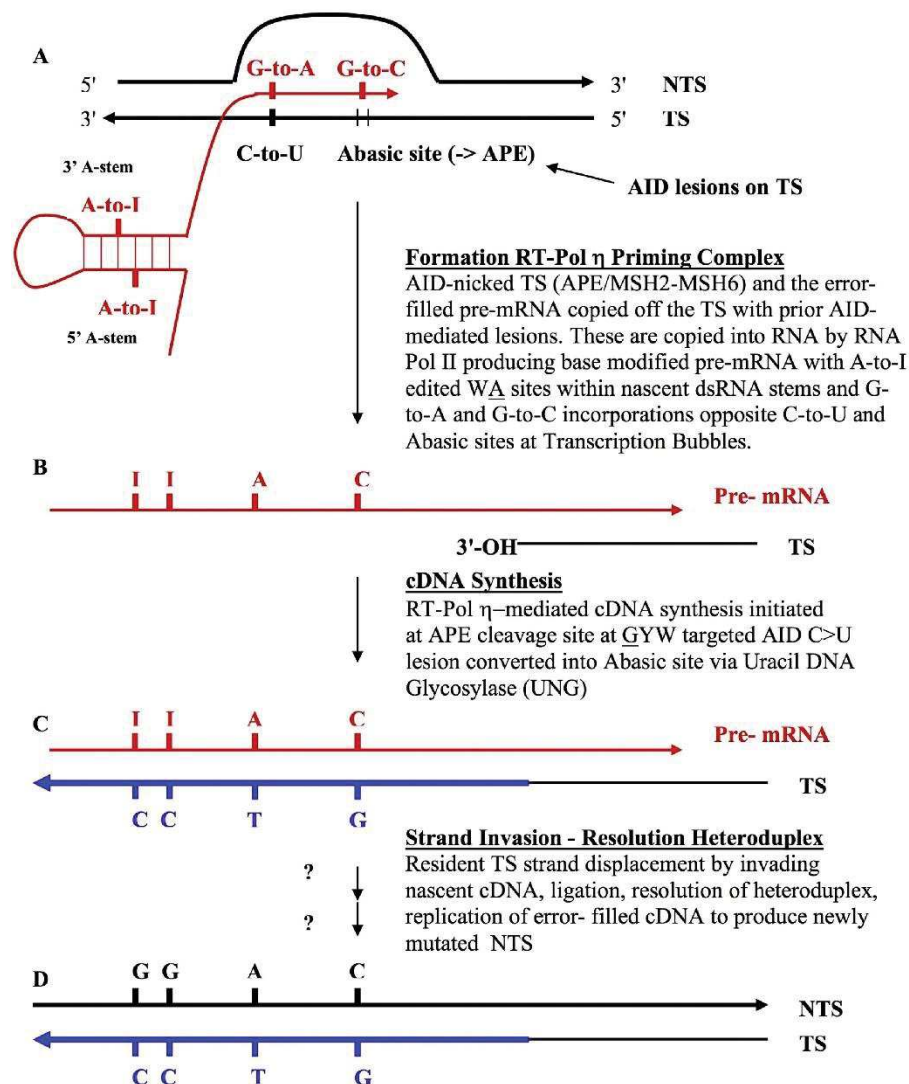


Figure 18: Somatic hypermutation - RNA based model or reverse transcriptase model as shown in Steele, 2016.

Implications of pol η in non-canonical mismatch repair

As described above in the DNA deamination model for somatic hypermutations, pol η interacts with the mismatch repair pathway proteins MSH2-MSH6. This mechanism occurs also during a non-canonical mismatch repair pathway (Hsieh, 2012). Pol η interacts with transient ubiquitinated PCNA-induced at oxidative lesions in an S-phase independent manner. This modification requires the presence of MSH2-MSH6 and allows the repair of oxidative or alkylated DNA damage in a “non-canonical” MMR dependent pathway (Zlatanou et al., 2011). Interestingly, mismatch repair proteins and pol η recruitment at active chromatin was shown

to be the cause of the clustered mutations (over a distance of less than 100 nucleotides) observed in several types of tumors (Peña-Díaz et al., 2012; Supek and Lehner, 2017). Thus, in tumor cells experiencing replication stress, pol η participates in tumorigenesis by inducing mutagenesis, including mutations responsible for the activation of oncogene.

Other functions of pol η

Pol η demonstrates further function, such as, its requirement for the stability of common fragile sites (CFSs) (Bergoglio et al., 2013; Rey et al., 2009). CFSs are typically several hundred kb regions of chromosomal DNA that are replicated in late S-phase, which are intrinsically difficult to replicate. CFSs rearrangements are considered a driving force of oncogenesis (Durkin and Glover, 2007). Furthermore, it was shown that pol η travels with the replication fork in unperturbed S-phase when it is sumoylated and thereby preventing under-replicated DNA during mitosis (Despras et al., 2016a). Interestingly, pol η was recently shown to compete with the replicative pol α and pol δ for the synthesis of the lagging strand genome-wide in yeast, with a preference for T-T in the DNA template (Kreisel et al., 2019). Furthermore, it was shown that there is a strand bias for WA>WG transitions in human melanomas due to pol η activity, suggesting an evolutionary conservation of strand specificity. Recently, it was shown that pol η extends RNA primers, yet, in humans with a 10^3 -fold lower rate than when incorporating dNTPs. This RNA synthesis can also bypass 8-oxoguanine and thymine dimer DNA lesions error-free (Mentegari et al., 2017; Su et al., 2016). This activity was recently shown to be implicated in transcription in *Saccharomyces cerevisiae* (Gali et al., 2017).

A recent study has shown that pol η is involved in the alternative lengthening of telomeres (ALT) (Garcia-Exposito et al., 2016). A proximity-dependent biotinylation assay revealed that multiple DNA repair pathways are present at ALT telomere. The author hypothesize that pol η is involved in the managing of replicative stress at ALT telomeres by restarting stalled replication forks and stimulating DNA synthesis by pol δ . Additionally, pol η is involved in the recombination-associated DNA synthesis during HR (Buisson et al., 2014). PALB2 and BRCA2 interact with pol η to sustain the recruitment of pol η at blocked replication forks. This interaction stimulates pol η -dependent DNA synthesis on D loop substrates (Buisson et al., 2014). PALB2 is also involved in telomere ALT mechanisms (Martinez et al., 2017; Pan et al., 2019). The pol η /PALB2 interaction might stabilize pol η during replicative stress at telomeres.

Regulation of pol η by protein interactions and posttranslational modifications

Proteins interacting with pol η

As described above pol η has diverse functions, which can be contradictory in terms of error prone and error free mechanisms. Therefore, pol η needs to be regulated by several means. One way of regulating its activity is by protein interactions. Some of the most studied interactions are depicted in Figure 14 with the site of interaction on pol η . Figure 19 shows interactions with pol η , which have been demonstrated by at least two methods. Examples are for instance the above described interaction of pol η with PCNA as a scaffold protein during TLS and replication. Pol η also interacts with other polymerases, one of them is the replicative pol δ POLD2 as a possible way to facilitate the polymerase switch (Baldeck et al., 2015). Additionally, pol η interacts with the translesional polymerases pol ι and REV1 (Boehm et al., 2016; Kannouche et al., 2003)(Paragraph "Polymerase switch"). Pol η was also described to interact with ubiquitinated H2B and FANCD2, helping pol η to be tethered to DNA (Fu et al., 2013; Northam and Trujillo, 2016). Also ubiquitinated H2A was proposed to recruit the Rad18/pol η complex (Despras et al., 2016a). Furthermore, pol η interacts with the homologous recombination proteins PALB2 (Partner and localizer of BRCA2) and BRCA2 (breast cancer type 2 susceptibility protein), which is crucial in the initiation of recombination-associated DNA synthesis (Buisson et al., 2014) (Paragraph "Other functions of pol η "). Pol η also interacts with Rad18, building a bridge between pol η and the PIAS1 SUMO ligase to promote pol η SUMOylation. Further examples are given throughout the introduction.

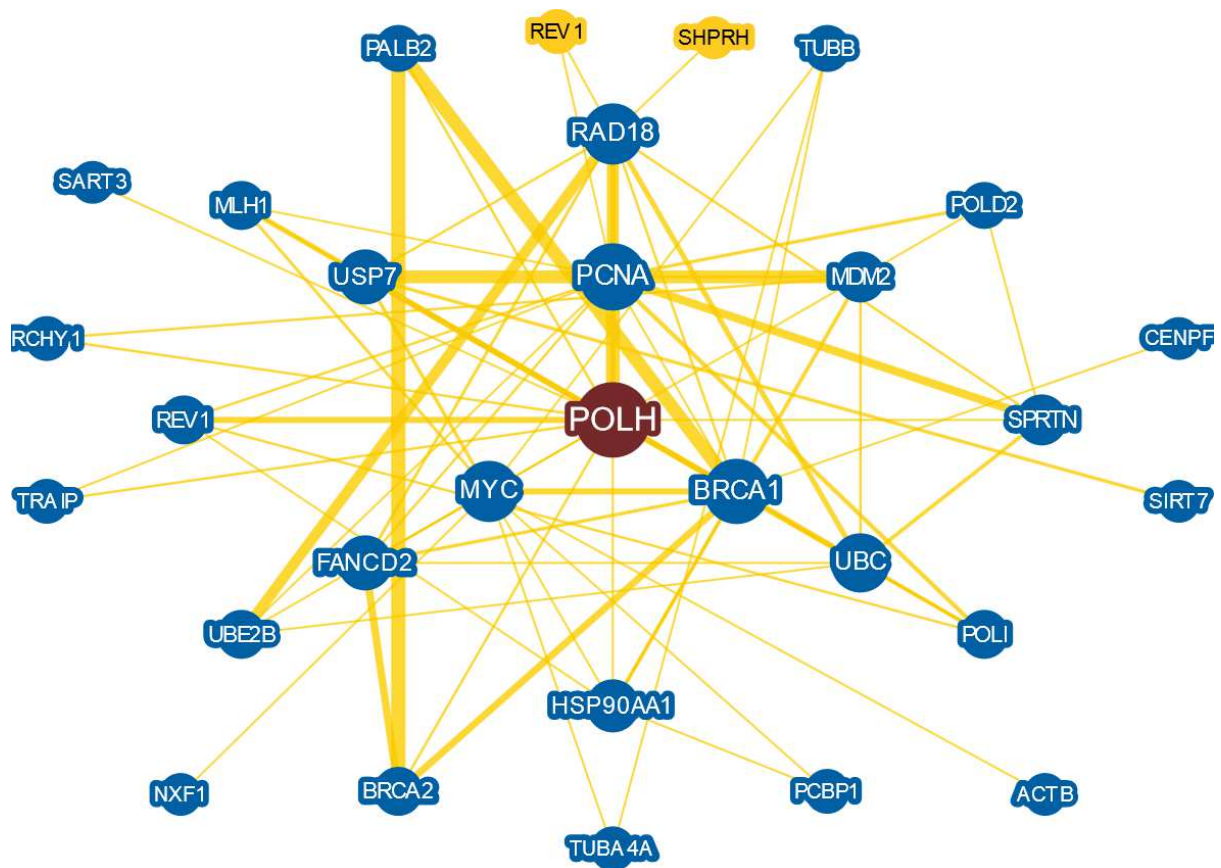


Figure 19: Proteins interacting with pol η from BioGrid3.5 software. Pol η interaction network presenting protein interactions with at least two experimental proofs of interaction. Proteins highlighted in blue are human proteins; Proteins highlighted in yellow are originated from other organisms. Greater node size represents increased connectivity and thicker edge sizes represent increased evidence supporting the association.

Posttranslational modification of pol η

Another mean of regulating the activity of pol η is by posttranslational modifications. There are several posttranslational modifications reported for pol η (Sumoylation, Ubiquitination, GlcNAcylation and Phosphorylation) (Figure 20).

Firstly, pol η **sumoylation** on K163 is necessary for pol η to travel with the replication fork during unperturbed S-phase. Cells expressing pol η mutants which cannot be SUMOylated present with replication defects in response to mild replication stress, leading to chromosome fragments in mitosis (Despras et al., 2016a). Rad 18 builds a bridge between pol η and PIAS1 SUMO ligase, promoting the sumoylation of pol η . Recently, it was shown that pol η is PIAS1-dependent polysumoylated when it is associated with monoubiquitinated PCNA at DNA

damaged site. This sumoylation recruits SUMO-targeted ubiquitin ligase (STUbL) promoting the extraction of pol η (Guérillon et al., 2020a).

Secondly, pol η can be **mono- and polyubiquitinated** at several sites. For instance, pol η is monoubiquitinated at K682 within the NLS sequence. Interestingly, if this site is mutated so that it cannot be ubiquitinated, the neighboring lysines would become a targeted for ubiquitination. Thus, pol η interacts with PCNA at two sites: the pol η PIP box interacts with PCNA and also the UBZ domain of pol η interacts with monoubiquitin attached to PCNA, forming an extended pol η -PCNA interaction surface. Furthermore, Jung et al. showed that Pirh2 mediated pol η monoubiquitination inhibits the interaction between pol η and PCNA, leading to decreased viability of UV-damaged cells (Jung et al., 2011). Bienko et al. propose a model where monoubiquitinated pol η can interact intramolecular between monoubiquitin and the UBZ domain, hindering pol η from interacting with PCNA during replication. Following UV irradiation, PCNA is monoubiquitinated and pol η is deubiquitinated, which in turn facilitates the interaction between pol η and PCNA. Consistently, monoubiquitination of pol η is downregulated by various DNA-damaging agents (Bienko et al., 2010). Additionally, Mdm2 is polyubiquitinating pol η promoting its degradation under basal condition in response to UV irradiation (Jung et al., 2012). Pol η K462 is polyubiquitinated by CRL4^{CDT2} promoting pol η degradation (see O-GlcNAcylation) (Ma et al., 2017). Several more ubiquitination sites have been found in pol η , however, their relevance is not yet known (Akimov et al., 2018; Beltrao et al., 2012; Rose et al., 2016).

Thirdly, pol η can be **O-GlcNAcylated** by O-GlcNAc transferase at T457. Pol η O-GlcNAcylation is induced by both UV and cisplatin exposure. Non O-GlcNAcylated pol η shows a significantly decreases K48-linked polyubiquitination at the adjacent K462. The following model is proposed: pol η is recruited to stalled replication forks and is O-GlcNAcylated. After pol η completes its task in TLS, O-GlcNAcylated pol η is ubiquitinated by CRL4^{CDT2} E3 ligase. This pol η polyubiquitination is recognized by the p97-UFD-NPL4 complex, resulting in its dissociation from replication forks and degradation. Thereby, O-GlcNAcylation of pol η is promoting its removal from replication forks by facilitating polyubiquitin-induced degradation and subsequent polymerase switches (Ma et al., 2017).

Fourthly, pol η can be **phosphorylated** at several sites, by different kinases. Phosphorylation of pol η is increased after UV irradiation. Preventing phosphorylation of S587 and T617 by mutation hinders nuclear foci formation induced by UV irradiation or treatment with gemcitabine/cisplatin (Chen et al., 2008). Furthermore, pol η phosphorylation at S601 by the ATR kinase depends on the interaction of pol η with Rad18 but not with Ub-PCNA after UV irradiation. This phosphorylation contributes to UV survival and is involved in the checkpoint response to UV damage (Göhler et al., 2011). Additionally, S687 is phosphorylated by Cyclin Dependent Kinase 2 (CDK2) in the absence of damage in a cell cycle-dependent manner. Phosphorylation on S687 protects pol η from degradation, which allows pol η to accumulate in late S and G2 when productive TLS is crucial for cell survival (Bertoletti et al., 2017). The S687 phosphorylation by CDK2 is also increases upon UV irradiation. This phosphorylation in the NLS sequence results in diminished interaction of pol η with PCNA in vitro, which is proposed to promote the polymerase switch from pol η to pol δ (Dai et al., 2016). Furthermore, cells expressing pol η with mutations of the phosphorylation sites S510, S512 and S514 are more sensitive to UV irradiation (Bertoletti et al., 2017).

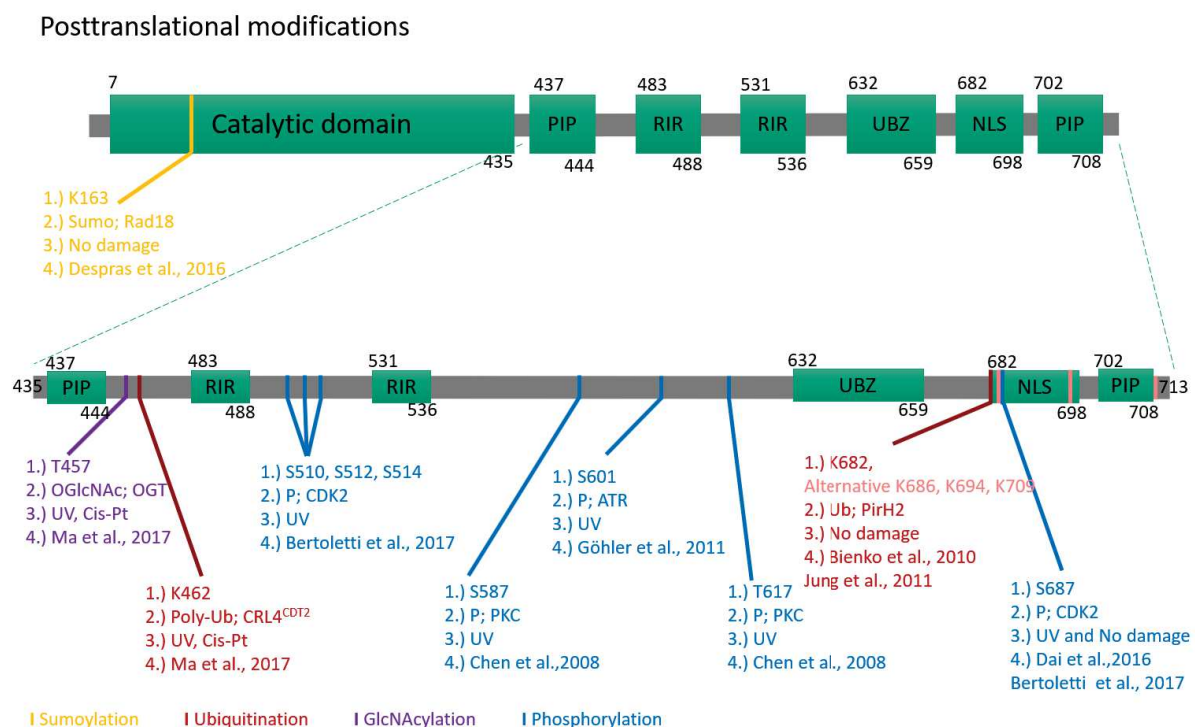


Figure 20: Posttranslational modifications of pol η . 1.) = site of modification; 2.) = enzyme of modification; 3.) = in dependence of which lesion; 4.) = reference.

Calpains

Yeast two-hybrid screening, conducted in our laboratory, identified CAPNS1 as a putative pol η binding protein. CAPNS1 is a regulatory subunit essential for the stability and function of calpain 1 and 2. Calpains (EC 3.4.22.17; Clan CA; Family C2,) are a family of non-lysosomal cysteine proteases, which are evolutionarily well-conserved from bacteria to mammals. These proteases modulate the structure and function of their specific substrate through limited proteolysis rather than complete degradation. The importance of calpain 2 and CAPNS1 is demonstrated by the fact that their mutations are embryonic lethal (Arthur et al., 2000; Dutt et al., 2006; Takano et al., 2011; Zimmerman et al., 2000). Genetic mutations in the calpain 1 gene *CAPN1* lead to spastic paraplegia, platelet dysfunction and spinocerebellar ataxia (Azam et al., 2001; Gan-Or et al., 2016; Wang et al., 2016a).

Calpains were shown to be involved in the same pathways or mechanisms as pol η . It was shown that calpain can be activated by UV light irradiation (Gulati et al., 2004), whereas pol η is needed for UV lesion bypass. Furthermore, it was shown that, during replication stress the Ca^{2+} level in the nucleus is elevated (Li et al., 2019). The calcium flux leads to phosphorylated Exo1, which cannot be recruited to stressed replication forks and thus circumventing unscheduled fork resection. This finding reveals a direct link between Ca^{2+} signaling and the DNA damage cell response.

The calpain family

Humans possess 15 calpain genes (*CAPN1-3*, *CAPN5-16*) and two calpain subunit genes (*CAPNS1* and *2*). The 15 calpain proteins expressed by these genes are classified by their domain structure or tissue distribution as depicted in Figure 21 A and B (Ono and Sorimachi, 2012; Sorimachi et al., 2011). Humans have 9 classical calpains and 6 non-classical calpains (Figure 21B). Whereas most calpains are expressed ubiquitously, at least six calpains are tissue specific as highlighted in black in Figure 21 A.

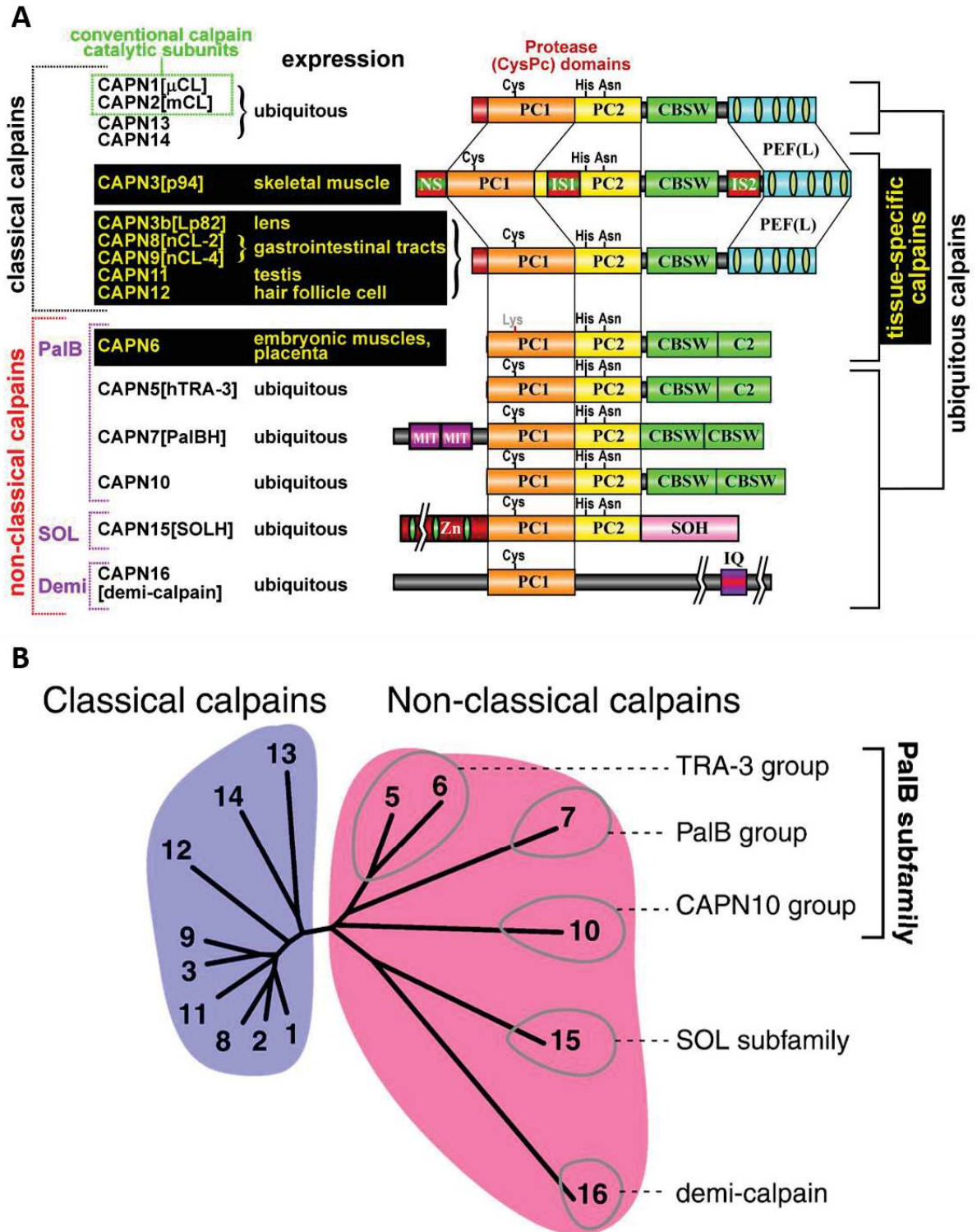


Figure 21: Calpain family classification as described in Ono and Sorimachi, 2012

Interestingly, some human calpains, particularly those with non-classical domain structures, are very similar to calpain homologs identified in evolutionarily distant organisms (Table 1). Whereas, the classical calpains are mostly conserved in vertebrates and until now homologs

have not been found in *Caenorhabditis elegans* (nematode), trypanosomes, plants, fungi, or *Saccharomyces cerevisiae* (budding yeast) (Table 1).

Table 1: Calpains across species as shown on the calpain research portal “calpain.net”. **TM**: transmembrane domain, **MIT**: microtubule interacting and transport motif.

Classification	Subfamily	Subgroup	Consensus structure (see Fig. 1)	Human	Mouse	Chicken	Frog	Fish	Mosquito	Fly	Schistosome	Nematode	Fungi	Yeast	Plant	Bacteria	
classical calpain			CysPc-C2L-PEF	CAPN1[μCL]	✓	✓	✓	✓									
				CAPN2[mCL]	✓	✓	✓	✓									
				CAPN3[p94]	✓	✓	✓	✓									
				CAPN8[nCL-2]	✓	✓	✓	✓	✓								
				CAPN9[nCL-4]	✓	✓	✓	✓	✓	✓ ^{b)}	✓	✓					
				CAPN11[μ/mCL]	✓	✓		✓									
				CAPN12	✓												
				CAPN13	✓	✓											
				CAPN14			✓		✓								
	non-classical calpain	PalB		PalB	MIT-CysPc-C2L-C2L	CAPN7[PalBH]	✓	✓	✓	✓	✓		✓	✓	✓	✓	✓ ^{d)}
PalB		TRA-3	CysPc-C2L-C2	CAPN5[hTRA-3]	✓	✓	✓	✓		✓	✓	✓					
		CAPN10	CysPc-C2L-C2L	CAPN10	✓	✓	✓	✓									
SOL			Zn-CysPc-SOH	CAPN15[SOLH]	✓	✓	✓	✓	✓	✓		✓	✓				
demi			part(s) of PC1 and/or PC2	CAPN16[demi-calpain]	✓	✓	✓	✓	✓		✓	✓					
DEK1			(TM)-CysPc-C2L	CAPN10:ex1-7 9 10B[calpain-10b] ^{b),c)}						✓ ^{b),c)}		✓ ^{b)}				✓	
bacterial			CysPc	CAPN8:ex1-9 10B[nCL-2'] ^{b),c)}		✓ ^{c),d)}											✓

^{a)} Insects and schistosomes have classical calpains that are not significantly similar to specific human classical calpains, but show equal similarity to the human proteins.

^{b)} These calpains do not contain a TM domain.

^{c)} These are alternative splicing products of the genes that encode classical calpains, and other species without a ✓ may also have such gene products.

^{d)} Yeast Rim13/Cpl1 does not contain a sequence that can be explicitly identified as an MIT motif.

In mammals, the two best-characterized members of the calpain family are calpain 1 and calpain 2 (μ-calpain and m-calpain, respectively). Calpain 1 and 2 bind to CAPNS1 in a calcium (Ca²⁺) dependent manner. Calpain 1 and 2 contain calpain-type beta-sandwich domain (CBSW) and penta-EF-hand domain (PEF) in addition to the calpain-like cysteine protease domain (CysPc). The CysPc domain consists out of the two protease core domains 1 and 2 (PC1 and PC2) (Figure 21A).

Regulation of calpain by calcium and calpastatin

Activation of calpain

Most calpains are Ca²⁺ activated. The Ca²⁺ ion is a ubiquitous signaling system in various cell types, and is involved in a vast range of cellular processes. Intracellular free Ca²⁺ concentration varies depending on its location. About 10⁻⁷ M in the cytoplasm, nuclear matrix and mitochondrial matrix whereas the Ca²⁺ concentration the extracellular milieu is much

higher with $\sim 10^{-3}$ M (Bagur and Hajnóczy, 2017). The Ca^{2+} homeostasis is kept through calcium transporters (e.g. plasma membrane Ca^{2+} transport ATPase (PMCA) and $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX)) and transmembrane Ca^{2+} pumps in the plasma membrane and the membrane of intracellular Ca^{2+} stores. The main intracellular Ca^{2+} store is the endoplasmic reticulum (ER), which is continuous with the nuclear envelope, maintains a Ca^{2+} concentration of $1\text{-}5 \times 10^{-4}$ M (Mauger, 2012). Excessive Ca^{2+} is transported into the extracellular milieu, or stored within endoplasmic reticulum. The release of these Ca^{2+} molecules generate cell signals. High intracellular Ca^{2+} concentration can lead to many pathological mechanisms because of its reactivity with carboxyl groups (protein and nucleic acid aggregation, instability of plasma membrane, precipitation of phosphates, sulphates, and carbonates).

Calpain 1 and calpain 2 need to heterodimerize with CAPNS1 in a calcium dependent manner, forming functional proteases. Calpains can bind up to ten Ca^{2+} atoms during activation, resulting in several conformational changes. In particular, the two PEF domains are closer to the core and have displaced the N-terminal anchor helix (see Figure 22: Calcium dependent activation of calpain) (Moldoveanu et al., 2008). Activated calpain 1 can undergo autoproteolysis from the 80 kDa catalytic subunit into a 78 kDa and 76 kDa subunit, resulting in a lower Ca^{2+} concentration requirement for its activity (Li et al., 2004). Calpain 2, also 80 kDa, is autoproteolysed into a 79 kDa and 78 kDa protein. It was also shown that CAPNS1 autolysis, from 28 kDa to 20 kDa and 18 kDa, is associated with the dissociation from calpain (Daniel et al., 2003; Hosfield, 1999). Calpain 1 and 2, are also termed μ - and m-calpain in accordance with the Ca^{2+} concentration (μM and mM respectively) required for their catalytic activity *in vitro* (Goll et al., 2003; Storr et al., 2011). *In vitro* activation of calpain requires Ca^{2+} concentrations in the micromolar range, which is relatively high for an *in vivo* activation, as cells have a free Ca^{2+} concentration of about 10-100 nM. Phospholipids, a major component of plasma membrane, can lower the required Ca^{2+} concentration for calpain activation (Beltran et al., 2011; Saido et al., 1992; Shao et al., 2006). Interestingly, a Ca^{2+} influx induced by ionomycin promotes also the nuclear entry of the mostly cytosolic calpain 2 (Baek et al., 2016; Chou et al., 2011).

Interestingly, calpains can be activated by different pathways, although the Ca^{2+} activation is considered the main activator. The epidermal growth factor (EGF) activates calpain 2 via phosphorylation by ERK/MAP kinase signaling pathway (Glading et al., 2000, 2001). Furthermore, it was shown that inhibition of phospholipid synthesis in membranes blocks

EGF-mediated calpain 2 activity (Shao et al., 2006). Lastly, the p94/calpain 3 protease can be activated by Na^+ in the absence of Ca^{2+} (Ono et al., 2010).

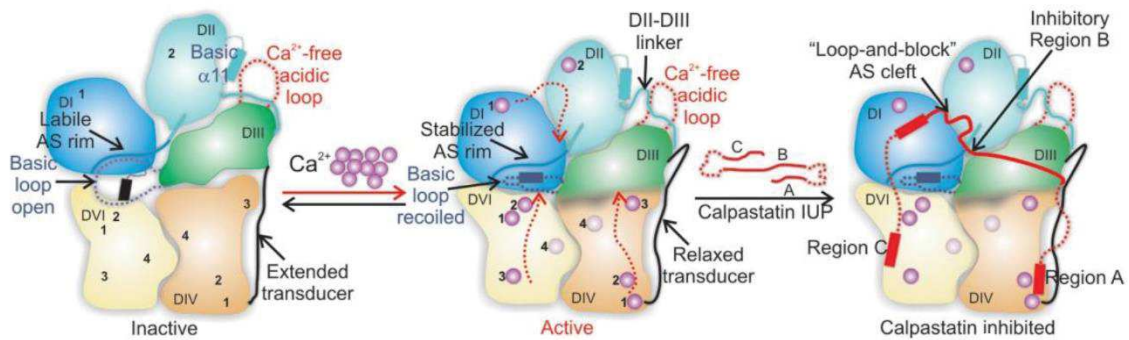


Figure 22: Calcium dependent activation of calpain as shown in Moldoveanu et al., 2008.

Inhibition of calpain by the endogenous inhibitor calpastatin

Calpastatin is an endogenous specific inhibitor for the calpains CAPN1, CAPN2, CAPN8[nCL-2], and CAPN9[nCL-4] (Hata et al., 2007; Kiss et al., 2008; Lee et al., 1999). The single calpastatin gene can produce at least eight calpastatin polypeptide variants, ranging from 17 kDa to 85 kDa by use of different promoters and alternative splicing events (Lee et al., 1992; Parr et al., 2000). Calpastatin is very specific to classical calpains, which is not the case for many of the low molecular weight inhibitors that show some inhibition of cysteine proteases (Neffe and Abell, 2005). One full length calpastatin molecule contains four inhibitor units, each unit inhibiting one calpain molecule with variable efficiency (Emori et al., 1987; Maki et al., 1987) (Figure 23). Interestingly, calpastatin is poorly conserved between species, only the region A, C and parts of B within the inhibitor units are conserved. Co-crystallization of CAPN2/S1 with one of the inhibitory units of calpastatin and Ca^{2+} revealed that an intrinsically unstructured sequence of calpastatin enables tight binding to calpain, while calpastatin forms a loop which hinders proteolysis by calpain (Figure 22). Calpastatin recognizes calpain in its Ca^{2+} activated and subsequently conformation changed form. The regions A and C target the penta-EF-hand domains of CAPN1/2/8/9 and CAPNS1 and thereby increase the affinity between calpain and calpastatin. Region B occupies the substrate-binding cleft and is consequently inhibiting calpain (Hanna et al., 2008; Moldoveanu et al., 2008; Wendt et al., 2004). However, calpastatin can interact with non- Ca^{2+} activated calpain with the non-inhibitory L-domain (Melloni et al., 2006).

Intracellular reversible phosphorylation of calpastatin is regulating the level of cytosolic calpastatin which is in proximity to the nuclear membrane in an aggregated state. This phosphorylation is removed upon Ca^{2+} increase, relocating the calpastatin to the cytoplasm (Averna et al., 2001; De Tullio et al., 1999). Recent studies have found that calpastatin expression can be regulated by the nuclear factor I (NFI): hypophosphorylation of NFI leads to the expression of an alternative splicing variant of CAST lacking the N-terminal XL domain and is localized predominantly in the perinuclear region of the cell (Vo et al., 2019a). As a consequence, calpain remains active in other compartments of the cytoplasm. Interestingly, calpain activates the phosphatase calcineurin, which in turn activates NFI by dephosphorylation. Hypophosphorylated NFI upregulates FABP7, which is associated with malignant glioma cell migration (Vo et al., 2019b). It was shown that in diseases characterized by Ca^{2+} dysregulation, overexpression of calpastatin inhibitory units successfully prevents the excessive digestion of calpain 1 targets (Rao et al., 2016; Ye et al., 2015).

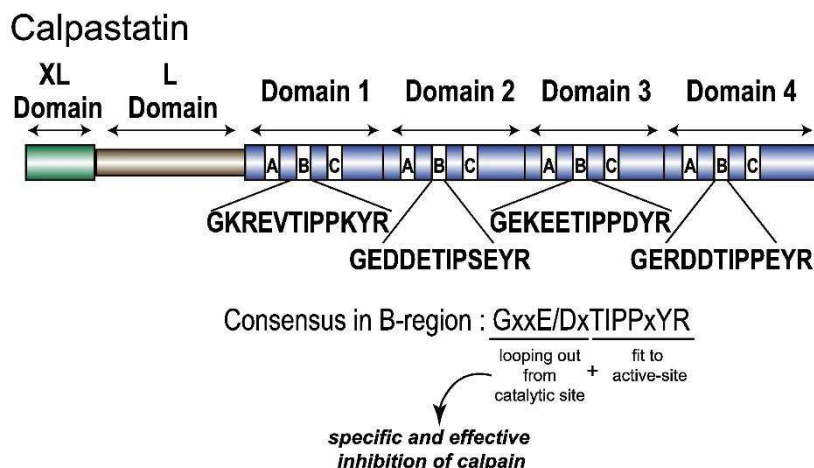


Figure 23: Calpastatin domains as described in Ono and Sorimachi, 2012

Cleavage site prediction of calpain 1/2

To understand the physiological functions of calpain, it is essential to elucidate their substrate specificity and selectivity, clarifying which proteins are proteolytically processed by calpain and at which position(s). CAPN1/2 preferentially recognize the PEST sequence (rich in proline (P), glutamate (E), serine (S), and threonine (T)) (Tompa et al., 2004). Yet, it was shown in several studies that the PEST sequences is not required for cleavage (Carillo et al., 1996; Molinari et al., 1995). The prediction of the cleavage sites of calpain 1/2 is complex because of the diverse cleavage site amino acid (aa) sequences. Nevertheless, one approach to predict

the cleavage site is based on the sequence of the substrate, a position-based residue preference (Banik et al., 1994; Cuerrier et al., 2005; Stabach et al., 1997; Tompa et al., 2004). The second approach is based on the tertiary structure of the substrate as well as the sequence (Sorimachi et al., 2012). Two of the calpain cleavage site computational tools, which were used for pol η cleavage site prediction are Calpacchopper (<http://calpain.org/predict.rb?cls=substrate>) and DeepCalpain (<http://deepcalpain.cancerbio.info/>) (duVerle et al., 2011; Liu et al., 2019).

Sequence comparisons of the cleavage sites of calpain 1 and 2 revealed similar catalytic efficiencies for most cleavage sites (Shinkai-Ouchi et al., 2016). Nonetheless, distinctive preferential sites could be revealed for each calpains at the positions P-9, P-8, P-7, P-2 and P+5 around the cleavage site as shown in Figure 24.

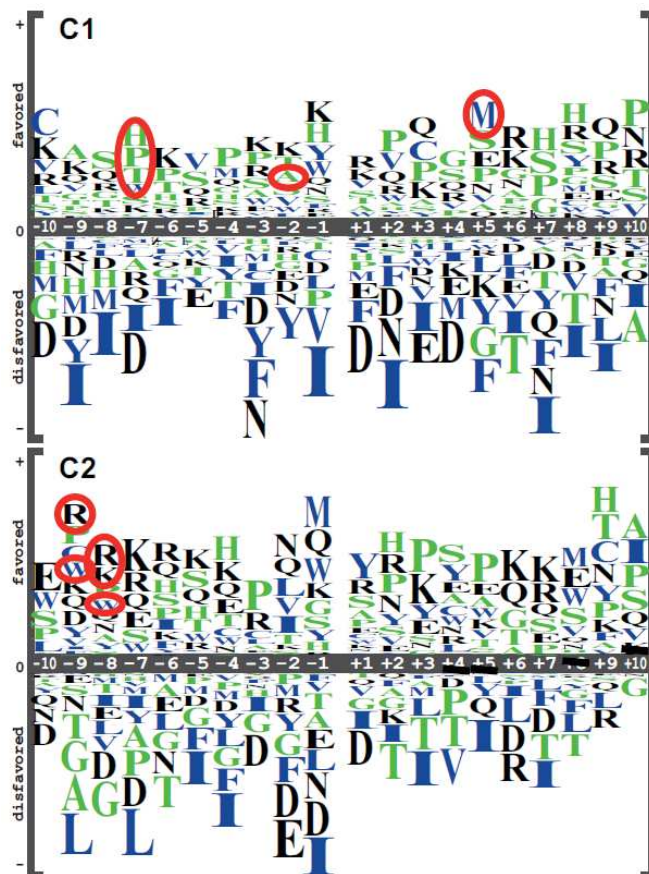


Figure 24: Frequency of amino acids in proximity to CAPN1/2 cleavage site as presented in Shinkai-Ouchi et al., 2016. C1 = CAPN1; C2 = CAPN2. The color of the amino acid letter indicates whether it is hydrophilic in black (Arg (R), Lys (K), Asp (D), Glu (E), Asn (N), or Gln (Q)), neutral in green (Ser (S), Gly (G), His (H), Thr (T), Ala (A), or Pro (P)), or hydrophobic in blue (Tyr (Y), Val (V), Met (M), Cys (C), Leu (L), Phe (F), Ile (I), or Trp (W)). Red circles around aa show significant differences between CAPN1 and 2.

Calpains in disease

Unregulated calpain in human pathologies

Calpains are implicated in several and very various diseases (table 2). Intracellular Ca^{2+} changes are implicated in a number of pathological states such as cardiac ischemia, muscular dystrophy, neuronal damage during cerebral ischemia and hypoglycemia (Guo et al., 2009b). It has been demonstrated that persistently altered Ca^{2+} homeostasis can lead to pathological disfunctions of calpain through an extensive intracellular degradation of specific proteins (Kanazawa et al., 2008). Calpain overactivation has been linked to several mis-regulated mechanisms causing diseases: aberrant angiogenesis, apoptosis, cytoskeletal degradation, inflammation and platelet dysfunction (Potz et al., 2016). Baseline calpain function is thought to be neuroprotective, while heightened calpain activity has been observed in several neurological injuries and disorders, where an inhibition of calpain is beneficial. This is described in ischemic (Cao et al., 2007; Chen et al., 2020; Stys and Jiang, 2002) and traumatic brain injuries (Gan et al., 2019; Kampfl et al., 1996; Liu et al., 2006), strokes (Etehad Moghadam et al., 2018; Sun et al., 2009), Alzheimer's (Di Rosa et al., 2002; Getz, 2012; Tsuji et al., 1998), Huntington's (Gafni and Ellerby, 2002; Weber et al., 2016) Parkinson's diseases (Alvira et al., 2008; Hassen et al., 2018; Samantaray et al., 2008; Shams et al., 2019) and multiple sclerosis (Das et al., 2008; Shields et al., 1999; Trager et al., 2014).

Moreover, calpain 2 is overexpressed and over activated in muscular dystrophy (Kumamoto et al., 1995; Shanmuga Sundaram et al., 2006) and calpain 3 is over activated in cataracts (Muralidharan et al., 2015; Tang et al., 2007). Calpains are also often dysregulated in cancer (Mamoune et al., 2003; Shiba et al., 1996; Yang et al., 2008)(see "calpain as prognostic marker in cancer"). For example in triple-negative breast cancer, an aggressive type of breast cancer, where the calpain system plays an important role in apoptosis and proliferation (Al-Bahlani et al., 2017). Many of the above described diseases are caused or are worsened by inflammation mechanism, which is also a hallmark of cancer (Hanahan and Weinberg, 2011)(see calpain functions chapter).

Calpain inhibitors

Calpains can cause or worsen several human pathologies as describe above, which is why the calpain system has been studied as a target for treatment since decades (table 2). First calpain

inhibitors have been published in the 1970s, since then more than 50 calpain inhibitors have been analyzed *in vivo* and in clinical trials. One such a calpain peptide inhibitor is cysteic–leucyl–argininal (CYLA). Following an acute ischemia calpain is activated, resulting in necrosis and apoptosis of retinal ganglion cells contributing to their degeneration. Treatment with CYLA in a rat model of retinal ischemia provides significant preservation of retinal function (David et al., 2011). Alicapostat (ABT-957) is a calpain 1/2 inhibitor which just completed successfully clinical trial phase 1 studies against Alzheimer disease (Lon et al., 2019). The calpain 1 and 2 inhibitor E-64d was tested in clinical trial phase 3 studies against muscular dystrophy (Satoyoshi, 1992)

Indirect inhibitors have shown the most success so far. Olesoxime is in clinical trials against Huntington disease, multiple sclerosis, spinal muscular atrophy and amyotrophic lateral sclerosis (clinicaltrials.gov). It is thought to inhibit calcium and cytochrome c release from mitochondrial and thereby reduces calpain activation (Weber et al., 2019a). Cyclosporine A is tested in clinical trial phase 2 studies against traumatic brain injuries. One of its mechanisms of action is the regulation of calcium release similar to olesoxime (Ferrand-Drake et al., 2003).

Table 2: Calpain in diseases as presented in Ono et al., 2016

Disease	Related calpain protein or gene (source)	Effect of activity	Calpain-related therapeutic candidates
Disease with a primary cause other than calpains			
Atherosclerosis	Calpain-1 and -2 (human, mouse)	Aggravating or causative	Calpain inhibitors
Brain ischaemia	Calpain-1 and -2 (human, mouse)	Aggravating or causative	Calpain inhibitors
Cardiovascular disorders	Calpain-1 and -2 (human, mouse)	Aggravating or causative	Calpain inhibitors
Cataracts	Calpain-1 and -2 (human, mouse)	Aggravating or causative	Calpain inhibitors
Neurodegenerative disorders (e.g. Parkinson disease, amyotrophic lateral sclerosis, spinocerebellar ataxia type 3 and lissencephaly)	Calpain-1 and -2 (human, mouse)	Aggravating or causative	Calpain inhibitors
Retinitis pigmentosa	Calpain-1 and -2 (human, mouse)	Aggravating or causative	Calpain inhibitors
Cancers	Calpain-1, -2, CAPN3 and CAPN9 (human, mouse)	Preventive, aggravating or causative	Calpain inhibitors and/or gene therapy
Alzheimer disease	Calpain-1 and -2 (human, mouse)	May be aggravating or causative	Potentially calpain inhibitors
Muscular dystrophies	Calpain-1 and -2 (human, mouse)	May be aggravating or causative	Potentially calpain inhibitors
Disease model			
Cardiac scar	Calpain-1 and -2 (human, mouse)	Preventive	Potentially gene therapy
Dilated cardiomyopathy	Calpain-1 and -2 (human, mouse)	Preventive	Potentially gene therapy
Infectious disease			
Fungal infection (opportunistic infection)	PalB/Rim13 (fungus)	Aggravating or causative	Calpain inhibitors
Malaria	Calpain-1 and -2, Pf-calpain (human*, mouse*, parasite)	Aggravating or causative	Calpain inhibitors
Periodontitis	Tpr (bacteria)	Aggravating or causative	Calpain inhibitors
Trypanosomiasis and leishmaniasis (e.g. African sleeping sickness)	Calpain-1 and -2, ClpGM6 (human*, mouse*, parasite)	Aggravating or causative	Calpain inhibitors
Schistosomiasis	Smp-157500 (parasite)	Aggravating or causative	Vaccine (rSm-p80)
Calpainopathies			
Autosomal dominant neovascular inflammatory vitreoretinopathy	CAPN5 (human, mouse)	Aggravating or causative	Calpain inhibitors
Eosinophilic oesophagitis	CAPN14 (human, mouse)	Preventive	Potentially gene therapy
Gastric ulcer	CAPN8, CAPN9 (human, mouse)	Preventive	Potentially gene therapy
Limb-girdle muscular dystrophy type 2A	CAPN3 (human, mouse)	Preventive	Potentially gene therapy
Spastic paraplegia 76	CAPN1 (human, mouse)	Preventive	Potentially gene therapy
Type 2 diabetes	CAPN10 (human, mouse)	Not definable	Unclear

Calpain in infectious disease

Calpains are involved in diseases caused by infections with pathogenic microorganisms, such as malaria (Chandramohanadas et al., 2009; Li et al., 2007; Olaya and Wasserman, 1991), trypanosomiasis (Ersfeld et al., 2005), schistosomiasis (Karmakar et al., 2014), candidiasis (Xu et al., 2016) and periodontitis (Staniec et al., 2015). Calpain inhibitors are also developed against conditions caused by such pathogens. *Candida albicans* and *Cryptococcus neoformans* use the Rim101 pathway for infection that can cause candidiasis and meningitis. Rim101 is a zinc-finger transcription factor, which is activated under alkaline conditions activating the

expression of several proteins mainly involved in the adaptation to environmental pH change. Rim13, a homologous to human calpain 7, is part of this pathway. Rim13 dependent filamentation is important for *C. albicans* virulence. It was shown that *C. albicans*, expressing nonfunctional Rim13, is hindered in infection. Thus inhibition of the calpain like Rim13 could be used against diseases which form consequently of *C. albicans* infection (Sorimachi et al., 2011; Xu et al., 2015). Malaria infections depend also on its pf-calpain, which can be targeted with conventional calpain inhibitors ALLN and ALLM suppressing the erythrocyte invasion (Olaya and Wasserman, 1991). *Leishmania amazonensis* can be treated with the calpain inhibitor MDL28170, efficiently suppressing their growth and viability (Marinho et al., 2014).

Calpain mutations causing diseases

In opposition to the mostly negative effects of overexpressed calpain, tissue specific expressed calpains and their dysfunction or absence are associated with diseases in humans. For example, mutations in *CAPN1* cause autosomal-recessive hereditary spastic paraplegia as well as alterations in cerebellar development and cerebellar ataxia (Gan-Or et al., 2016; Wang et al., 2016b). Loss of *CAPN2* or *CAPNS1* is embryonic lethal (Arthur et al., 2000; Dutt et al., 2006; Takano et al., 2011; Zimmerman et al., 2000). Furthermore, mutations in the *CAPN3* gene transcribed in muscle cells, causes the limb-girdle muscular dystrophy type 2A (LGMD2A or LGMDR1) a neuromuscular disorder (Lasa-Elgarresta et al., 2019; Lostal et al., 2019). Mutations in the *CAPN10* gene contribute to the development of type II diabetes (Harris et al., 2006; Horikawa et al., 2000; Salazar et al., 2019) and *CAPN5* mutations cause autoimmune uveitis, retinal neovascularization, and photoreceptor degeneration (Mahajan et al., 2012). Moreover, mutations in *CAPN12* lead to congenital ichthyosis, a rare and often life-threatening condition (Bochner et al., 2017). For those conditions, gene therapy could be a possible treatment option. However, tissue specificity and intricated expression levels complicate this endeavor.

Calpain as prognostic marker in cancer

Calpains evolve to be used as prognostic markers in several cancer types, for both a favorable and unfavorable outcome. In ovarian cancer for example high calpain 2 expression is associated with a negative overall survival, as well as low calpastatin and calpain 4 expression, whereas calpain 1 demonstrates no prognostic value (Zhang et al., 2019b). High expression of calpain 4 is linked to metastasis and poor prognosis in esophageal squamous cell carcinoma

(Wu et al., 2018). Low expression of calpain 9 is associated with poor outcome for patients with gastric cancer (Peng et al., 2016).

Furthermore, the human protein atlas project analyzed several calpains and their prognostic value for cancers. Calpain 1 expression is favorable in renal cancer and unfavorable in pancreatic cancer. Calpain 2 expression is similar to calpain 1 favorable in renal cancer and unfavorable in pancreatic cancer, as well as unfavorable in urothelial cancer. Moreover, high expression of calpain 5 results in poor outcome for endometrial cancer patients, whereas colorectal cancer patients with a high calpain 7 expression have a better outcome. Calpain 10 expression is associated with both outcomes, favorable for pancreatic cancer and head and neck cancer, yet unfavorable in liver cancer (Uhlen et al., 2017). Taken together, this demonstrates the importance of calpain in cancers as a prognostic marker.

Functions of calpain

Calpains do not require markers for substrate recognition, unlike other proteasomal degradation systems that required ubiquitin or chaperons. Due to their limited proteolytic activity and intrinsic substrate specificity, calpains are considered as modulator proteases rather than degradative proteases. Cleavage by calpains allows to regulate protein functions involved in various cellular pathways, such as apoptosis, autophagy and inflammation, which will be discussed in the following as examples on how the calpain system can work. However, calpains are also involved in cytoskeletal remodeling, cell cycle progression, gene expression, cell migration, insulin secretion, synaptic function and muscle homeostasis.

The role of calpain in inflammation

Calpain is involved in inflammatory processes (Figure 25). (Shumway et al., 1999). TNF- α indirectly activates cytosolic calpain 2 by redistributing it from the particulate to the cytosol. The activated calpain 2 then degrades I κ B and this in turn activates NF- κ B (Han et al., 1999). Subsequently, NF- κ B promotes the transcription of TNF- α and other cytokines and thereby amplifying the inflammation response. Inhibition of calpain activity promotes neutrophil migration associated with the activation of distinct signaling molecules (Katsube et al., 2008). Moreover, calpain can cleave pre-IL-1 α , localizing IL-1 α within the nucleus and increasing its affinity for IL-1 receptor 1 (Kobayashi et al., 1990). Calpain activity promotes the release of other cytokines, such as IL-6, IL-12 and IL-17 (Iguchi-Hashimoto et al., 2011; Smith et al.,

2011). Conversely, the cytokine IL-13 may activate calpain by increasing the endoplasmic reticulum stress (Pan et al., 2013). Furthermore, calpain also reduces the activity of glucocorticoids by cleavage of HSP90 and therefore reduces the anti-inflammatory effects of the hormone (Bellocq et al., 1999). Calpain has been shown, additionally, that it can up- and down-regulate nitric oxide concentrations, which in turn can be pro- and anti-inflammatory (Cui et al., 2005; Dong et al., 2009; Wang et al., 2008). All these mechanisms illustrate the very intricate calpain system and its versatile role in inflammation.

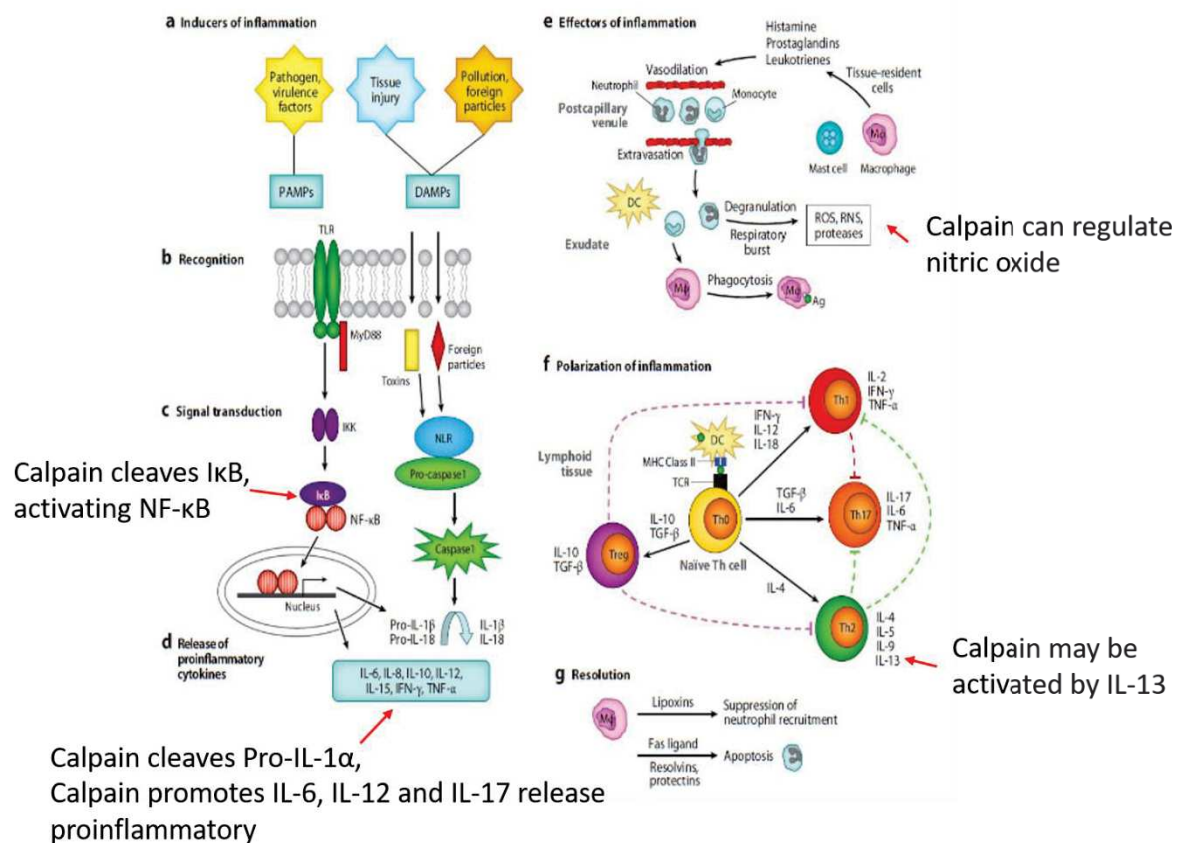


Figure 25: Inflammatory mechanisms and calpain adapted from Patel and Patel, 2015

Autophagy and calpain

Calpain has been shown to negatively regulate autophagy on multiple levels as shown in Figure 26 (Weber et al., 2019b). Autophagy is a self-degradative process, removing aggregated proteins, damaged organelles and intracellular pathogens. Calpain is involved in autophagy through inactivating ATG5 by cleavage, hindering the expansion of the phagosome membrane (Xia et al., 2010; Yousefi et al., 2006). Remarkably, the cleaved ATG5 relocates within the mitochondria, triggering the switch from autophagy to apoptosis (Yousefi et al., 2006). Furthermore, $G_{s\alpha}$ cleavage by calpain activates adenylyl cyclase, which leads to an

accumulation of cAMP, inhibiting the autophagosome formation (Rivero-Ríos et al., 2016; Williams et al., 2008). It has been shown that ischemia-reperfusion leads to overactivation of calpain and the inhibition of autophagy, which can be prevented with calpain knockdown in test models (Kim et al., 2008; Russo et al., 2011; Zhao et al., 2016).

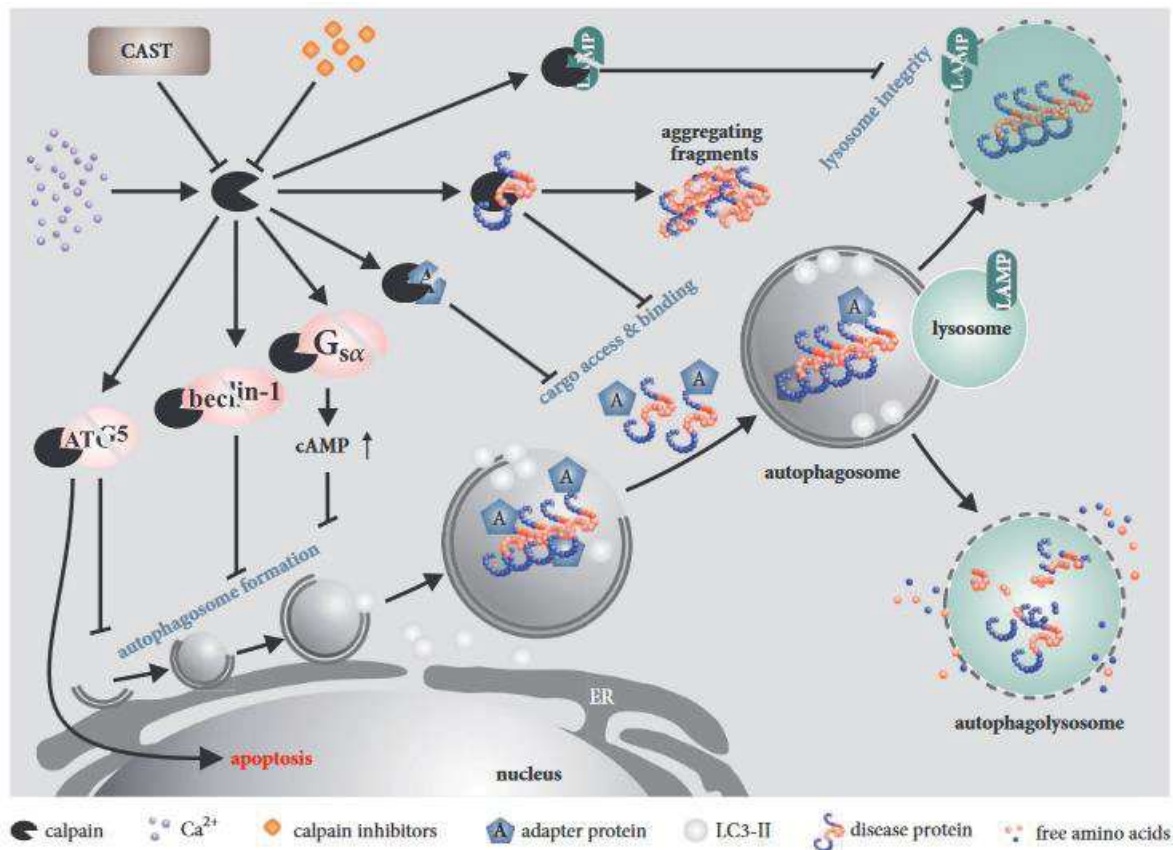


Figure 26: Calpain targets in the autophagic machinery as presented in Weber et al., 2019

Calpain activity during apoptosis

Ca^{2+} level increase during apoptosis, activating calpain and driving apoptosis progression (Figure 27) (Guo et al., 2009b). Calpain can cleave Bax, generating a potent proapoptotic fragment that promotes cyt c release and therefore fosters apoptosis (Choi et al., 2001; Gao and Dou, 2001). Similarly, calpain can cleave Bid and the new generated fragment releases cyt c from mitochondria (Mandic et al., 2002). Interestingly, this cleavage was observed in human melanoma cells treated with cisplatin (Chen et al., 2001a). Furthermore, it was shown that caspase 12 activity is dependent on active calpain (Imai et al., 2014; Nakagawa and Yuan, 2000). Calpain further promotes apoptosis through cleaving cain/cabin1, the endogenous inhibitor of the proapoptotic protein calcineurin. The caspase 3 inhibitor XIAP is degraded by calpain, enhancing the apoptotic progress (Yamada et al., 2012). Furthermore, PARP1

hyperactivation during apoptosis results in a Ca^{2+} flux which can activate calpain (Morales et al., 2014). Interestingly, CAPN3 was reported as anti-apoptotic factor in skeletal muscles by altering the regulation of the expression of NF-kappa B-dependent survival genes (Benayoun et al., 2008).

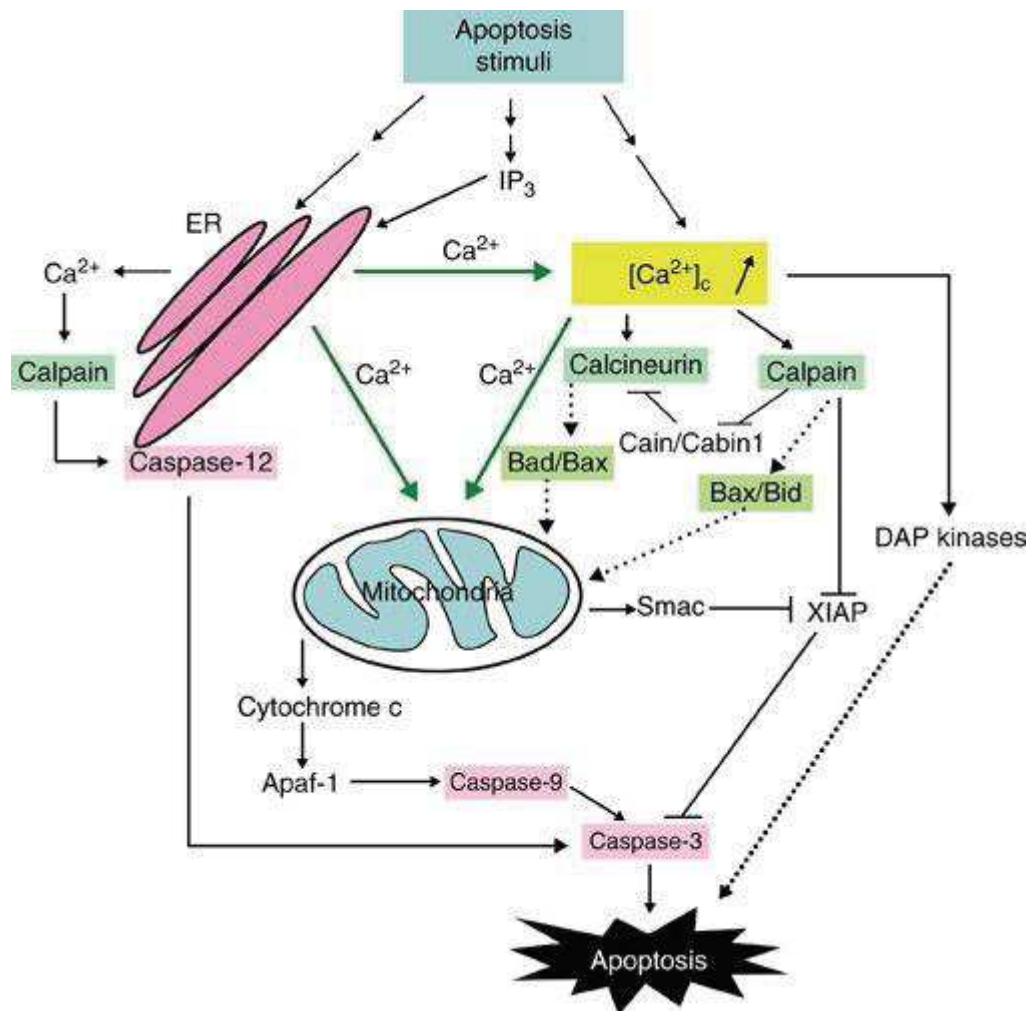


Figure 27: Calpain activities during apoptosis as presented in Guo et al., 2009b

Calpain activity on proteins involved in the same pathways as pol η

Calpain activity was reported on several proteins involved in genome stability, such as, replicative polymerases, USP1 and p53. Firstly, calpain 1 cleaves POLD4 the smallest subunit of the replicative DNA polymerase pol δ when cells are calcium triggered. This POLD4 cleavage occurs shortly after a chemical induced calcium surge, then after 24h the level is restored, followed by a renewed decrease of POLD4 after 36h, whereas the other pol δ subunits stay stable (Fan et al., 2014). Furthermore, calpain cleaves the catalytic subunit of

pol ϵ (POLE1), resulting in a 140 kDa fragment, which is slightly more active than the full-length enzyme, yet it loses its capability of interacting with PCNA (Liu and Linn, 2000). Both replicative polymerase subunits (POLD4 and POLE1) were cleaved under apoptotic conditions. Interestingly, calpain inhibitors could partially suppress UV-induced apoptosis in HeLa cells (Guo et al., 2009b). Another example is the cleavage of USP1 by calpain, which increases the stability of USP1. USP1 is deubiquitinating PCNA (see TLS) and thus destabilizing the interaction of TLS polymerases with PCNA. CAPNS1-depleted cells show an increase of ubiquitinated PCNA in unstressed conditions, favoring pol η on chromatin and mutagenesis. Additionally, the tumor suppressor p53 is cleaved by calpain independently from proteasomal degradation (Chen et al., 2018; Kubbutat and Vousden, 1997). Zhang et al. describes that degradation of p53 by a calpain-like protease is necessary for G₁-to-S-phase transition (Zhang et al., 1997). The proteolysis of p53 was shown to be dependent on the digestive organ expansion factor (Def) protein and calpain 3 (Tao et al., 2013).

Thesis objectives

Pol η has several functions preventing genome instabilities but also promoting mutations during somatic hypermutation. This contradictory potentials of pol η need to be carefully regulated. Yeast two hybrid studies showed that pol η interacts with CAPNS1, the small subunit of calpain 1 and 2. Therefore, we investigated the hypothesis that CAPNS1/pol η interaction is involved in the regulation of pol η .

To verify this hypothesis, we sought to answer the following questions:

1. Is calpain co-localized with pol η *in cellulo*?
2. Which pol η part interacts with CAPNS1?
3. Can calpain cleave pol η *in vitro* and *in cellulo*? Where is pol η cleaved?
4. Can UV activated calpain cleave pol η ?
5. Which effect has calpain inhibition on pol η foci formation after UV irradiation?

Preliminary Article

Calpain is involved in the pol η -dependent UV response

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Abstract

DNA polymerase η (pol η) is specifically required for translesion DNA synthesis (TLS) across ultraviolet (UV) irradiation-induced DNA lesions. Recruitment of this error prone DNA polymerase is tightly regulated during replication to avoid mutagenesis and perturbation of fork progression. Here we report that pol η interacts with the small subunit of calpain, a calcium-dependent cysteine protease. This interaction is functional as demonstrated by the ability of endogenous calpain to mediate calcium-dependent cleavage of pol η in cell-free extracts and in living cells treated with a calcium ionophore. The proteolysis of pol η is found to occur at position 465 leading to a truncated protein encompassing the catalytic domain and the PIP1 motif. Unexpectedly, inhibiting calpain by treatment with the specific calpain inhibitor calpeptin decreases pol η foci formation after UV irradiation and pol η -dependent UV survival. Together, these data suggest a positive role for calpain in the pol η -dependent UV response.

Introduction

Cells are continuously exposed to exogenous and endogenous genotoxic agents that create damaged DNA bases or adducts. If the lesions have not been repaired before replication initiation, they may stall replicative DNA polymerases, inducing cytotoxic DNA double-strand breaks, gross chromosomal rearrangements, and even cell death. Translesion synthesis (TLS) across sites of base damage is one important mechanism that ensures the completion of genomic replication, contributing greatly to cell survival. TLS is carried out by specialized DNA polymerases able to accommodate a distorted DNA template owing to the open conformation of their catalytic site. Nevertheless, the recruitment of these error-prone DNA polymerases during replication have to be tightly regulated in order to avoid an increased mutagenesis.

One key event to the activation of TLS is the mono-ubiquitination of the sliding clamp PCNA, mediated by the Rad6-Rad18 complex upon exposure of ssDNA at sites of arrested replication in response to DNA damage. Y-family DNA polymerases (including REV1, pol η , pol ι , and pol κ) interact with monoubiquitinated PCNA (Ub-PCNA) through their ubiquitin-binding (UBM/UBZ) and PCNA-interacting motifs (PIP) (Bienko et al., 2005; Guo et al., 2006, 2008; Kannouche et al., 2004; Plosky et al., 2006; Watanabe et al., 2004). These domains are necessary for the accumulation of the TLS polymerases in replication factories in cells treated with agents stalling the replication forks. After lesion bypass, timely dissociation of TLS polymerases is essential for the switch back to replicative polymerases, thereby preventing mutagenesis. Deubiquitination of PCNA by USP1 (Huang et al., 2006), or by USP10 promoted by PCNA ISGylation (Park et al., 2014), has been invoked to limit the extension of bypass events.

DNA polymerase η (pol η) is specifically required for the accurate replicative bypass of cyclobutane pyrimidine dimers (CPDs) induced in DNA by UV radiation (Johnson et al., 1999; Masutani et al., 1999). Consistently, loss of pol η in humans is responsible for the *Xeroderma pigmentosum variant* (XPV) disease, characterized by sensitivity to sunlight and a predisposition to skin cancer radiation (Johnson et al., 1999; Masutani et al., 1999, 2000). Additionally, pol η is able to replicate across other types of DNA damages including cisplatin-induced GpG adducts (Pt-GG), (Vaisman et al., 2000; Zhao et al., 2012) and 8-oxoguanine (Patra et al., 2014).

Besides TLS, pol η plays also important roles in somatic hypermutation (Zeng et al., 2001; Zhao et al., 2013), telomere maintenance (Garcia-Exposito et al., 2016; Pope-Varsalona et al., 2014), and common fragile site stability (Bergoglio et al., 2013; Despras et al., 2016; Rey et al., 2009). Association with different partners and several post-translational modifications such as phosphorylation (Bertoletti et al., 2017; Chen et al., 2008; Dai et al., 2016; Göhler et al., 2011; Peddu et al., 2018), ubiquitination (Bienko et al., 2010; Jung et al., 2011), SUMOylation (Despras et al., 2016; Guérillon et al., 2020) and O-GlcNAcylation (Ma et al., 2017) control pol η activity for these diverse functions.

In a search for pol η binding partners that could be involved in the regulation of the protein, we identified CAPNS1 as a pol η interacting protein by a yeast two-hybrid screening. CAPNS1 is the regulatory subunit essential for the stability and function of the ubiquitous calpain 1 and 2. These proteases, belonging to the calcium-dependent calpain family, regulate a wide

range of key cellular physiological processes through limited proteolysis of their specific substrate.

The finding that pol η interacts with CAPNS1 prompted us to examine whether calpains participate in TLS. Here we demonstrate that pol η is a substrate for calpains and that pol η and CAPNS1 are colocalized in the cell nucleus. Unexpectedly, inhibition of calpain results in a decreased UV survival of MRC5 cells and a reduced amount of pol η foci in UV-irradiated cells demonstrating a positive role of calpain in pol η relocalisation or maintenance in replication foci. These results unveil a key role for calpains in regulating TLS and open new avenues to a novel role of calcium signaling in the DNA damage response.

Material and Methods

Cell lines and plasmids

Cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal bovine serum (Eurobio or Dutcher). The XP30RO cell line (SV40-transformed *Xeroderma pigmentosum* variant human fibroblasts) has a homozygous deletion in the pol η gene resulting in a truncated protein of only 42 amino acids (Masutani et al., 1999). MRC5-V1 cells (called MRC5 in this paper) are SV40-transformed normal human lung fibroblasts (Huschtscha et al., 1983).

MRC5 cell lines expressing either eYFP or eYFP-pol η were generated by transfection with the plasmid peYFP (Clontech) or peYFP-pol η (Tissier et al., 2004). Transfected cells were selected for two weeks in medium containing 0.6 mg/ml G418 (Gibco). Cells were sorted using a FACSAria cell sorter (BD) to yield a population of cells moderately over-expressing pol η .

Plasmids pcDNA, pGBKT7 or peYFP encoding wild type and mutant pol η proteins, and pACT2-ubi, have been described previously (Schmutz et al., 2010; Tissier et al., 2004, 2010). Plasmid expressing eGFP-tagged pol κ was a kind gift from M.J. Pillaire (CRCT, Université de Toulouse, Inserm, CNRS).

Mutations in the coding sequence of pol η were generated by site-directed mutagenesis. Full oligonucleotide sequences are available from the authors on request.

Two-hybrid analysis (Y2H)

Two-hybrid analysis was performed in *S. cerevisiae* AH109, using plasmids pGBKT7-pol η as described previously (Tissier et al., 2004). pACT2-CAPNS1 (residues 27-268) identified as a positive clone during a screening using pol η as the « bait », lacked the extreme N-terminal part of CAPNS1.

In vitro calpain cleavage assay

In vitro transcription/translation was performed using a TnT Quick coupled lysate system (Promega) and the pGBKT7 or pcDNA plasmids encoding wild type or mutant pol η . ³⁵S-labeled protein (2.5 μ l) was mixed on ice with cleavage buffer (30 mM Tris-HCl pH7.5, 250 μ M CaCl₂, 1.5 mM dithiothreitol in a final volume of 20 μ l). The mixture was incubated at 30°C in the presence of purified calpain 1 (Calbiochem; 380 U/ml) as indicated. The reactions were

stopped in Laemmli buffer and analyzed by SDS- PAGE, immunoblotting and phosphorimaging (GE Healthcare).

Translesion synthesis assay

The construction of single-stranded plasmids containing a single CPD lesion (pUCTT-CPD.ss) has been extensively described (Napolitano and Fuchs, 1997). Primer extension assays were performed as previously described (Cordonnier et al., 1999). Briefly, the reaction mixture (6.25 μ l) containing 10 fmoles of primed circular single-stranded DNA and an XP30RO cell extract (20 μ g) was incubated at 37°C for 10 min in 50 mM Hepes-KOH (pH 7.8), 7 mM MgCl₂, 1 mM DTT, 4 mM ATP, 200 μ M of dNTPs, 40 mM creatine phosphate, 100 μ g/ml creatine kinase. The XP30RO nuclear extract was supplemented with wild type or mutated pol η (0.1 μ l) produced using a TnT Quick coupled lysate system (Promega) with the pcDNA plasmids. Replication products were digested with EcoRI and PvuII restriction enzymes and analyzed by electrophoresis on a 8% polyacrylamide-7 M urea denaturing gel. The quantification of TLS levels were determined using the ImageQuant™ TL software after phosphorimaging (GE Healthcare). The percentage of TLS was calculated as the ratio of the intensity of the bands of TLS to the sum of the intensity of the TLS and L-1 bands.

RNA interference transfection

All RNA interference (siRNA) experiments were performed at the concentration of 40 nM using INTERFERin transfection reagent according to the manufacturer instructions (Polyplus). All siRNA used in this study were ON-TARGETplus SMART pools (Dharmacon; Non-Targeting siRNA #1 D-001810-01-05, Human CAST siRNA R-017433-00-0005). The cells were synchronized 48 h after siRNA transfection with 2 mM thymidine for 17 h. After a 2 h release cells were treated either with 15 J/m² UV (254 nm) or with 10 μ M ionophore (Sigma) for indicated times. If stated, cells were treated with 20 μ M calpeptin 30 min before the UV or ionophore treatment. Afterwards, cells were fixed and stained for immunofluorescence microscopy or lysed for immunoblotting.

Whole cell extracts and Immunoblotting

Cells were harvested at indicated times by scrapping and washed twice with PBS. Half of the cells were resuspended in one pellet volume fresh lysis buffer (50 mM Tris pH 7.5, 20 mM

NaCl, 10 mM MgCl₂, 0.1% SDS, anti-proteases (Roche)) for 30 min on ice. Aliquots were used as whole cell extract (W).

After electrophoresis on SDS/polyacrylamide gels, separated proteins were transferred onto a PVDF or low fluorescence PVDF membrane (Biorad) and probed with antibodies. Antibodies used in this study included: PCNA-PC10 (SC56, Santa Cruz, 1/2000); pol η (custom made by Biotem, raised against three peptides: 1. CGEEPQRDEEEAELELLRQ, 2. CRAKQMGLEPPPEVWQVLKT, 3. PEVWQVLKTHPGDPRFQC, 1:1000); pol η (ab17725 1/500); pol η (C-terminal part 414-713; H300 sc-5592, Santa Cruz 1/1000); Myc (9E10, sc-40, 1/3000, Santa Cruz Biotechnology); POLD1 catalytic subunit (A9, sc-1777, Santa Cruz, 1/1000), POLD2 (C-50, sc8800, Santa Cruz, 1/500), GFP JL8 (632381, Clontech, 1/500), Calpain (25565, Cell signaling technology, 1/1000) and Calpastatin (A-1, sc376547, Santa Cruz, 1/500).

Clonogenic assay cell survival

2x10³ cells were plated in 100 mm dishes in triplicates for each condition. 48 h after, cells were treated with calpeptin for 30 min, washed in PBS and UV irradiated as indicated. Medium containing calpeptin or DMSO was added to the cells and incubated at 37°C for 10 days. Cells were fixated with 4% formaldehyde in PBS for 15 min at room temperature. Subsequently, colonies were stained with a 0.1% crystal violet solution for 30 min at room temperature and washed with water 3-4 times. Colonies were counted.

Fluorescence Microscopy

Cells grown on glass coverslips were transfected using the jetPEI or the *TransIT*[®]-LT1 reagents according to the manufacturer's protocols (Polyplus and Mirus, respectively). Transfected cells or MRC5 cell lines expressing either eYFP or eYFP-pol η were UV-irradiated at 15 J/m², 48 h after transfection and were processed at the indicated times. Cells were washed twice with phosphate buffered saline (PBS) and treated for 5 min with CSK 100 buffer (100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 10 mM Pipes pH 6.8, 1 mM EGTA) supplemented with 0.2% Triton-X100 and a protease inhibitor cocktail (Complete, Roche). Cells were then fixed for 20 min in 4% formaldehyde/PBS, permeabilized with methanol at -20°C for 10 sec and stained by overnight incubation at 4°C with a primary antibody (CAPNS1, clone 3C4, Merck-Sigma, 1:100 dilution; PCNA ab15497, abcam 1:200 dilution, PCNA PC10 Santa Cruz, 1/300). Finally, after washing with PBS the coverslips were incubated for at least 2 h at room temperature with a secondary antibody (Alexa Fluor 594 goat anti-

mouse IgG, 1:1000; Alexa Fluor 488 goat anti-rabbit IgG, 1:1000; all Invitrogen) before mounting onto slides using Fluorescent mounting medium (Southern Biotech containing DAPI).

The proximity ligation assay (PLA) kit was purchased from Sigma. Cells were washed twice with PBS and treated for 5 min with ice-cold CSK 100 buffer and fixed as described above. The primary antibodies mouse anti-CAPNS1 (clone 3C4, Merck-Sigma, 1:100 dilution) and rabbit anti-PCNA (ab15497, abcam 1:200 dilution) were incubated overnight at 4°C. The secondary antibodies conjugated to the PLA-oligonucleotide probes were used (Duolink II PLA probe anti-mouse MINUS, anti-rabbit PLUS) according to the manufacturer's instructions. Slides were analyzed on a Leica DM5500B microscope equipped with a LeicaDFC350FX camera and the capture software LASAF. Analysis of Images in ImageJ (see appendix for script).

Statistical Analyses

Differences in survival assays were analyzed by two-way ANOVA. Statistical differences in all cases were determined by Student's t-test. IF image analysis of foci formation was tested for significance by Wilcoxon signed-rank tests. In all cases: NS (not significant) $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and **** $p < 0.0001$.

Results

Pol η interacts with CAPNS1

A yeast two-hybrid screen identified CAPNS1, the regulatory subunit of the calcium-dependent protease calpain, as a putative pol η interacting protein. In contrast, interaction of CAPNS1 with pol κ , another Y-family DNA polymerase, was not detected by the Y2H assay suggesting that CAPNS1 specifically interacts with pol η (Figure 1A). By using deletion constructs we determine that the region of pol η spanning amino acids 596-662 is sufficient for the binding to CAPNS1 (Figure 1B). This region encompasses the UBZ domain of pol η that drives its interaction with Ubiquitin. We therefore investigated the impact of point mutations in this region. The mutations in the UBZ domain of pol η that abolish the zing finger structure (H650A/H654A) or the interaction with ubiquitin (D652A) do not change the pol η ability to interact with CAPNS1. This suggests that CAPNS1 and ubiquitin may utilize independent interaction interfaces to bind to pol η within the same region (Figure 1C).

The interaction of CAPNS1 with pol η was further explored *in vivo* by assessing their subnuclear localization. Previous work showed that pol η is uniformly distributed through the nucleus but accumulates during S phase in replication foci, which are resistant to a mild extraction with a CSK buffer. UV-irradiation results in an increase in S-phase cells containing polymerase foci, which represent replication factories containing stalled replication forks. Since the endogenous levels of pol η are insufficient to be detected by any commercial antibody, we used an MRC5 cell line stably overexpressing eYFP-pol η . As expected from previous studies, pol η accumulates in replication foci in a low proportion of unperturbed cells, and this fraction increases after UV irradiation (Figure 2A, 2B). Remarkably, at least 75% of cells containing eYFP-pol η foci displayed colocalizing CAPNS1 foci (Figure 2B, lower histogram) whereas the accumulation of endogenous CAPNS1 in replication foci is not detected in the control cells expressing eYFP alone. Transient transfection of MRC5 cells with a construct encoding eYFP fused to the minimal domain required for pol η foci formation (aa 594 to 713; Kannouche 2001) shows that it is sufficient for CAPNS1 relocalization (Figure 2A, panel g). In contrast, CAPNS1 does not colocalize within UV-induced GFP-pol κ foci

(Figure 2C), consistent with the previous observation that this related TLS polymerase does not interact with CAPNS1 in Y2H.

To further probe for a localization of CAPNS1 in replication factories we used the Proximity Ligation Assay technology with antibodies directed against CAPNS1 and PCNA. The proximity between PCNA and CAPNS1 is unveiled in the cells displaying eYFP-pol η foci (Figure 2D). An increase in PLA signals after UV irradiation is observed, which correlate with the number of pol η foci per cell (Figure 2E). MRC5 cells stably expressing eYFP exhibit also a small but significant increase of PCNA/CAPNS1 PLA signals after UV irradiation which could be due to the relocalization of endogenous pol η to the replication foci. All together, these data suggest that the CAPNS1 interaction within the C-terminal part of pol η may be responsible for CAPNS1 localization in replication foci. However, the proximity of CAPNS1 with PCNA is observed only in a subset of replication foci after UV irradiation. Whether UV irradiation merely promotes CAPNS1 interaction with PCNA remains to be resolved.

Pol η is a calpain substrate *in vitro*

Purified calpain 1 was used to investigate whether pol η could be a substrate of calpain. The cleavage of myc-pol η protein results in a major cleavage product (about 50 kDa) that is recognized by the anti-myc antibody but not by the antibody H300 (Santa Cruz) which was raised against a C-terminal peptide (414-713) of pol η (Figure 3A). A time course experiment with calpain 1 shows that the reaction is almost complete within 20 sec (Figure 3B). A truncated portion of pol η corresponding the extreme C-terminus is observed at early times and disappeared thereafter while the major product of 50 kDa remains stable over time.

We next sought to determine whether pol η could be cleaved by endogenous calpain in a cell-free extract. Figure 3C shows that incubating a cell-free extract expressing eYFP-pol η in the presence of CaCl_2 is sufficient to induce the cleavage of eYFP-pol η (lanes 1-4). The cleavage product is specifically recognized by an anti-GFP antibody and has a similar electrophoretic mobility on SDS-PAGE compared to the recombinant eYFP-pol η (1-465) transiently expressed in MRC5 cells (lane 9). The cleavage is dependent upon CaCl_2 addition and correlates with the activation of calpain 1, as shown by its autoproteolytic conversion of the 80-kDa large subunit to a 78-kDa "intermediate" and a 76-kDa fully autolyzed form. Figure 3C shows that similarly to eYFP-pol η , p53 an already known substrate of calpain, can be cleaved upon incubation of cell-free extracts in the presence of CaCl_2 to a distinct 46-kDa form, as already published

(Kubbutat and Vousden, 1997). The calpain inhibitor calpeptin significantly abolished eYFP-pol η and p53 cleavages, as well as calpain activation (Figure 3C; lanes 5-8). Collectively, these findings suggest that pol η is a target of endogenous calpain in cell-free extract, leading to a truncated protein devoided of the regulatory domains located at the C terminus of the protein.

Using two different calpain cleavage prediction computer programs (calpain.org and ccd.biocuckoo.org), we found that although several cleavage sites are predicted, the highest score is at the position 465. This is consistent with the size of the major cleavage product obtained *in vitro* with purified calpain 1 and in cell extracts (Figure 3 A-C). To determine the cleavage site of pol η precisely, eYFP-pol η was isolated from MRC5 expressing cells by pull-down using anti-GFP coupled agarose beads and was subsequently incubated with purified calpain 1 in the presence of calcium. The products were separated by SDS-PAGE (Figure 3D) and subjected to mass spectrometry analysis after digestion with AspN and chymotrypsin endopeptidases. This analysis revealed that the truncated eYFP-pol η is the N-terminal part of pol η , with peptides ending at Gly 465, Ser 459 and Ser 458 (Figure 3D). Relative abundances of these peptides suggest that the preferred cleavage site is between amino acids Gly 465 and Ser 466 as predicted by cleavage prediction programs. These residues are conserved in mammals whereas the proline localized three residues after the major cleavage site is conserved among most vertebrates except *Danio rerio*. Notably, it has been reported that calpain 1 and 2 have a significant preference for proline at P3' position of their substrates even though the cleavage site specificity of calpain is rather weak and is defined by both primary and higher-order structures (Shinkai-Ouchi et al., 2016). In short, eYFP-pol η is preferentially cleaved after the amino acid 465, a site which is conserved among mammals.

Pol η is a substrate of calpain *in vivo*

To determine whether pol η could be cleaved by calpain *in vivo*, we treated eYFP-pol η expressing MRC5 cells with calcium ionophore, which causes an increase in intracellular calcium levels. In addition, the expression of calpastatin, the endogenous inhibitor of calpains was knocked down by siRNA transfection (siCAST). The appearance of the truncated form of eYFP-pol η correlates with a substantial activation of calpain, as evidenced by the autocleavage of calpain 1 (Figure 4A left panel). Similarly, the disappearance of endogenous pol η is observed in MRC5 cells co-treated with ionophore and siCAST, indicating that

endogenous pol η is also a substrate for calpains in these conditions (Figure 4A right panel). Treatment with the calpain inhibitor, calpeptin, abrogates eYFP-pol η cleavage as well as calpain activation. The cleaved product comigrates with a in MRC5 expressed eYFP-pol η 1-465 protein (Figure 4B). To check a possible link of the observed truncation of pol η with apoptosis, we tested the cells for signs of apoptosis under conditions inducing the truncation. The non-cleavage of the 115-kDa PARP 1 indicates that the cells are not undergoing PARP1-dependent apoptosis in these conditions (data not shown). Collectively, these data show that increased calcium and subsequent calpain activation are responsible for pol η cleavage *in vivo* at the same site than *in vitro*.

Translesion synthesis efficiency of truncated pol η across a TT-CPD lesion

Overall, the data described above show that the C-terminal domain of pol η is very sensitive to proteolytic degradation by calpain both *in vitro* and *in vivo*. The resistant truncated pol η (1-465) encompasses the catalytic domain (1-435) and a small adjacent region containing a PCNA-binding motif, named PIP1 (437-444). It has been shown that PCNA binding to this motif is involved in the stimulation of DNA polymerase activity (Acharya et al., 2008; Masuda et al., 2015). To determine whether pol η (1-465) could function in TLS, we expressed wild type and truncated pol η mutants in rabbit reticulocyte lysates. The catalytic activities of these proteins are all equivalent as assessed by the measure of the primer extension efficiencies on undamaged DNA templates (Figure 5B). We then tested the ability of these proteins to complement an XP30RO cell extract for the bypass of a TT-CPD lesion. We have previously established that the PIP2 (701-708), PIP3 (483-484) and UBZ domains localized in the C-terminal region of pol η contribute to the bypass reaction in this assay (Schmutz et al., 2010). Accordingly, pol η (1-435) containing only the catalytic domain is defective in TLS. Interestingly, pol η (1-465) still functions in TLS, albeit with a lower efficiency than the wild type protein. As shown in Figure 5B, mutational inactivation (F443A/L444A) of the PIP1 motif within this region abrogates the residual TLS ability of pol η (1-465), which thus relies on the presence of a functional PIP1. Altogether, the truncated pol η (1-465) can complete TLS in dependence of its PIP1 domain, although less efficiently than the full-length pol η .

Calpain is involved in pol η foci formation

To investigate further the influence of calpain on pol η during UV irradiation response, we irradiated MRC5-eYFP-pol η cells with UV and compared the level of pol η foci formation when the calpain inhibitor calpeptin is used. As published in the literature (Kannouche and Lehmann, 2006), the foci formation increases over time and decreases at 9 h. Interestingly, cells treated with calpeptin form less foci per cell than untreated cells 6 and 9 h after UV irradiation (Figure 6A). This difference could be explained if calpeptin changes the cell cycle or lowers the level of PCNA ubiquitination. Both were tested and revealed no differences between calpain treated and untreated cells (Supplementary Figure 1). Furthermore, when cells are calpastatin depleted with siRNA, MRC5-eYFP-pol η cells show an increase of pol η foci formation after 24 h, whereas no changes in the cell cycle and PCNA ubiquitination could be detected under these experimental conditions (Supplementary Figure 2). Taken together this demonstrates that calpain activity promotes pol η foci formation. In addition, in order to investigate if calpain is involved in the pol η -dependent UV survival, XP30RO and MRC5 cells were irradiated with UV light while treated with different concentrations of calpeptin (Figure 6B). In non-irradiated cells, calpeptin treatment alone reduces cell survival in XP30RO and MRC5 equally (Figure 6C). In contrast, while calpeptin only modestly affects UV survival of XP30RO cells, it affects significantly MRC5 cells, suggesting that calpain is involved in the pol η -dependent UV response.

Discussion

Pol η has several functions preventing genome instabilities but also promoting mutations during somatic hypermutation. This contradictory potentials of pol η need to be carefully regulated. Yeast two-hybrid studies showed that pol η interacts with CAPNS1, the small subunit of calpain 1 and 2. Therefore, we investigated the hypothesis that CAPNS1/pol η interaction is involved in the regulation of pol η .

In this paper we demonstrate that the translesion DNA pol η interacts with CAPNS1, the small regulatory subunit of calcium-dependent proteases calpain 1 and 2. More specifically CAPNS1 interacts with the amino acids 596-662 of pol η . Furthermore, CAPNS1 is colocalized with pol η and PCNA in replication foci. *In vitro* assays confirmed that pol η is cleaved by calpain in a calcium-dependent manner and mass spectrometry analysis of the cleaved fragment demonstrated its cleavage between the amino acids 465-466, leaving the catalytic domain of pol η intact with an alternative PIP domain, PIP1 (1-465). Interestingly, also the C-terminus of pol η is initially conserved after cleavage induction and is degraded *in vitro* just after 30 min. This could indicate that the C-terminus could exhibit a function, which needs to be further investigated. The TLS assay shows that the truncated pol η fragment (1-465) containing the catalytic domain is still active, although less efficient. This result is in accordance with previous studies showing that a truncated version of pol η (1-475) is responsive to PCNA stimulation *in vitro*, promotes efficient TLS opposite a *cis-syn* TT dimer *in vivo* and confers UV resistance to XP30RO cells (Acharya et al., 2010). We conclude from this result that the PIP1 motif plays a role in TLS *in vitro*, in accordance with previous studies (Acharya et al., 2008; Masuda et al., 2015). However, the loss in efficiency shows that this motif cannot completely substitute for the other pol η PIP motifs that have been shown to exert distinct functions *in vivo* (Masuda et al., 2015). In addition, the truncated pol η protein, devoided of the UBZ and PIP2 motifs, can no longer relocalize into replication foci in UV irradiated cells (Kannouche et al., 2001). Moreover, simultaneous invalidation of both these motifs confers cells sensitivity to UVC (Despras et al., 2012) and is responsible for the carcinogenesis in XPV patients (Broughton et al., 2002). We thus anticipate that the cleavage of pol η by calpain would impair its relocalization *in vivo*. However, if the cleavage occurs on the chromatin after relocalization at blocked replication forks, the cleaved pol eta could retain TLS ability.

Treatment of cells with the calcium transporter ionophore confirmed the cleavage of pol η by calpain *in vivo*. However, the endogenous inhibitor of calpain, calpastatin, inhibited most of the ionophore-induced activity of calpain. When down regulating calpastatin with siRNA, calpain is strongly activated and cleaves most of the overexpressed eYFP-tagged pol η as well as about 50% of the endogenous pol η (MRC5). This suggests that about half of the endogenous pol η (or an equal amount of the tagged pol η) is protected from the cleavage due to posttranslational modification or interactions with other proteins. Some activities of calpain occur during apoptosis (Cao et al., 2007; Fan et al., 2014; Liu and Linn, 2000), therefore we analyzed PARP1 as an apoptosis marker (Chaitanya et al., 2010; Soldani and Scovassi, 2002). We observed that PARP1 is not cleaved and therefore PARP1-dependent apoptosis is not active in the conditions where pol η is cleaved.

It was shown that during replication stress the Ca^{2+} level in the nucleus is elevated (Li et al., 2019), which could activate calpain. Furthermore, it was shown that calpain can be activated by UV irradiation (Gulati et al., 2004). Thus, we investigated the influence of calpain on the UV damage response. We could demonstrate that pol η -dependent UV survival is reduced when cells are treated with calpeptin. To further investigate the influence of calpain on UV-induced translesion synthesis, replication foci formation was investigated in MRC5 cells expressing eYFP-pol η . Surprisingly, the inhibition of calpain with calpeptin decreases the amount of pol η foci per cell, indicating that calpain activity facilitates pol η foci formation. These results can be explained by several hypothesis: First hypothesis: the cleavage of pol η by calpain could protect pol η from proteasomal degradation, a common outcome for calpain cleaved proteins (Cataldo et al., 2013). The cleavage site on pol η by calpain lies next to the O-GlcNAcylation site T457 (Ma et al., 2017). When pol η is O-GlcNAcylated and subsequently polyubiquitinated, pol η is degraded by the proteasome. The cleavage of pol η could hinder the O-GlcNAcylation and therefore degradation. Consistently, it was shown that when the O-GlcNAcylation site T457 is mutated, pol η foci persist longer. The remaining truncated pol η could complete TLS with the help of its remaining PIP1 domain. It is described that mutations in the UBZ of pol η greatly impairs the localization of pol η in replication foci (Bienko et al., 2005; Plosky et al., 2006). The truncated pol η does not possess the UBZ or PIP domain, therefore, we suggest that this cleavage occurs when pol η is already interacting or in vicinity with PCNA. Second hypothesis: calpain is needed to cleave other proteins that compete with pol η for the interaction with PCNA at the replication fork. The cleavage of the

POLD4 subunit of the replicative pol δ by calpain in calcium triggered apoptotic HeLa cells has been described by Fan et al., 2014. It has been proposed that this could be an alternative pathway to the cleavage of POLD4 after UV by CRL4^{Cdt2} (Terai et al., 2013; Zhang et al., 2013) allowing the space for pol η and therefore the polymerase switch to occur. Similarly, UV-induced degradation of p21^{cip1} through CRL4^{Cdt2} in response to DNA damage by UV has been shown to be required for efficient pol η recruitment to DNA lesions (Bendjennat et al., 2003; Soria and Gottifredi, 2010; Soria et al., 2006). p21^{cip1} was also shown to be degraded by calpain (Chen et al., 2001). Calpain could be here as well an alternative pathway to regulate p21^{cip1}. Third hypothesis: it was described that USP1 is cleaved by calpain (Cataldo et al., 2013). USP1 is a modulator of TLS and DNA repair, partly through deubiquitination of FANCD2 and PCNA (Huang et al., 2006; Nijman et al., 2005; Oestergaard et al., 2007). The cleavage of USP1 by calpain in non-stressed cells, leads to a stabilization of USP1, but impairs its deubiquitination function of FANCD2 (Arkinson et al., 2018). The USP1 deficient dependent enrichment of monoubiquitinated FANCD2 recruits pol η in response to DNA damage (Fu et al., 2013).

Calpain inhibitors are in clinical trials for several cardiovascular and neuronal diseases and have shown promising results under short term treatments. Such calpain inhibitors could be used as a supportive treatment with cisplatin to reduce or even inhibit the resistance caused by pol η . Taken together we found that CAPNS1 colocalizes and interacts with pol η and we uncovered a positive role for calpain in pol η foci formation and cell survival after UV irradiation. Further work should seek to decipher the mechanism underlying the mechanism of calpain in TLS.

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Figure legends

Figure 1. Pol η interacts with CAPNS1, the small subunit of calpain

A and B: Schematic representation of pol η and pol κ proteins and several truncated forms of pol η (remaining residues are indicated into brackets). Their ability to interact with CAPNS1 is indicated. The catalytic and regulatory domains of pol η are depicted as well as the UBZ motif.

C: Yeast transformants expressing both CAPNS1 and wild type or pol η mutant fusion proteins are selected on double drop out medium (-W-L). Positive interactions are indicated by growth on quadruple drop out medium (-W-L-A-H). The ubiquitin which interacts with the UBZ domain of pol η was used in parallel as a control.

Figure 2. Association of CAPNS1 and eYFP-pol η within the nucleus

A: Representative images of MRC5 cells expressing eYFP, eYFP-pol η (1-713) or eYFP-pol η (594-713), immunostained with CAPNS1 antibody 6 hours after mock or UV irradiation (15 J/m²). The panels d, f and g show the magnification of one nucleus. Scale bars, 10 μ m.

B: MRC5 cells expressing eYFP-pol η (1-713) were processed as in A. The percentage of cells with either eYFP-pol η (green) or CAPNS1 (red) foci are presented in the upper histogram. The lower histogram displays the percentage of cells containing pol η foci with colocalizing CAPNS1 foci. Data are the mean +/- SD of three independent experiments (n > 180 for each experiment).

C: Representative images of MRC5 cells expressing eGFP-pol η or eGFP-pol κ , immunostained with CAPNS1 antibody 6 hours after UV irradiation (15 J/m²). The panels show the magnification of one nucleus. Scale bars, 10 μ m.

D: Localization of CAPNS1 in replication factories: representative images of a Proximity Ligation Assay (PLA: CAPNS1/PCNA) in MRC5 cells expressing eYFP or eYFP-pol η , using antibodies against CAPNS1 and PCNA, 6 hours after mock or UV irradiation (15 J/m²). Scale bars, 10 μ m.

E: The upper boxplot represents the distribution of the number of PLA signals (CAPNS1/PCNA) per nucleus in each sample in one representative experiments. The lower boxplot shows the amount of pol η foci per cell cells in the indicated cell lines before or after UV irradiation (15J/m²) (n > 400 cells; **** = p<0.0001, unpaired Wilcoxon test).

Figure 3. pol η is a substrate of calpain 1 *in vitro*

A: *In vitro* cleavage of ^{35}S -labeled myc-pol η by various amounts of purified calpain 1 was carried out as described in Materials and Methods for 30 min at 30°C. After electrophoresis on a 8-15% polyacrylamide/SDS gel and transfer on a nitrocellulose membrane, samples were analyzed by phosphorimaging (left panel) or probed either with an anti-myc antibody (middle panel) or an anti-pol η antibody against the C-terminal domain of pol η (right panel). The positions of the full-length myc-pol η protein (arrowhead) and preferential cleavage products (*) are indicated.

B: Time course experiment of the cleavage of wild type pol η using purified calpain 1 (80 mU) as indicated. After SDS-PAGE and transfer, the nitrocellulose membrane was analyzed by phosphorimaging and probed with an antibody against the C-terminal part of pol η . Asterisk indicates the major cleavage product.

C: Cell-free extracts from cells expressing eYFP-pol η were incubated in the presence of increasing amounts of CaCl_2 and calpeptin, as indicated. After a 10 min incubation at 30°C, cell extracts were assessed for eYFP-pol η or p53 cleavages by immunoblotting with an anti-GFP or anti-p53 antibodies, respectively. Positions of the full-length and truncated proteins are denoted by an arrow and an asterisk respectively. POLD1 is used as a loading control. A MRC5 extract overexpressing eYFP-pol η (1-465) is loaded on the same gel (lane 9) as a molecular weight control.

D: Left panel: eYFP-pol η proteolyzed with calpain 1 *in vitro* was subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. The cleavage product (asterisk) was excised and analyzed by mass spectrometry. Right panel: A summary of the MS-detected peptides after digestion with AspN or chymotrypsin. The sequence of pol η (430-470 aa) and the peptides covering this region are shown. The cleavages sites are highlighted with arrows and the numbers of the peptides identified by MS-LC-MS are indicated.

E: Sequence alignment using COBALT (Constraint-based Multiple Alignment tool) centered on region 454-476 of human pol η sequence. The calpain cleavage site conserved in mammalian species and the proline residue important for cleavage are highlighted. NCBI RefSeq identifiers are as follow: Homo sapiens: NP_006493.1 ; Pan troglodytes: XP_518497.1 ; Bos

taurus: NP_001029622.1 ; Mus musculus: NP_109640.1 ; Alligator mississippiensis: gb KYO23933.1 ; Gallus gallus: NP_001001304.1 ; Xenopus laevis: NP_001086552.1.

Figure 4. Ionophore induces eYFP-pol η cleavage

A: MRC5 cells expressing eYFP-pol η (left panel) or MRC5 cells (right panel) were treated with siRNA against calpastatin or with non-targeting siRNA for 72 h. After treatment with ionophore and/or calpeptin as indicated, proteins were extracted and analyzed by western blotting with the indicated antibodies. Cleavage products are indicated with an asterisk *.

B: SDS-PAGE migration profile comparison of eYFP pol η (1-465) with the calpain cleavage product of eYFP-pol η after ionophore and siRNA against Calpastatin treatment as described in A.

Figure 5. Catalytic and TLS activity of wild type and truncated mutant pol η

A: Scheme of the wild type and truncated pol η proteins. Amino acid sequence (438-447) located just after the catalytic domain, is indicated. The FL residues of the PIP1 motif which are mutated to AA in the FL*mutant, are underlined.

B: Time course of DNA synthesis catalyzed by wild type (WT) or mutant pol η (0.04 μ l of the reticulocyte lysate) using a primed single-stranded template (pUC118.ss). DNA products were subjected to electrophoresis on a 20% polyacrylamide–7 M urea denaturing gel. Below the gel: Immunoblot of the different forms of pol η produced in rabbit reticulocytes lysates (1 μ l), using an antibody against the N-terminal part of pol η .

C: TT-CPD bypass efficiency of wild type and mutant pol η

Upper panel: Outline of the experiment and diagram of the modified plasmid. The length of the strand produced upon elongation of the [³²P]-labeled primer, up to the lesion site, is indicated. nts: nucleotides.

Middle panel: Monomodified DNA substrates (10 fmoles) were incubated 10 minutes at 37°C in the presence of XP30RO cell-free extracts (20 μ g) complemented with an equal amount (0.1 μ l) of different forms of pol η produced *in vitro* (as shown panel B). Products were analyzed by electrophoresis through a 8% denaturing polyacrylamide gel after restriction analysis. Product L-1 is generated when synthesis is blocked one nucleotide before the lesion.

Lower panel: Quantitative analysis of TLS efficiency with the different versions of pol η . Error bars denote the standard deviation (SD) of three experiments performed with independent pol η samples.

Figure 6. Calpain is involved in pol η foci formation and UV survival

A: Synchronized MRC5 cells expressing eYFP-pol η were treated with 20 μ M calpeptin and irradiated with 15 J/m² UV (254 nm). The boxplot is a representative of three experiments, showing the amount of foci formation per cell in the different conditions as indicated. IF image analysis of foci formation was tested for significance by Wilcoxon signed-rank tests. In all cases: NS (not significant) $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and **** $p < 0.0001$.

B: Colony survival assay with MRC5 and XP30RO cells irradiated at 5 J/m² and 2 J/m² respectively in the presence of calpeptin as indicated. UV survival was assessed in three independent experiments (mean +/- SD). Normalized on non-UV irradiated but calpeptin treated cells to distinguish from solely calpeptin effect. Differences in survival assays were analyzed by two-way ANOVA. Statistical differences in all cases were determined by Student's t-test. NS (not significant) $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and **** $p < 0.0001$.

C: Cell survival after treatment with calpeptin alone at indicated concentrations using MRC5 and XP30RO cells. UV survival was assessed in three independent experiments (mean +/- SD).

Figure 7. Hypothesis of the regulation of pol η by calpain

The center part of the figure shows the dogma of TLS: Firstly, the DNA is replicated by replicative DNA polymerases until the lesion. Secondly, the replication fork stalls and recruits TLS polymerases η , which insert nucleotides across the lesion. Thirdly, a second switch occurs back to replicative polymerases. The red squares represent the three hypothesis debated in the discussion. Hypothesis 1: pol η is stabilized by cleavage of calpain, protecting against O-GlcNAcylation-dependent degradation. Hypothesis 2: Calpain cleaves proteins which compete with pol η for the access to the replication site. Hypothesis 3: USP1 is cleaved by calpain, inhibiting deubiquitination of FANCD2. Ubiquitinated FANCD2 recruits pol η to the replication site.

Supplementary figure 1. Cell cycle and PCNA ubiquitination after calpeptin treatment

A: MRC5 cells expressing eYFP-pol η were treated with 20 μ M calpeptin and irradiated with 15 J/m² UV (254 nm). The cell cycle was synchronized with 2 mM thymidine and cells treated

1.5 h after release. The cell cycle was analyzed in ethanol fixed cells 3, 6 and 9h after UV irradiation. Cells were stained with propidium iodide (4 $\mu\text{g}/\text{ml}$; Sigma) using the BD Accuri™ C6 Plus flow cytometer (BD Bioscience).

B: Cells treated in the same conditions as in A were analyzed on western blot.

Supplementary figure 2. Foci formation after UV irradiation in calpastatin depleted cells

A: MRC5 cells expressing eYFP-pol η were treated with siRNA against calpastatin 72 h before UV irradiation with 15 J/m^2 (254 nm). The cell cycle was synchronized with 2 mM thymidine and cells were treated 2 h after release. The boxplot is representative of triplicates, showing the amount of foci formation per cell in the different conditions as indicated. IF image analysis of foci formation was tested for significance by Wilcoxon signed-rank tests. In all cases: NS (not significant) $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and **** $p < 0.0001$.

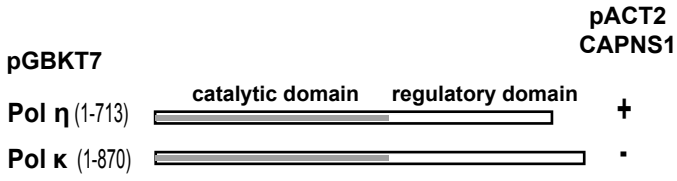
B: Cells treated in the same conditions as in A were analyzed on western

C: The cell cycle was analyzed in ethanol fixed cells treated in in the same conditions as in A. Cells were stained with propidium iodide (4 $\mu\text{g}/\text{ml}$; Sigma) using the BD Accuri™ C6 Plus flow cytometer (BD Bioscience).

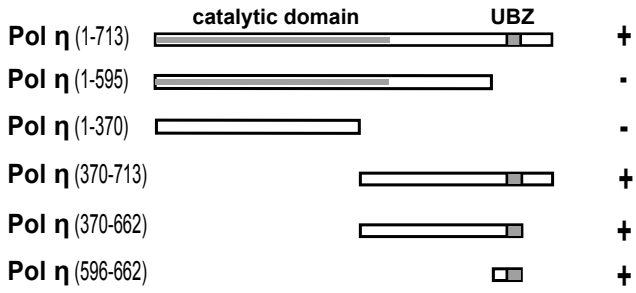
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Figure 1

A



B



C

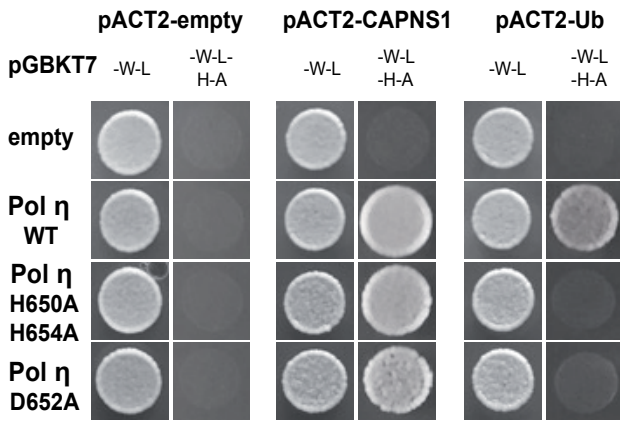


Figure 2

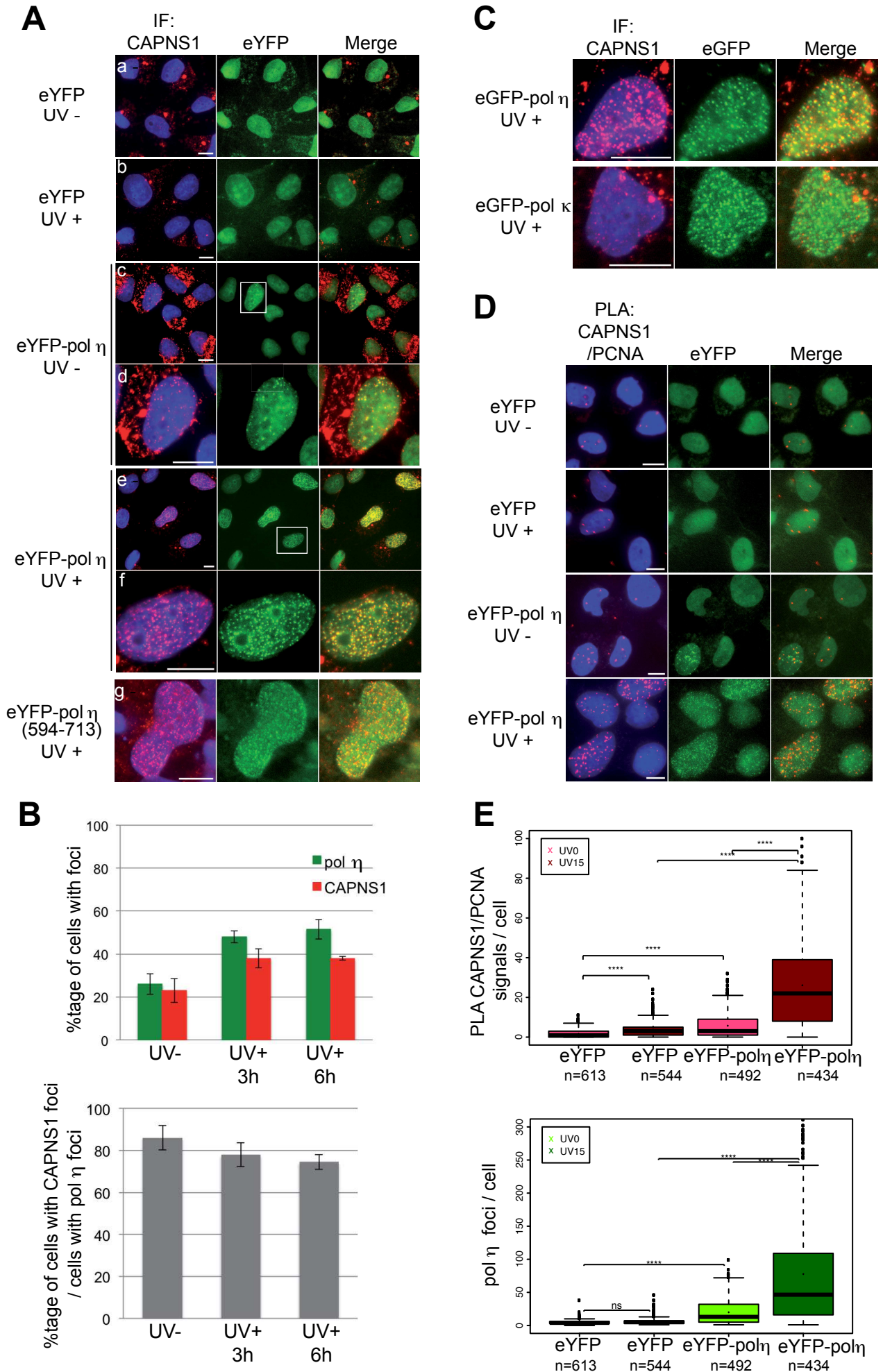


Figure 3

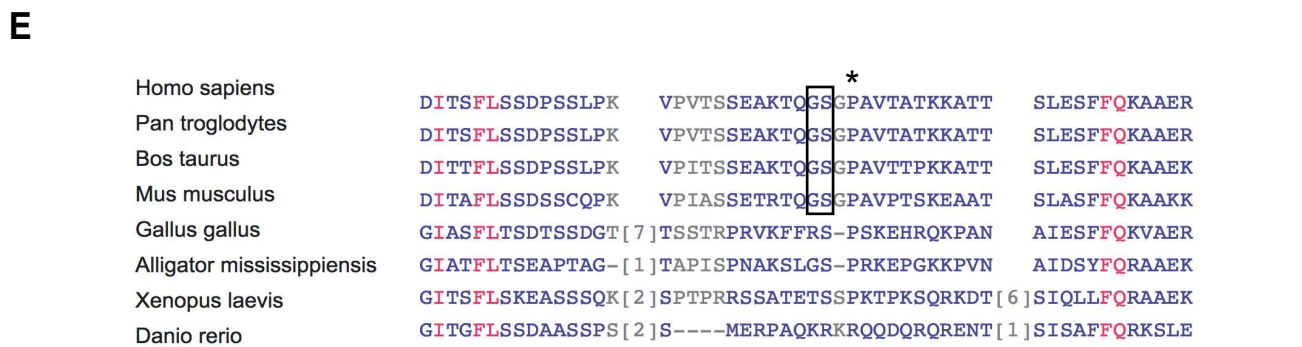
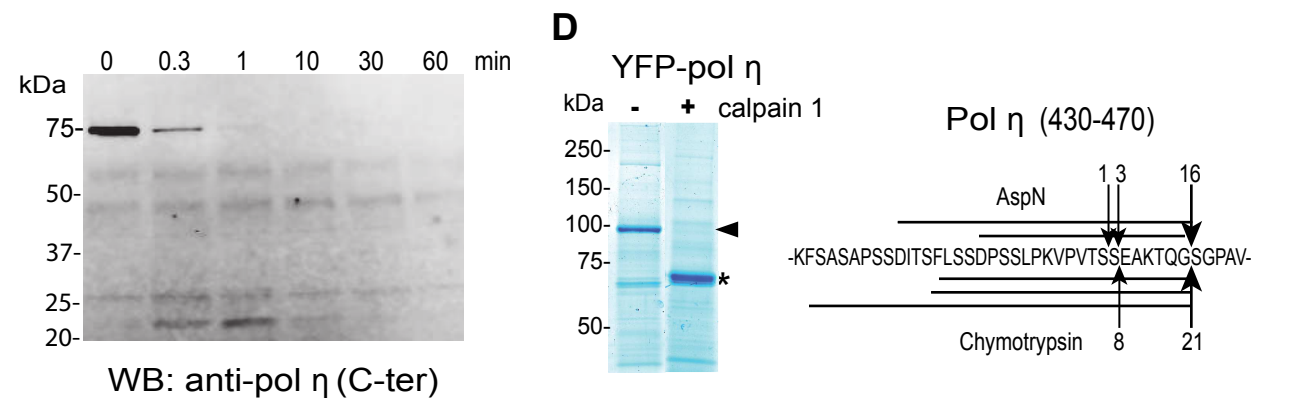
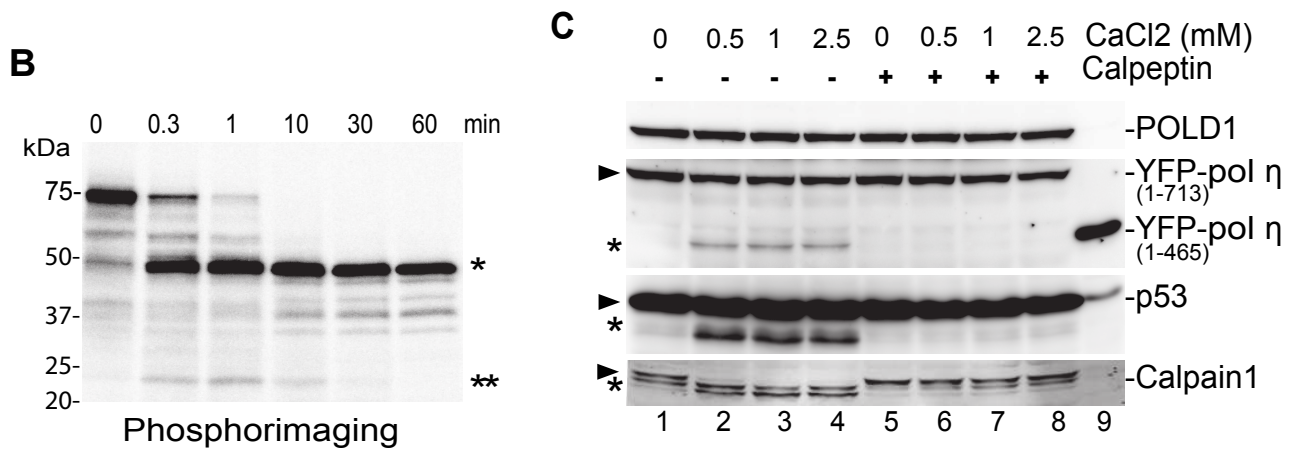
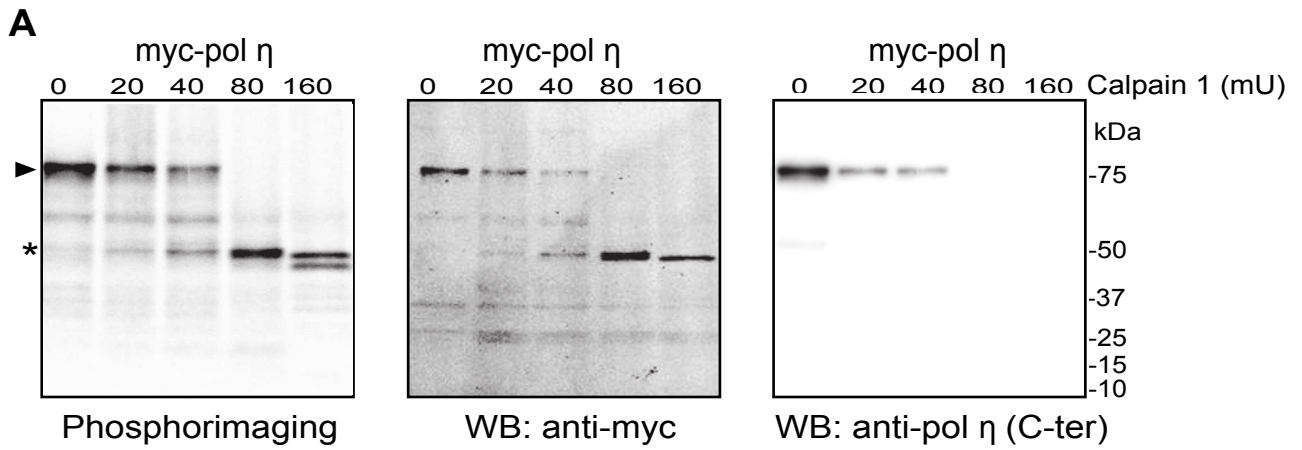
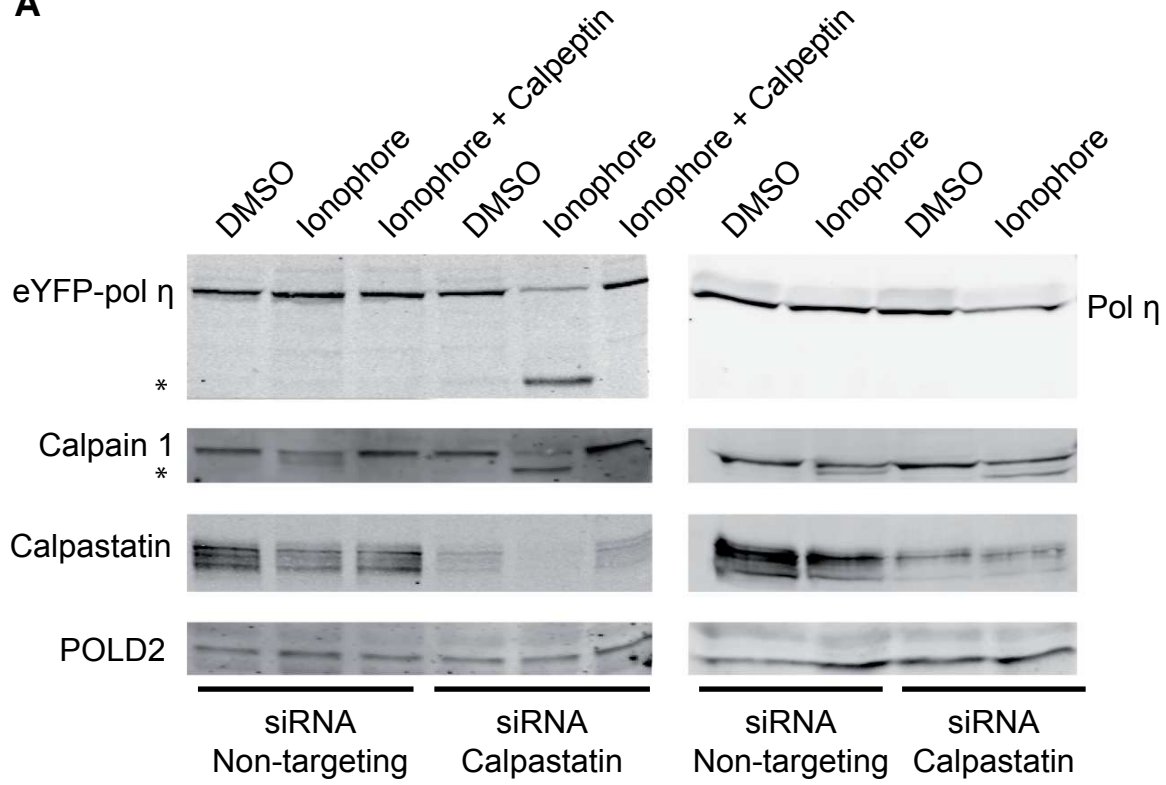


Figure 4

A



B

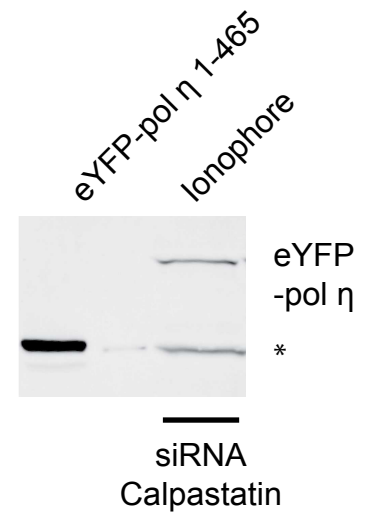
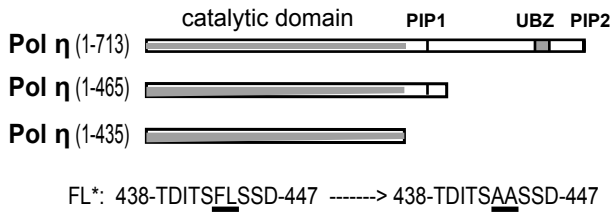
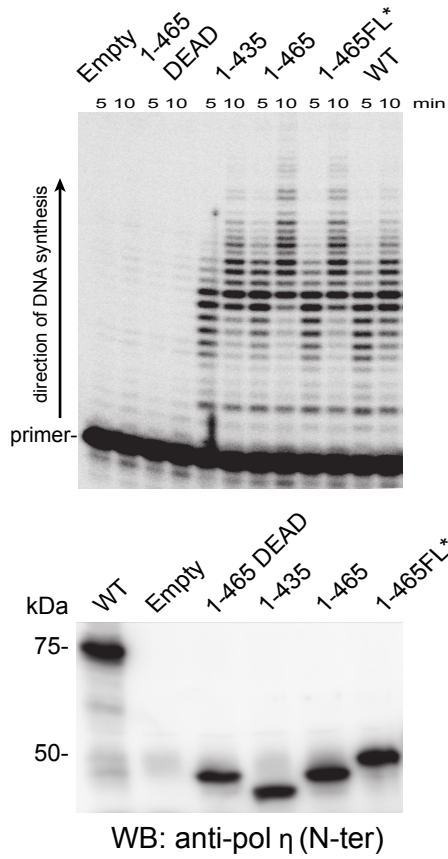


Figure 5

A



B: Catalytic activity of WT and mutant pol η



C: TT-CPD bypass efficiency of WT and mutant pol η

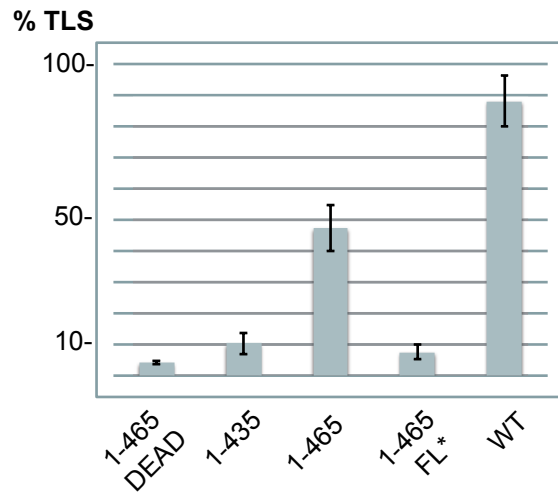
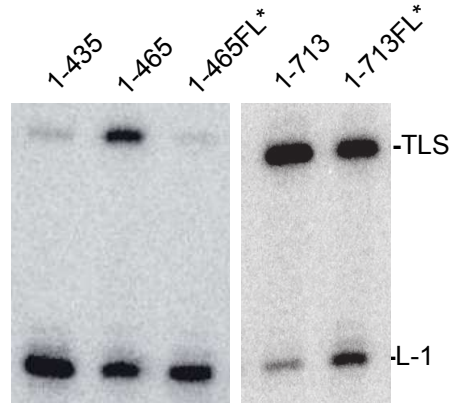
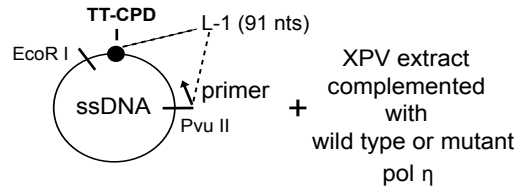
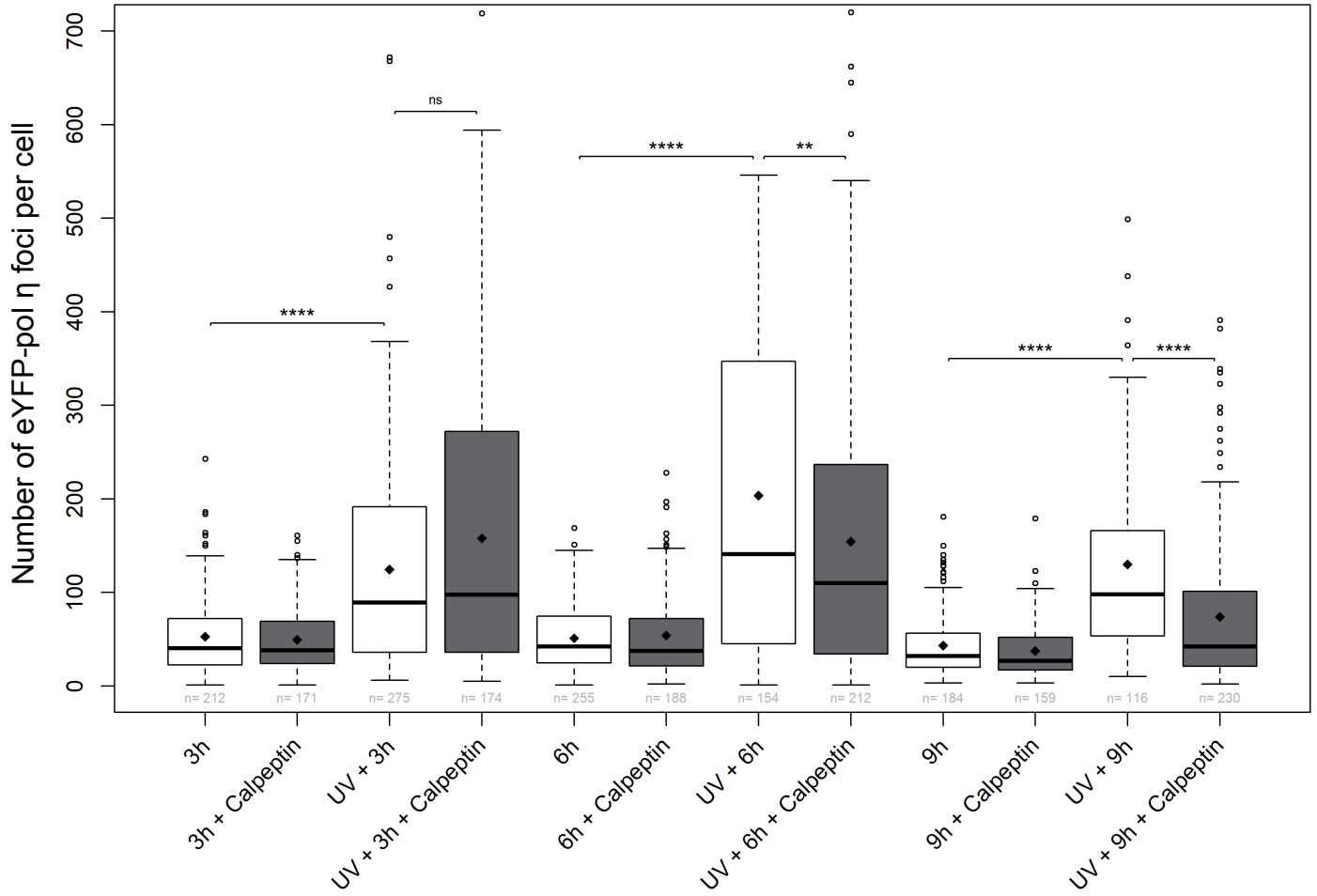
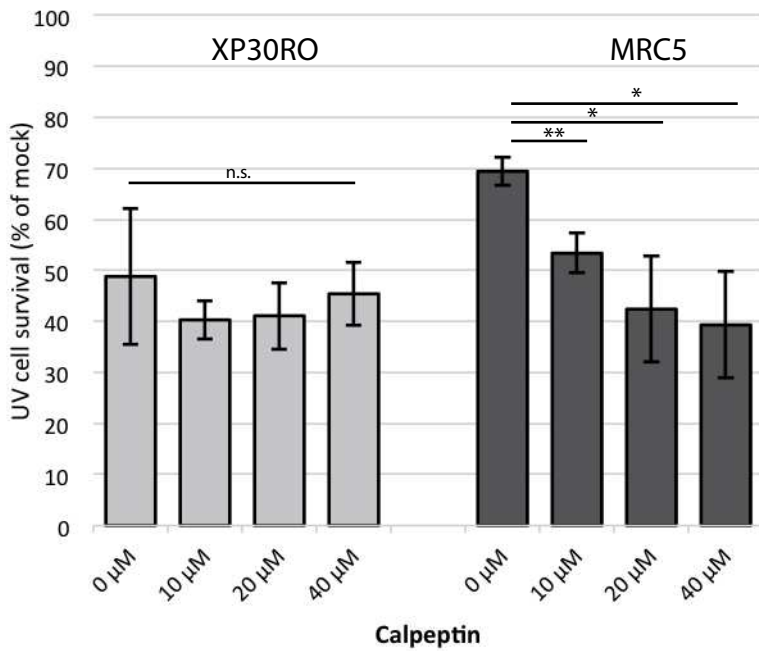


Figure 6**A**eYFP-pol η foci formation after UV irradiation and calpeptin treatment**B**

UV cell survival in dependence of calpeptin

**C**

Cell survival after calpeptin treatment

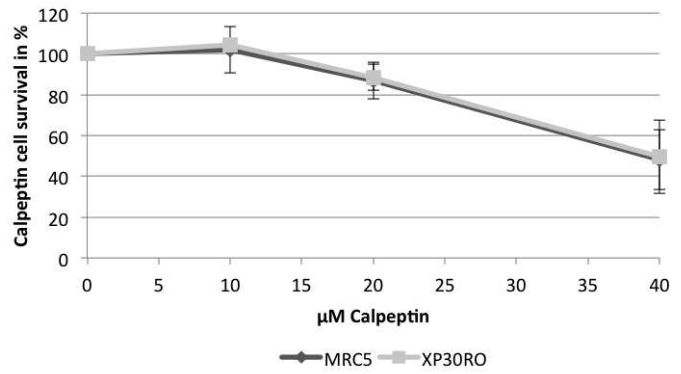
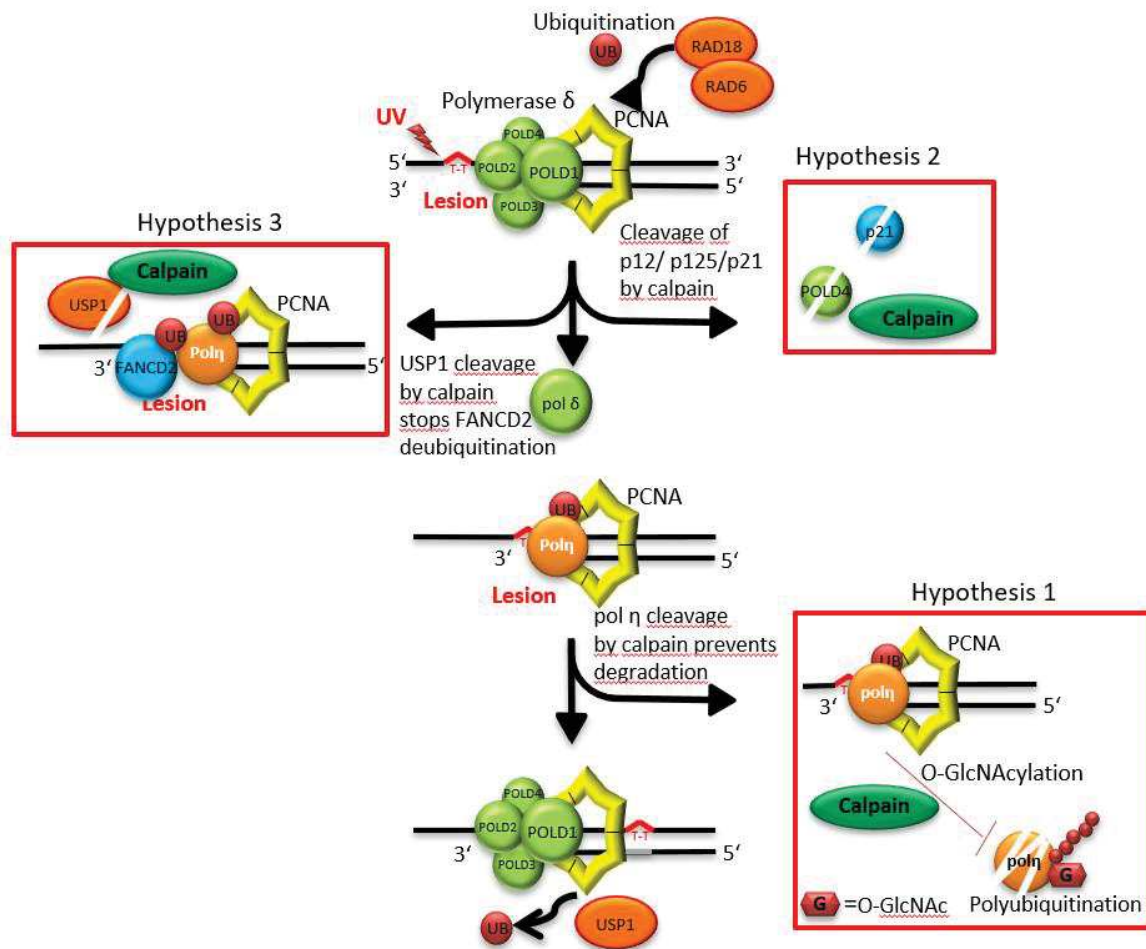
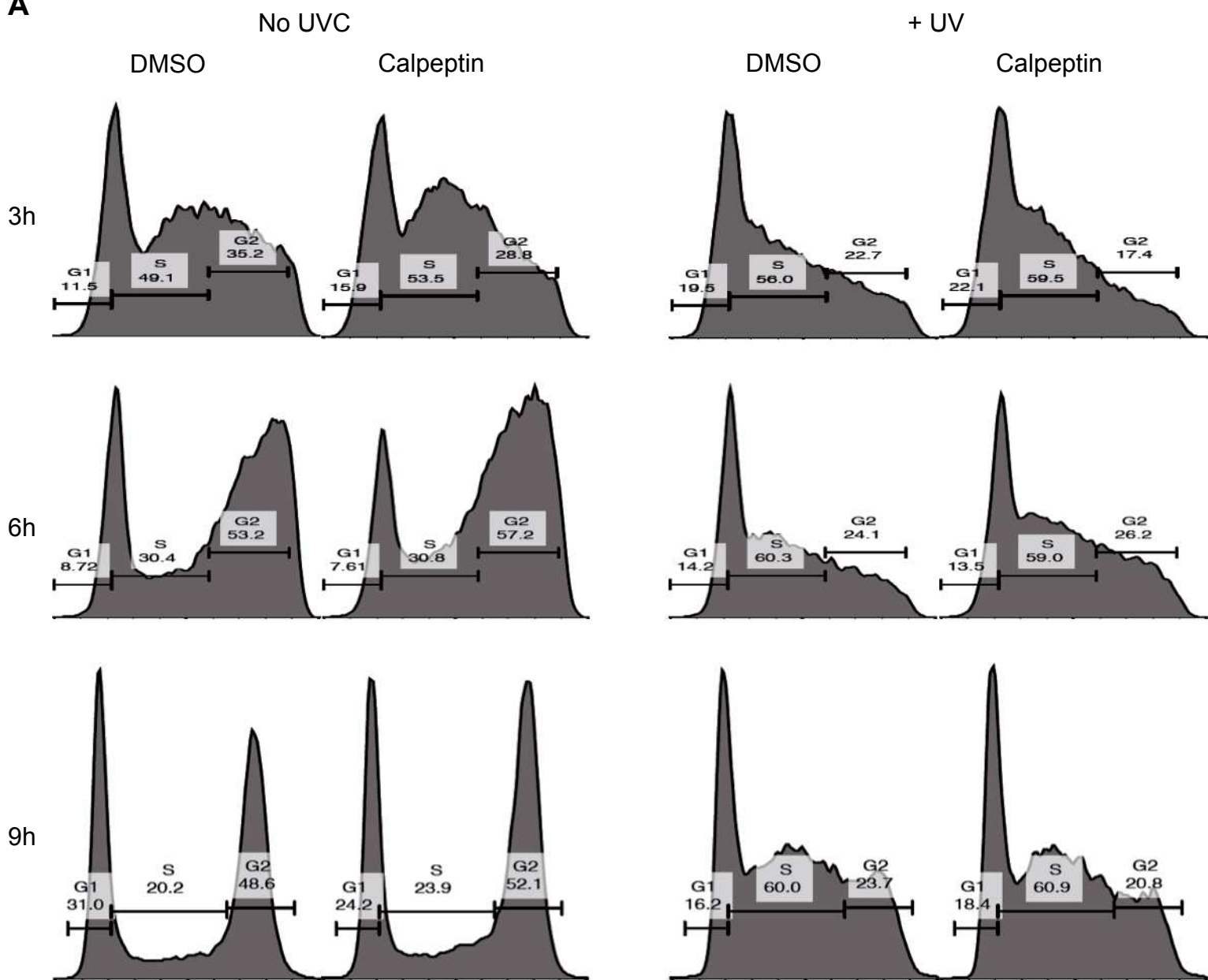


Figure 7

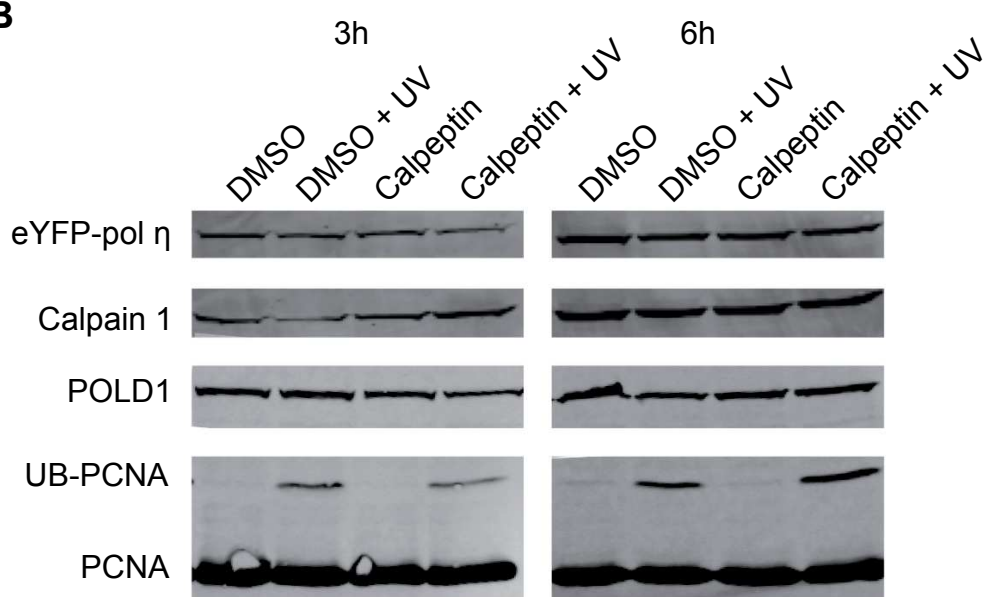


Supplementary Figure 1

A

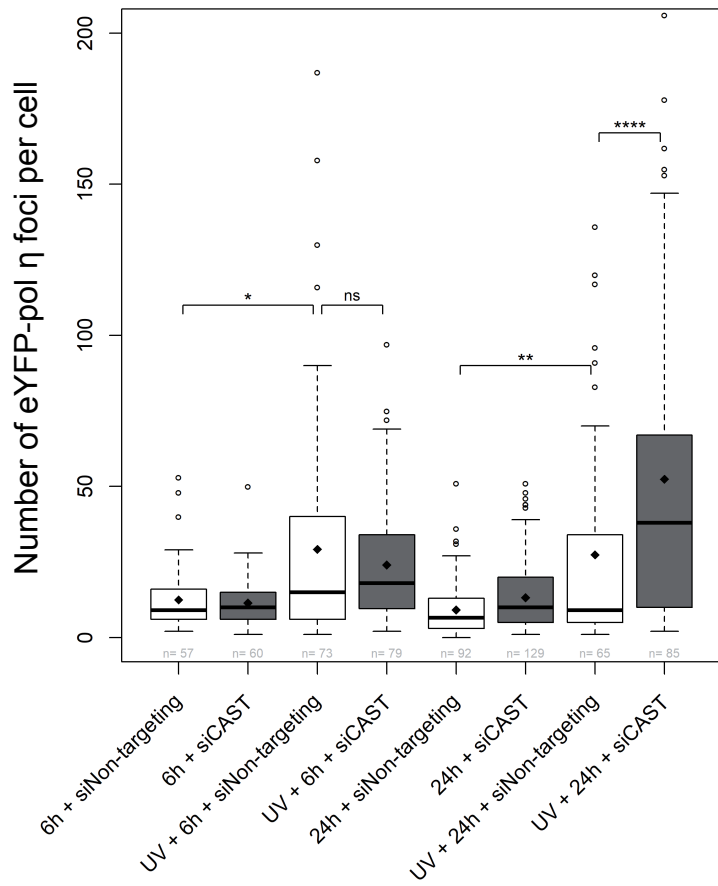


B

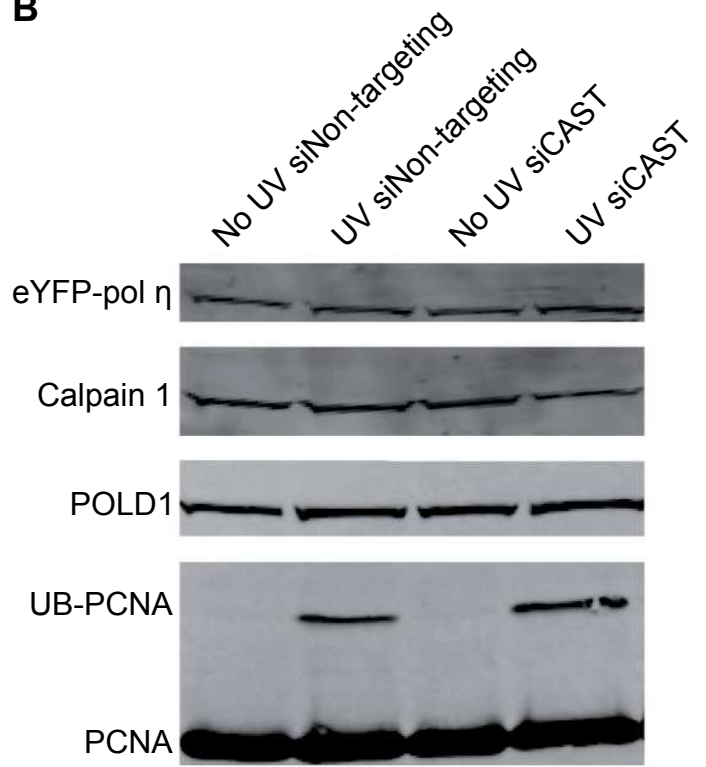


Supplementary Figure 2

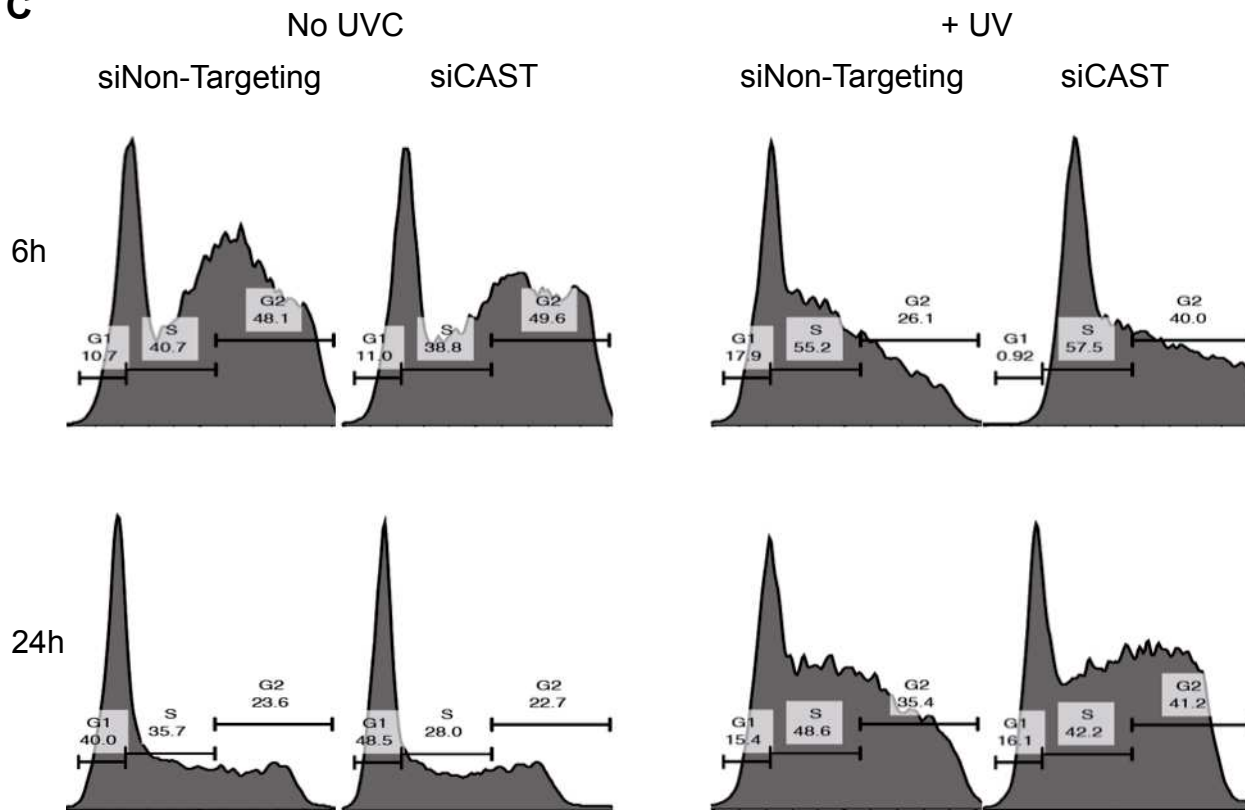
A eYFP-pol η foci formation after UV irradiation in Calpastatin depleted cells



B



C



Additional results and discussion

Ionophore induces eYFP-pol η and POLD1 cleavage

During the analysis of the cleavage of pol η in cells after treatment with ionophore and siCAST (Paper Figure 4), we observed that POLD1 can also be cleaved by calpain (Figure 28 A). Interestingly, the cleavage of POLD1 occurs also in different cell lines stably over-expressing tagged pol η (XP30RO-eGFP-pol η and U2OS-Flag-pol η) (Figure 28 B). Furthermore, the cleavage of pol η occurs as well in these cell lines overexpressing pol η with different tags, demonstrating that pol η is cleaved independently from the GFP-tag *in vivo*. Compellingly, the cleavage of POLD1 would open up the site of replication and allows access for pol η , enabling the first polymerase switch during TLS. This mechanism could explain the decreased foci formation of pol η after UV irradiation when calpain is inhibited (Paper Figure 6). However, the cleavage of POLD1 is not visible in MRC5 cells (Figure 28A), whereas the endogenous pol η is cleaved. This might occur because the activation of calpain appears to be stronger in MRC5 cells over expressing pol η than in MRC5 cells in which higher calpastatin levels and lower autolysis of calpain are observed. Alternatively, pol η could bring calpain together with CAPNS1 to the replication foci. The increased pol η level in the stable cell lines could lead to a higher activity of calpain in the replication forks leading to the increased cleavage of POLD1. The cleavage of POLD1 presents an interesting mechanism on how pol η can access the DNA, which needs to be further investigated.

Interestingly, in addition to the band corresponding to the major cleavage N-ter product another band was detected after the induction of cleavage by ionophore and siCAST treatment in MRC5, MRC5-eYFP-pol η cells (Figure 28 B right panel) and U2OS-Flag-pol η (Figure 28 B middle panel) cells when using an antibody raised against the C-ter of pol η . This band appears at about 37 kDa which is not the expected size for the cleaved C-ter (the fragment 465-713 is about 27 kDa). This could indicate that C-ter ubiquitinated or sumoylated pol η is cleaved by calpain. The only sumoylation on the pol η C-ter described is a polysumoylation occurring at positions all over pol η after UV irradiation, leading to the displacement of pol η from the DNA damaged site (Guérillon et al., 2020b). The observed band presents as single band at about 37 kDa, suggesting only a monosumoylation. Ubiquitination of pol η was described at several positions at the C-ter with its main site at K682 in unstressed cells (Jung et al., 2011). It is proposed that the monoubiquitinated pol η interacts intramolecular between monoubiquitin and the UBZ domain, hindering pol η from

interacting with PCNA during replication. Consistently, UV irradiation induces PCNA monoubiquitination and pol η deubiquitination, which in turn facilitates the interaction between pol η and PCNA (Bienko et al., 2010). The reduced pol η ubiquitination occurs 6 h after UV irradiation, which is the time frame during which calpain appears to be important during pol η foci formation. This suggests that calpain could cleave ubiquitinated pol η after UV irradiation, removing it from the vicinity of the replication fork and thereby improving the interaction of non-ubiquitinated pol η with the replication fork.

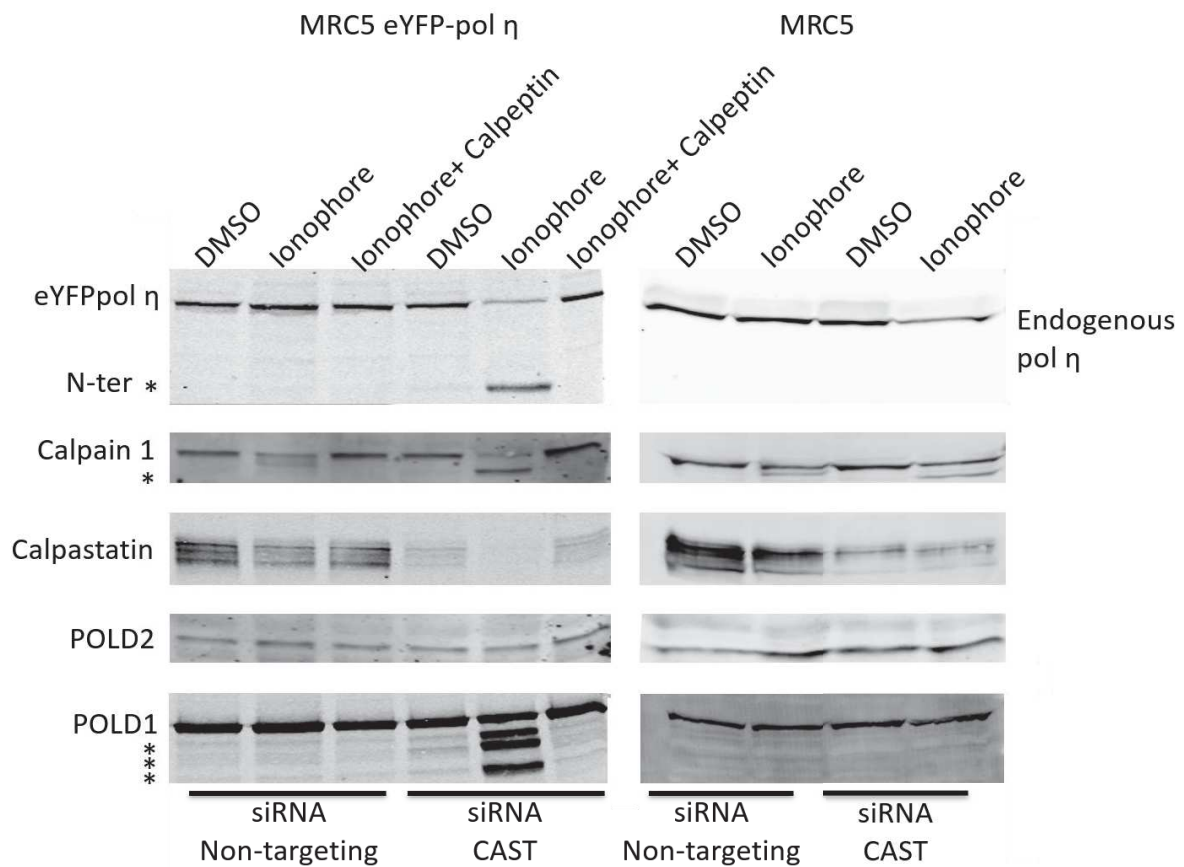
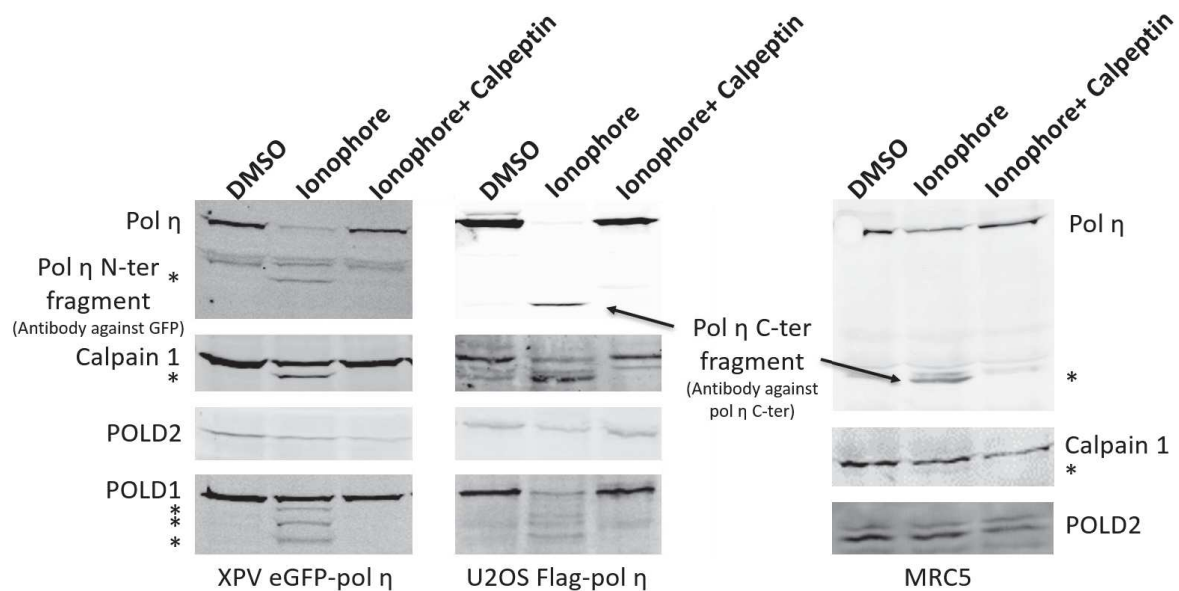
A**B**

Figure 28: Ionophore induced cleavage of pol η and POLD1. **A:** MRC5 cells expressing eYFP-pol η (left panel) or MRC5 cells (right panel) were treated with siRNA against calpastatin or with non-targeting siRNA for 72 h. After treatment with ionophore and/or calpeptin as indicated, proteins were extracted and analyzed by western blotting with the indicated antibodies. Cleavage products are indicated with an asterisk *. Pol η was visualized

with an anti-GFP antibody on MRC5 eYFP-pol η cell samples revealing the pol η N-ter and an anti-pol η C-ter antibody was used for MRC5 cells revealing the endogenous pol η . **B:** XP30RO cells expressing stably eGFP-pol η and U2OS cells expressing stably Flag-pol η were treated as in A. Depicted are the results of cells treated with siRNA against calpastatin. Arrows highlight the C-ter cleaved products. Pol η was visualized with an anti-GFP antibody on XP30RO eGFP pol η cell samples revealing the pol η N-ter and an anti-pol η C-ter antibody was used for MRC5 and U2OS Flag-pol η cells.

Cleavage of pol η by calpain after UV irradiation

Calpain is activated by an increase of the calcium concentration, which was shown to occur during replication stress in the nucleus (Li et al., 2019). Furthermore, UV irradiation can activate calpain (Gulati et al., 2004). Therefore, we investigate if UV activated calpain cleaves pol η , since pol η is the main translesion polymerase replicating across UV-induced lesions. For this, we analyzed the cleavage of pol η in calpastatin depleted cells, irradiated with 15 J/m^2 at 254 nm. The results (Figure 29) show that, eYFP-pol η is cleaved at the 1 h time point after irradiation while at 9 h the cleaved pol η product disappears, suggesting that pol η cleavage occurs early after UV irradiation. The cleaved eYFP-pol η migrates similar as the engineered eYFP-pol η 465 control. Interestingly, POLD1 cleavage could not be detected, indicating that it may not be triggered by UV irradiation. The cleaved pol η is mainly present in the soluble fraction, suggesting that it is either cleaved directly in this fraction or pol η detaches from the chromatin after cleavage. This detachment might appear because the interacting regions are separated from the catalytic domain (connected with eYFP). However, the cleavage of pol η occurred only in a small proportion of the protein after UV irradiation since the band appearing after the cleavage is close to the detection limit. Therefore, other cell lines can be used like XP30RO eGFP-pol η or U2OS as the activation with Ionophore was stronger in these cell lines, this might also be the case for UV activation.

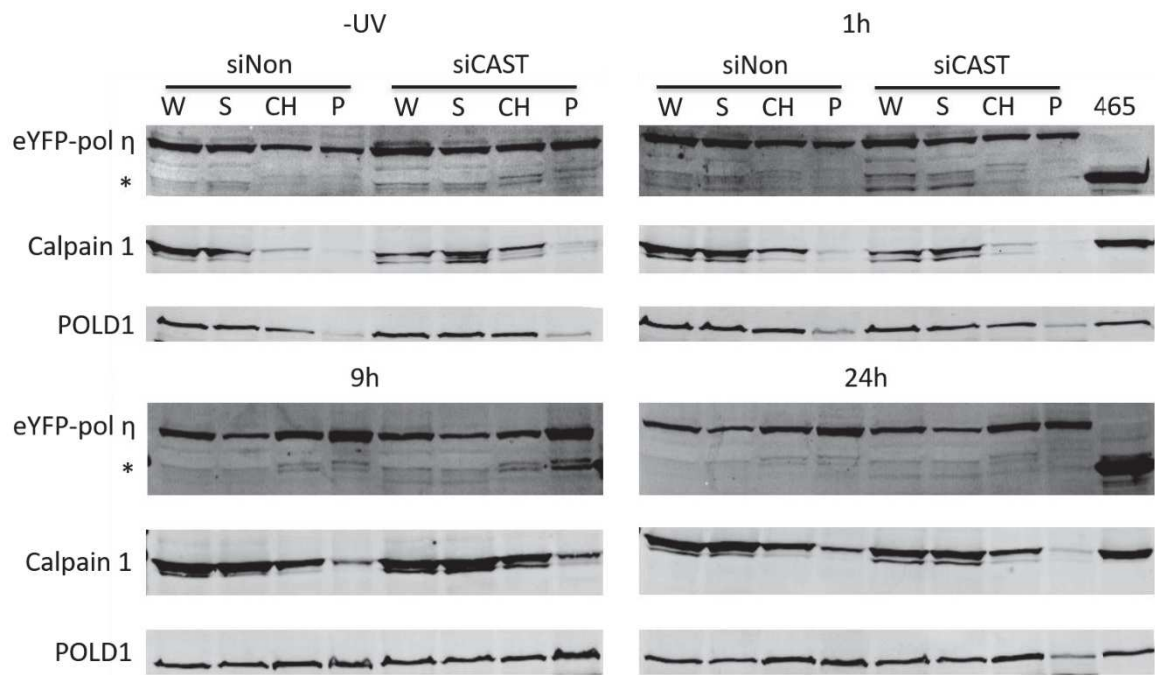


Figure 29: Cleavage of pol η by calpain after UV irradiation. MRC5 expressing eYFP-pol η were treated with siRNA against calpastatin or with non-targeting siRNA for 72 h. The cell cycle was synchronized with 2 mM thymidine and cells were UV irradiated 2 h after release with 15 J/m² (254 nm). Cell extracts were assessed for eYFP-pol η cleavage by immunoblotting with an anti-GFP antibody. Cleavage products are indicated with an asterisk *. Whole cell extract (W) was obtained as described in the paper. For cell fractionation, the second half of the collected cells were used. The cell pellet was resuspended in one volume of CytoSkeleton (CSK) 100 buffer (100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 10 mM PIPES pH 6.8, 1 mM Ethyleneglycol -tetraacetic Acid (EGTA), 0.2% Triton \times 100, anti-proteases) and incubated 15 min on ice. Sample was centrifuged at 4500 g for 5 min at 4°C. Supernatant was kept as soluble protein fraction (SF). The pellet was resuspended in one volume of CSK 50 (50 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 10 mM PIPES pH 6.8, 1 mM Ethyleneglycol -tetraacetic Acid (EGTA), 0.2% Triton \times 100, anti-proteases) containing 250 units/ml Benzonase (Merck Millipore) and was incubated for 15 min on a wheel at room temperature to give the chromatin fraction (CH). The pellet was resuspended in one volume of lysis buffer (50 mM Tris pH 7.5, 20 mM NaCl, 10 mM MgCl₂, 0.1% SDS, anti-proteases) and was lysed for 10 min on a wheel at room temperature to give the insoluble protein fraction.

Discussion

Discussion

In this thesis we discovered an interaction between the C-ter of pol η and CAPNS1, the small subunit of calpain 1 and 2. Interestingly, Y2H experiments revealed that CAPNS1 does not interact with pol κ and ι (data not shown), demonstrating the selectivity of its substrates. Within cells, CAPNS1 colocalizes with pol η in the nucleus and forms foci after UV irradiation. Thus, we investigated if calpain can cleave pol η . Our results show that pol η is cleaved in vitro in a time and concentration-dependent manner, as well as in cells by ionophore activated calpain. Furthermore, we showed that calpain activity is needed to form pol η foci after UV irradiation and the downregulation of the inhibitor calpastatin leads to more foci formation. As discussed in the paper this could be explained by several hypothesis.

Cataldo et al. described that USP1 is stabilized through cleavage by calpain and CAPNS1 depleted cells show an increase of pol η foci formation in unstressed cells. Surprisingly, CAPNS1 depletion has no impact in UV irradiated cells. However, the depletion shown for CAPNS1 is not complete and leaves a noteworthy amount of CAPNS1 within the cells. The remaining CAPNS1 could still interact with calpain and induce its interaction. This could explain why Cataldo et. al. did not find a difference in UV irradiated cells, whereas we found that calpain inactivity reduces the pol η foci formation after UV irradiation.

Alternative to the UV damage related activity of the pol η /calpain interaction, we theorize that pol η could be cleaved by calpain during its function in somatic hypermutation in B-cells. Calcium signaling is a common regulation in B-cells (Scharenberg et al., 2007), which could activate calpain. The truncated pol η containing the catalytic domain with the alternative PIP domain could increase its mutagenicity by omitting regulations of pol η through the C-terminus and thereby its retention time. This could promote antibody diversity, which would be interesting to investigate.

Perspectives

To fully understand the function of calpain during pol η recruitment and completion of TLS, the different hypothesis discussed above need to be investigated. To identify which proteins could be targeted by calpain during the UV response in an unbiased approach, we suggest to analyze protein changes by 2D gel analysis. For this, cells need to be irradiated with 15 J/m² UV (254 nm) in the presence or absence of calpeptin in a time course experiment.

Extracted proteins are analyzed by 2D-DIGE (Differential In Gel Electrophoresis) to identify differential protein spots by mass spectrometry. Direct analysis by mass spectrometry to identify calpain targets during the UV damage response is not suited, because we expect several proteins to be cleaved, whereas a cleavage of a protein would not be detectable by mass spectrometry due to the unchanged spectral counts (assuming truncated proteins are not degraded). This analysis can also be used to analyze post translational modifications which potentially could hinder or enable calpain activity.

The pol η /CAPNS1 interaction could also play a role during somatic hypermutations in B-cell. Pol η is needed to induce A/T mutations in immunoglobulin genes during B-cell maturation. Firstly, the cleavage of pol η should be analyzed in B-cells. Secondly, it would be interesting to study if the truncated pol η 1-465 is more mutagenic than the full-length pol η , which would be preferential during somatic hypermutation. Therefore, mutation frequency would be assessed in XP30RO cells stably expressing either pol η full-length or pol η 1-465 using the supF shuttle vector system (Parris and Seidman, 1992). Increased pol η activity can be achieved with UV irradiation.

Lastly, the mutation patterns in B-cell immunoglobulin genes can be analyzed in mice treated with calpeptin. Immunization and analysis of immunoglobulin genes by sequencing could be performed as described in Cui et al., 2016. Calpeptin treatments of mice have been described in investigation of calpeptin as therapy against several diseases and could be adapted from these protocols e.g. Mani et al., 2009; Tabata et al., 2010 and Zhou and Cai, 2019. Assuming that pol η function is reduced or even inhibited when mice are treated with calpeptin, we expect less A/T mutations to occur and more G/C mutations.

Conclusion

Taken together, pol η interacts with CAPNS1 and is colocalized in the nucleus. Pol η is cleaved by calpain after the amino acid 465 in vitro and in vivo. Interestingly, UV-induced foci formation of pol η and the UV cell survival are reduced in calpain inhibited cells. These results, indicating a novel role of calpain in the UV-induced response, encourage further studies aimed at deciphering the exact mechanism calpain has during TLS by pol η .

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Appendix

Image analysis of foci formation script

```
pathFichier = "/Users/jo-ann/Desktop/FociLiffstack/";
listeFichierInDir = getFileList(pathFichier);//créé un Array contenant
les noms des fichiers

//*****Setup for Channels
marquageDapi = 1;
marquageFitc = 1;
marquageRhod = 1;

marquage1 = "dapi";
marquage2 = "fitc";
marquage3 = "rhod";
nbMarquage = marquageDapi + marquageFitc + marquageRhod + marquageIR;

//***** Order of channels *****
dapiZ = 2;
IRZ = 0;
RhoDZ = 1;
fitcZ = 3;

//***** Counting *****
fociRhod = 1;//Mettre 1 pour compter les foci
fociFitc = 1;//Mettre 1 pour compter les foci
colocalisation = 1; //Mettre 1 pour colocalisation des foci
limitMaximaRhod = 80;
limitMaximaFitc = 100;
limitMaximaColoc = 150000;

if (nbMarquage == 0){
    exit("Terminé");
}

//setBatchMode(true);

for (ii = 0; ii < listeFichierInDir.length; ii++){//Boucle sur tous les
fichiers images du dossier précisé .liff
    if (endsWith(listeFichierInDir[ii], ".liff")){//test la présence de
fichier .liff
        fichier = substring(listeFichierInDir[ii], 0
,lengthOf(listeFichierInDir[ii])-5);//créé le nom de fichier en
enlevant l extension
        cheminFichier = pathFichier + fichier + ".liff";
        if (marquageDapi == 0){
            exit("Terminé");
        }else{
            //*****Analyse des ROI et
sauve le fichier contenant les ROI
            run("Bio-Formats Importer", "open=" + cheminFichier + "
color_mode=Default rois_import=[ROI manager] specify_range
view=[Standard ImageJ] stack_order=Default z_begin=dapiZ
z_step=nbMarquage");
        }
    }
}
```

```

run("Set Measurements...", "area mean standard modal min
centroid center perimeter bounding fit shape feret's integrated median
skewness kurtosis redirect=None decimal=3");
run("Smooth", "stack");
run("Smooth", "stack");
run("Enhance Contrast...", "saturated=0.1 normalize
process_all");
run("Unsharp Mask...", "radius=10 mask=0.80 stack");
setOption("BlackBackground", true);
run("Convert to Mask", "method=Triangle background=Dark
calculate black");
run("Fill Holes", "stack");
run("Erode", "stack");
//run("Erode", "stack");
//run("Erode", "stack");
run("Set Scale...", "distance=0 global");
run("Analyze Particles...", "size=3000-Infinity
circularity=0.50-1.00 show=Outlines display clear summarize add in_situ
stack");

roiManager("Show All without labels");
//nROIs = roiManager("count");
fichierROI = pathFichier + "ROI_" + fichier + ".zip";
roiManager("Save", fichierROI);

close();
selectWindow("Results");
run("Close");

//*****Analyse Dapi -
Couleur 1 *****
run("Bio-Formats Importer", "open=" + cheminFichier + "
color_mode=Default rois_import=[ROI manager] specify_range
view=[Standard ImageJ] stack_order=Default z_begin=dapiZ
z_step=nbMarquage");

selectWindow(fichier + ".liff");
run("Enhance Contrast", "saturated=0.35");
//run("Set Measurements...", "area mean standard modal min
centroid center perimeter bounding fit shape feret's integrated median
skewness kurtosis redirect=None decimal=3");
run("Set Measurements...", "area mean standard min
integrated median redirect=None decimal=3");
roiManager("Show All without labels");
roiManager("Measure");

//*****rajoute les colonnes Label contenant fichier
et Couleur contenant couleur1
if (nResults != 0){
for (i=0; i < nResults; i++){
setResult("Label", i, fichier); //
set the text string for new label setResult("Marquage", i, marquage1);
// set the text string for Couleur
}
} else {
showMessage("No Result found in result table");
exit("Terminé");
}

```

```

updateResults();

selectWindow(fichier + ".liff");
run("Close");
} //fin test DAPI = oui *****
//***** Fin Dapi: Analyse
Roi et comptage Dapi *****

    if (marquageFitc == 1){

        //*****Analyse
Fitc *****

        run("Bio-Formats Importer", "open=" + cheminFichier + "
color_mode=Default rois_import=[ROI manager] specify_range
view=[Standard ImageJ] stack_order=Default z_begin=fitcZ
z_step=nbMarquage");

        selectWindow(fichier + ".liff");
run("Enhance Contrast", "saturated=0.35");
run("Unsharp Mask...", "radius=1 mask=0.50 stack");
run("Set Measurements...", "area mean standard min
integrated median redirect=None decimal=3");
roiManager("Show All without labels");
roiManager("Measure");

        //*****Partie 2 : ajoute
Couleur pour le deuxième marquage
        if (nResults != 0){
            for (i=0; i < nResults; i++){
                labeli = getResult("Label", i);
                couleuri = getResult("Marquage", i);
                if (couleuri == 0){
                    setResult("Marquage", i, marquage2);
                }
                setResult("Label", i, fichier);
            }
        } else {
            showMessage("No Result found in result table");
            exit("Terminé");
        }

        updateResults();

        selectWindow(fichier + ".liff");
run("Close");

        } //Fin Analyse marquage
fitc*****
*****

        if (marquageRhod == 1){

            //*****
Rhod *****

            run("Bio-Formats Importer", "open=" + cheminFichier + "
color_mode=Default rois_import=[ROI manager] specify_range

```

```

view=[Standard ImageJ] stack_order=Default z_begin=RhoDZ
z_step=nbMarquage");

    selectWindow(fichier + ".liff");
    run("Enhance Contrast", "saturated=0.35");
    run("Unsharp Mask...", "radius=1 mask=0.50 stack");
    run("Set Measurements...", "area mean standard min
integrated median redirect=None decimal=3");
    roiManager("Show All without labels");
    roiManager("Measure");

updateResults();
//*****
if (nResults != 0){
for (i=0; i < nResults; i++){
    labeli = getResult("Label", i);
    couleuri = getResult("Marquage", i);
    if (couleuri == 0){
        setResult("Marquage", i, marquage3);
        //setResult("Label", i, fichier);
    }
    setResult("Label", i, fichier);
}
} else {
    showMessage("No Result found in result table");
    exit("Terminé");
}

updateResults();

selectWindow(fichier + ".liff");
run("Close");
} //Fin Analyse marquage Rhod

//***** Rhod
*****
j = 0;
// couleuri = "";

for (i=0; i < nResults; i++){
    couleuri = getResultString("Marquage", i);
    if (couleuri == "rhod"){
        roiManager("Select", j); //
        run("Find Maxima...", "noise=limitMaximaRhod
output=[Point Selection]");
        getSelectionCoordinates(x,y);
        if (selectionType==10){
            nn = x.length;
            setResult("Comptage", i, nn);
        }
        j++;
    }
}

updateResults();
selectWindow(fichier + ".liff");
run("Close");
} //fin test si comptage de fociRhod

```

```

        if (fociFitc == 1){
            run("Bio-Formats Importer", "open=" + cheminFichier + "
color_mode=Default rois_import=[ROI manager] specify_range
view=[Standard ImageJ] stack_order=Default z_begin=fitcZ
z_step=nbMarquage");

            selectWindow(fichier + ".liff");
            run("Enhance Contrast", "saturated=0.1");
            run("Unsharp Mask...", "radius=1 mask=0.50 stack");
            roiManager("Show All without labels");

            //***** Fitc
*****
            j = 0;
            // couleuri = "";

            for (i=0; i < nResults; i++){
                couleuri = getResultString("Marquage", i);
                if (couleuri == "fitc"){
                    roiManager("Select", j); //
                    run("Find Maxima...", "noise=limitMaximaFitc
output=[Point Selection]");
                        getSelectionCoordinates(x,y);
                        if (selectionType==10){
                            nn = x.length;
                            setResult("Comptage", i, nn);
                        }
                        j++;
                    }
                }

            updateResults();
            selectWindow(fichier + ".liff");
            run("Close");
        } //fin test si comptage de fociFitc

        //***** Colocalisation foci Fitc
* Rhod
        if (colocalisation == 1){
            run("Bio-Formats Importer", "open=" +
cheminFichier + " color_mode=Default rois_import=[ROI manager]
specify_range view=[Standard ImageJ] stack_order=Default z_begin=fitcZ
z_step=nbMarquage");

                selectWindow(fichier + ".liff");
                rename(fichier + "(Rhod)" + ".liff");
                run("Smooth", "stack");
                run("Smooth", "stack");
                run("Unsharp Mask...", "radius=2 mask=0.50
stack");

                run("Bio-Formats Importer", "open=" +
cheminFichier + " color_mode=Default rois_import=[ROI manager]
specify_range view=[Standard ImageJ] stack_order=Default z_begin=RhoDZ
z_step=nbMarquage");

                    selectWindow(fichier + ".liff");
                    rename(fichier + "(Fitc)" + ".liff");
                    run("Smooth", "stack");

```



```

        run("Smooth", "stack");
run("Unsharp Mask...", "radius=2 mask=0.50
stack");
        imageCalculator("Multiply create 32-bit
stack", fichier + "(Fitc)" + ".liff", fichier + "(Rhod)" + ".liff");
selectWindow(fichier + "(Fitc)" + ".liff");
run("Close");
selectWindow(fichier + "(Rhod)" + ".liff");
run("Close");

        selectWindow("Result of " + fichier +
"(Fitc)" + ".liff");

        //*****Compte les
foci de colocalisation *****
        j = 0;
        // couleuri = "";

        for (i=0; i < nResults; i++){
            couleuri =
getResultString("Marquage", i);
            if (couleuri == "fitc"){
                roiManager("Select", j); //
                run("Find Maxima...",
"noise=limitMaximaColoc output=[Point Selection]");
                getSelectionCoordinates(x,y);
                if (selectionType==10){
                    nn = x.length;
                    setResult("Colocalisation", i,
nn);
                }
                j++;
            }
            }

            updateResults();
selectWindow("Result of " + fichier +
"(Fitc)" + ".liff");
            run("Close");
        }//fin test si comptage de Colocalisation

        cheminFichierExcel = pathFichier + fichier + ".csv";
selectWindow("Results");
saveAs("Results", cheminFichierExcel); //sauve les résultats
en .csv avec des virgules comme séparateurs

        roiManager("reset")
selectWindow("Results");
run("Close");

        }//du test de fichier .liff
}// fin de la boucle sur les fichiers

//selectWindow(fichier + ".liff");
//run("Close");
exit("Terminé");
setBatchMode(false);

```


Jo-Ann Nettersheim

Interplay between the translesion DNA polymerase η and the calpain system

Abstract

Cells are constantly exposed to DNA damaging agents causing lesions, which are repaired by a range of DNA repair pathways. If DNA damages prevail during replication, they can cause replication fork breakdowns and mutations. One mechanism to prevent this is the translesion synthesis. Pol η is a translesion DNA polymerase which is capable to circumvent UV-induced lesions, to be repaired at a later time. However, pol η is error prone on non-damaged DNA and, therefore, needs to be tightly regulated. In this thesis I present an interaction between pol η and CAPNS1 we have found in our laboratory and our investigation of its role in regulating pol η . CAPNS1 is the small subunit of the calcium dependent calpain 1 and 2. We demonstrate that CAPNS1 is colocalized with pol η in the nucleus and calpain 1/2 can cleave pol η *in vitro* and *in vivo*. The proteolysis of pol η is found to occur at position 465 leading to a truncated protein encompassing the catalytic domain and the PIP1 motif. Interestingly, inhibition of calpain leads to a perturbed pol η foci formation and decreased cell survival. Taken together, these results suggest an important positive role for calpain in pol η dependent TLS.

Résumé

L'ADN est constamment altéré du fait du métabolisme oxydatif de la cellule ou de l'exposition à des agents génotoxiques environnementaux. Malgré l'existence de systèmes de réparation de l'ADN efficaces, certaines lésions sont encore présentes lors de la phase S du cycle cellulaire et bloquent la progression des fourches de réplication. La synthèse translésionnelle permet la reprise de la réplication. Pol η est une ADN polymérase translésionnelles capable de franchir très efficacement et sans erreur les lésions induites par les UV, qui sont réparées ultérieurement. Cependant, pol η est mutagène lorsqu'elle réplique l'ADN non endommagé et, par conséquent, son activité doit être strictement régulée. Dans cette thèse, je présente l'étude de l'interaction entre pol η et CAPNS1 mis en évidence dans notre laboratoire. CAPNS1 est la sous-unité régulatrice des calpaïnes 1 et 2, des protéases ubiquitaires dépendantes du calcium qui régulent de nombreux processus cellulaires fondamentaux en effectuant une digestion contrôlée de leurs protéines cibles. Nous démontrons que CAPNS1 est colocalisée avec pol η dans le noyau et que la calpaïne peut cliver pol η *in vitro* et *in vivo*. La protéolyse de pol η s'effectue après l'acide aminé 465, laissant le domaine catalytique intact avec un motif PIP1 alternatif. De manière surprenante, l'inhibition de la calpaïne entraîne une diminution de la formation de foyers pol η et de la survie cellulaire. Notre étude permet de dévoiler l'implication de la calpaïne dans la TLS dépendante de pol η .