CHARACTERISATION OF TWO DEUBIQUITINATING ENZYMES IN THE DNA DAMAGE RESPONSE AND REPLICATION

By

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Abstract

Ub has an essential role within the DNA double strand break (DSB) response which is well documented. However the role of ubiquitin (Ub) in the regulation of replication is an emerging area of research. This thesis investigates how two deubiquitinating enzymes (DUBs), POH1 and USP50, regulate DSB repair and replication respectively.

A screen of 103 siRNAs against putative DUBs in the human genome, measuring the amount of conjugated Ub after release from HU-induced damage, identified the proteasome associated DUB, POH1 as being important in regulating Ub-conjuagtes after damage. Further work found that POH1 restricts the K63-linked Ub at DSBs and consequently 53BP1 foci formation. This appears to regulate repair of breaks by Non-homologous end-joining (NHEJ).

The DUB screen also identified USP50 as having significantly reduced levels of conjugated Ub after damage. USP50 is an inactive DUB with Ub-binding activities, which has a role in preventing formation of Mus81-dependent DSBs during replication, with depletion sensitising cells to replication-stress. Therefore this works demonstrates a role for USP50 in genomic stability during replication.

In this thesis I demonstrate the role of these two DUBs in DSB repair and replication respectively, providing potential therapeutic targets.

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Abbreviations

4HB	4 helix bundle
4OHT	4-hydroxytamoxifen
53BP1	p53-Binding protein 1
B-TRCP	β-transducin repeat containing E3-Ub ligase protein
AAA+ ATPase	ATPase associated with diverse cellular activities ATPases
AAD	Active adenylation domain
ADRM1	Adhesion regulating molecule 1
Amp	Ampicillin
AND1	Acidic nucleoplasmic DNA-binding protein 1
APC	Anaphase promoting complex
Aph	Aphidicolin
APS	Ammonium persulfate
Asn	Asparagine
Asp	Aspartic acid
ATG	Autophagy
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM and Rad4 related
ATRi	ATR inhibitor
ATRIP	ATR interacting protein
BAP1	BRCA1 associated protein 1
BARD1	BRCA1-associated RING domain protein 1
BCDX2	Rad51B/Rad51C/Rad51D/XRCC2
BLM	Bloom syndrome protein
BRCA1	Breast cancer associated gene 1
BRCA2	Breast cancer associated gene 2
BRCC36	BRCA1/BRCA2 containing complex 36
BRCT	BRCA1 C-terminus

BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
BER	Base excision repair
cDNA	Complementary DNA
CENPF	Centromere Protein F
Cdc6	Cell division cycle 6
Cdk	Cyclin dependent kinase
Cdt1	Chromatin licensing and DNA replication factor 1
ChIP	Chromatin immunoprecipitation
Chk1	Checkpoint kinase 1
Cis	Cisplatin
CK2	Casein kinase 2
CldU	5-Chloro-2'-deoxyuridine
CMG	Cdc45/MCM/GINS complex
Cpt	Camptothecin
CtIP	Ct-BP interacting protein
CUE	Coupling of ubiquitin conjugation to endoplasmic reticulum degradation
Cys	Cysteine
CX3	Rad51C/XRCC3
DDR	DNA damage response
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
Dna2	DNA replication helicase/nuclease 2
DNase	Deoxyribonuclease
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
dNTP	Deoxynucleotide triphosphate
Dox	Doxycycline
DSB	Double strand break

dsDNA	Double stranded DNA
DSS1	Deleted in split hand/split foot malformation gene 1
DTT	Dithiotheitol
DUIM	Double-sided Ub-interacting motif
DUB	Deubiquitinating enzyme
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase enzyme
ECL	Enhanced chemiluminescence
E.Coli	*Escherichia Coli*
EDTA	Ethylene diamine tetra-acetic acid
EdU	5-ethynyl-2'-deoxyuridine
ELG1	Enhanced level of genomic instability 1
Eme1/2	Essential meiotic structure-specific endonuclease 1/2
ESCs	Embryonic stem cells
Exo1	Exonuclease 1
FAAP24	Fanconi anemia-associated protein 24
FACs	Fluorescent-activated cell sorter
FACT	Facilitates chromatin transcription
FAN1	FANCD2/FANCI-associated nuclease 1
FANC	Fanconi anemia complementation group
FCCH	First catalytic cysteine half-domain
FCS	Foetal Calf Serum
FEN1	Flap structure-specific endonuclease 1
FHA	Forkhead associated
FITC	Fluorescein Isothiocyanate
FOXO4	Forkhead box O4
FPC	Fork protection complex
FUB	Fau Ub-like protein

G1/G2	Growth phase ½
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAT	GGA and TOM domain
Gen1	Gen endonuclease homologue 1
GFP	Green fluorescent protein
GINS	Go, Ichi, Nii, Sans (Sld5, Psf1, Psf2 and Psf3)
GLUE	GRAM-like Ub binding in EAP45
Gly	Glycine
H2A	Histone 2A
HCl	Hydrochloric acid
HECT	Homologous E6-AP C-terminus
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic acid
HERC2	HECT domain and RLD (renal cell carcinoma-like domain) 2
His	Histidine
HJ	Holliday junction
HR	Homologous recombination
HRP	Horseradish peroxidase
HU	Hydroxyurea
IAD	Inactive adenylation domain
ICL	Interstrand crosslink
IdU	Iododeoxyuridine
IF	Immunofluorescence
IKKγ	Inhibitor of Kappa-light polypeptide gene enhancer in B cells, kinase gamma
IL	Interleukin
Ile	Isoleucine
INO80	Inositol-requiring protein 80
IP	Immunoprecipitation
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IR	Irradiation

JAMM	Jab1/MPN/Mov34 metalloenzyme
JMJD	Jumonji domain containing
K	Lysine
Kan	Kanamycin
L3MBTL1	Lethal (3) malignant brain tumour like 1
LB	Luria Bertani
Lig1/3	Ligase1/3
M	Mitosis
MCM	Minichromosome maintenance complex
MDC1	Mediator of DNA damage checkpoint protein 1
Mdm2	Mouse double minute gene number 2
Merit40	Mediator of Rap80 interactions and targeting 40 kd
MJD	Machado-Joseph disease protein domain proteases
MoeB	Molybdopterin biosynthetic enzyme B
MRN	Mre11/Rad50/Nbs1
MUB	Membrane-anchored Ub-fold protein
MUI	motif interacting with Ub
Mus81	p6 ethyl-methansulfonate UV sensitive 81
NEDD8	neuronal precursor cell expressed developmentally down-regulated protein 8
NER	Nucleotide excision repair
NF-κB	nuclear factor Kappa-light-chain-enhancer of activated B cells
NFRKB	Nuclear factor related to Kappa B
NHEJ	Non-homologous end-joining
Ni^{+}	Nickel
NTC	Non-targeting control
NZF	Npl4 zinc finger
ORC	Origin recognition complex
OTU	Ovarian tumour proteases
P53	Protein 53

PAGE	Polyacrylamide gel electrophoresis
PALB2	Partner and localizer of BRCA2
PBS	Phosphate buffered saline
PAR	Poly-ADP ribose
PARP1	Poly-ADP ribose-polymerase 1
PAXX	Paralogue of XRCC4 and XLF
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PH	Pleckstrin homology
PI	Propidium Iodide
PIP	PCNA interacting peptide
PLK1	Polo-like kinase 1
PNKP	Polynucleotide kinase/phosphatase
POH1	Pad one homologue 1
Pol	Polymerase
PRC1	Protein regulator of cytokinesis 1
Pre-RC	Pre-replication complex
PRU	Pleckstrin-like receptor for Ub
PSMD	Proteasome (Prosome, macropain) 26S Subunit non-ATPase
PTM	Post-translational modification
PUP	Prokaryotic Ub-like protein
PVDF	Polyvinylidine difluoride
R	Arginine
Rad51	Radiation sensitivity gene 51
Rap80	Receptor associated protein 80
RFP	Red fluorescent protein
RIF1	RAP1 Interacting factor 1
RING	Really interesting new gene

RNF	Ring finger protein
ROS	Reactive oxygen species
RPM	Rotations per minute
RPN	Regulatory particle non-ATPase
RNA	Ribose nucleic acid
RPA	Replication protein A
RQC	RecQ C-terminal domain
RTK	Receptor tyrosine kinases
S-phase	Synthesis phase
SCCH	Second catalytic cysteine half-domain
SCE	Sister chromatid exchange
SDS	Sodium Dodecyl Sulfate
SENP	Sentrin-specific protease
Ser	Serine
shRNA	Short hairpin RNA
SIM	SUMO interacting motif
siRNA	Small interfering RNA
Slx	Structure-specific endonuclease subunit
SMARCAL1	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin A1
SNF2h	Sucrose non-fermenting 2 homologue
SNM1A	DNA cross-linking repair 1A
ssDNA	Single stranded DNA
TAE	Tris, Acetic acid, EDTA buffer
TEMED	Tetramethylethylenediamine
ThiF	Thiamine biosynthesis protein
TGN	*Trans*-Golgi network
Thr	Threonine
TLS	Translesion synthesis
Top2	Topoisomerase 2

TOP2B	Topoisomerase II beta
TOPBP1	DNA topoisomerase 2 binding-protein 1
Topo IIIα	Topoisomerase III alpha
TRIP12	Thyroid hormone receptor interactor 12
Tyr	Tyrosine
WRN	Werner Syndrome, RecQ-Helicase-like
WRNIP	WRN Interacting protein
Ub	Ubiquitin
UBA	Ub-like modifier activating enzyme
UBAN	Ub-binding in ABIN and NEMO domain
UBC	Ub-conjugated enzyme-related
UBD	Ubiquitin-binding domain
Ubl	Ubiquitin-like protein
UBM	Ub-binding motif
UBR5	Ub protein ligase E3 component n-recognin5
UBZ	Ub-binding Zinc finger
UCH	Ubiquitin C-terminal hydrolase
UCRP	Ub cross-reactive protein
UEV	Ub-conjugating enzyme E2 variant
UFD	Ub fold domain
UFM	Ub-fold modifier
UIM	Ub-interacting motif
UPS	Ubiquitin proteasome system
URM1	Ub-related modifier 1
USP	Ub specific protease
USP50	Ubiquitin specific protease 50
UV	Ultraviolet
Val	Valine
VCP	Valosin containing protein

VHS	Vps27/Hrs/STAM
WCE	Whole cell extract
WRN	Werners syndrome, RecQ-helicase like
WRNIP1	WRN interacting protein 1
WT	Wild type
XLF	XRCC4-like factor
XP	Xeroderma pigmentosum
XPC	Xeroderma pigmentosum complementation group C
XRCC	X-ray complementing Chinese hamster gene
Zn^{2+}	Zinc ion
ZnF	Zinc finger
ZRANB3	zinc-finger, RAN-binding domain containing 3

1. Introduction

Maintaining the integrity of the genome is vital in preventing diseases such as cancer (Jackson and Bartek 2009). DNA can be damaged by both endogenous mechanisms, such as reactive oxygen species (ROS), or through exogenous agents, including UV irradiation and drugs, for example, Hydroxyurea (HU) (Ravanat, Douki et al. 2001, Hakem 2008, Halazonetis, Gorgoulis et al. 2008, Jena 2012). The pathways required to repair damage and allow faithful replication of DNA are tightly regulated throughout cell divisions. Unfortunately, this process can be faulty with mutations arising which cause diseases to develop (Jackson and Bartek 2009). One method of regulating these pathways, such as DNA damage repair pathways including double strand break (DSB) repair, is by post-translation modifications (PTM) like ubiquitination, phosphorylation and methylation (Jackson and Durocher 2013).

PTMs change an aspect of the substrate, including its structure, interactions and activity. This can then be used to regulate a process in the cell, including DNA damage repair, cell cycle checkpoint regulation, replication fork maintenance and transcription (Berger 2002, Lavin and Gueven 2006, Branzei and Foiani 2010, Oberle and Blattner 2010, Lehmann 2011, Duan and Walther 2015). Many proteins contain domains that bind specifically to PTMs, for instance, the phosphorylation binding domain, BRCA1 C-terminus (BRCT) domain (Yu, Chini et al. 2003), methylation binding domains such as Tudor domains (Lu and Wang 2013) and ubiquitin-binding domains (UBDs) (Hurley, Lee et al. 2006). Therefore, certain protein-protein interactions only happen once a PTM occurs. Other proteins, including the DNA damage regulatory kinase, Ataxia telangiectasia mutated (ATM), requires phosphorylation in order to have enzymatic activity (Bakkenist and Kastan 2003). Within this project the regulation of the PTM Ubiquitin (Ub) has been studied due to its role in the DNA damage response (DDR) and

more recently in replication (Messick and Greenberg 2009, Lehmann 2011, Maric, Maculins et al. 2014, Moreno, Bailey et al. 2014).

1.1 Ubiquitin and Ubiquitin-like proteins

Ub is an integral part of the regulation of almost all pathways within eukaryotic cells (Hochstrasser 2000). Although not present in prokaryotes, the Ub cycle evolved from bacterial proteins (Lake, Wuebbens et al. 2001, Wang, Xi et al. 2001). Prokaryotic cells have a modification called Prokaryotic Ubiquitin-like Protein (PUP) which targets the substrate for degradation by the proteasome, likely giving rise to the eukaryotic Ub system (Pearce, Mintseris et al. 2008, Burns, Liu et al. 2009, Chen, Solomon et al. 2009).

Ub is an 8.5kDa protein that forms transient modifications on many different substrates (Goldstein, Scheid et al. 1975, Trempe 2011). These modifications can either be a single Ub, monoubiquitin (mono-Ub), several single Ub moietys (multi-mono-Ub), or as chains, called polyubiquitin (poly-Ub) (Fig. 1.1 A-D) (Hicke 2001). Polyubiquitin chains form through the seven conserved lysines within Ub, K6, K11, K27, K29 K33, K48 and K63 or via the N-terminal methionine (Peng, Schwartz et al. 2003, Pickart and Fushman 2004, Kirisako, Kamei et al. 2006). Depending on which linkage is formed, other linkages can be restricted due to certain lysines within the Ub no longer being accessible due to the orientation of the Ub molecule is bound (Trempe 2011). Different linkages have distinct chain structures to signal diverse processes, with K48 and K63-linked Ub being particularly important in the DDR (Fig. 1.2) (Komander 2009, Komander and Rape 2012).

The interaction of Ub with other proteins usually requires a hydrophobic patch on the surface of Ub known as the Ile44 patch. This hydrophobic domain is comprised of the

Leu8, Ile44 and Val70 residues on the Ub β-sheet (Sloper-Mould, Jemc et al. 2001). Most UBDs target this Ile44 hydrophobic patch in order to bind Ub, with many DUBs interacting with this patch (Hurley, Lee et al. 2006).

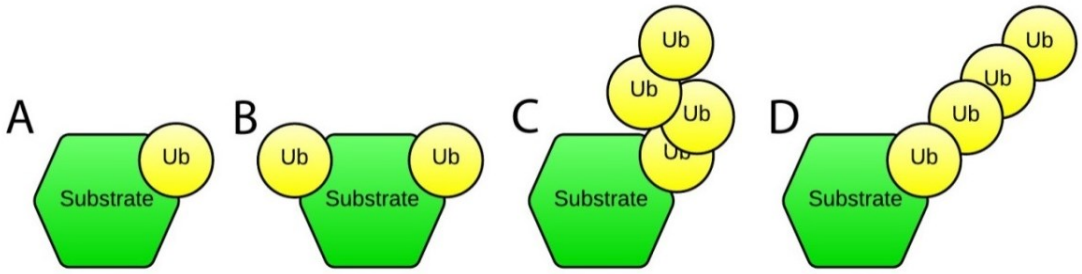

Figure 1.1. Representation of the different types of Ub modifications. Once modified with Ub a substrate can either have **A)** one Ub attached (mono-Ub). **B)** multiple single Ubs (multi-mono-Ub) **C and D)** or a poly-Ub chain. Chains take on different confirmations depending on how they are linked.

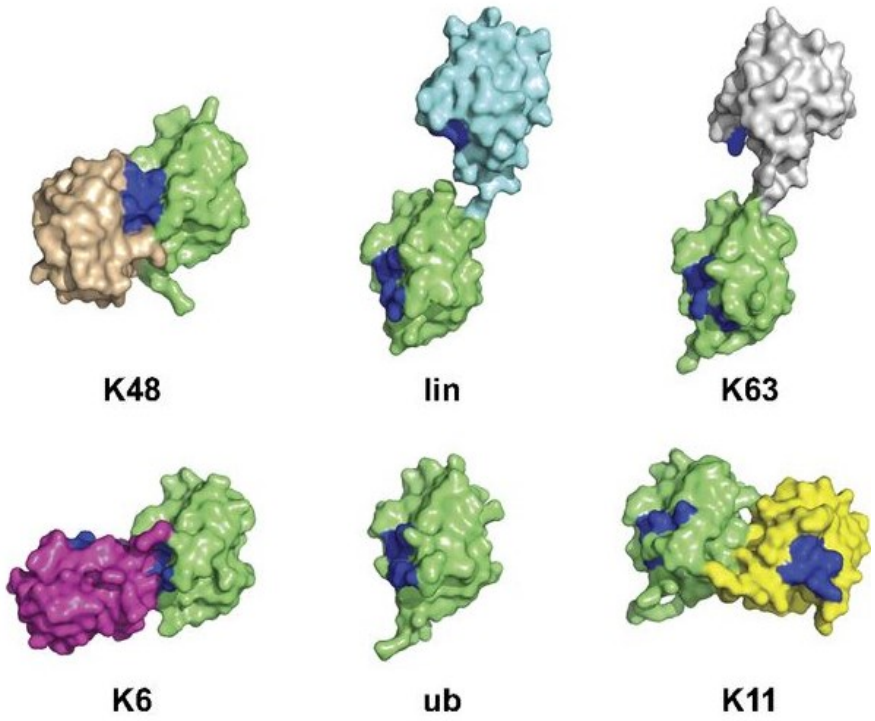

Figure 1.2. Protein structure of the isopeptide linkages formed through the different Ub lysines. The polyubiquitin chains have a different structure depending on which lysine the poly-Ub chain forms through. The green Ub is always in the same orientation, the Ile44 hydrophobic patch is highlighted in blue. K48-linked chains have a closed structure, where the Ub molecules bind closely together. The K6 and K11 chains have similar closed structures to K48 chains. K63-linked chains have an open conformation where the isopeptide bond is accessible between Ub moieties, mimicked by the linear linked Ub (Clague, Barsukov et al. 2013)

Ubiquitin-like proteins (Ubls) are structurally similar to Ub, with the same β-grasp fold structure although the similarity does not extend to the sequence (Cajee, Hull et al. 2012). There are 14 predicted Ubls encoded for in the human genome, of these the small ubiquitin-like modifier (SUMO) is the best studied (Hochstrasser 2009). Although not explicitly studied in this thesis the role of Ubls and their interaction with Ub is important in many cellular processes, with SUMO being shown to regulate factors involved in both DSB repair and replication (Branzei and Foiani 2010, Ulrich and Walden 2010, Bekker-Jensen and Mailand 2011).

The other Ubls include neuronal precursor cell expressed developmentally down-regulated protein 8 (NEDD8), which regulates a subset of Ub-ligases, the Cullin E3 ligases, which are implicated in regulating certain processes during replication (Pan, Kentsis et al. 2004). Other Ubls include Ub-Related Modifier-1 (URM1), Ub Cross-Reactive Protein (UCRP), Autophagy-8 (ATG8) and Autophagy-12 (ATG12), Fau Ub-like protein (FUB1), Membrane-anchored Ub-fold protein (MUB), Ub-fold Modifier 1 (UFM1) and ubiquitin-like protein-5 (UBL5) (Hochstrasser 2009). Although there is little similarity in sequence between Ub and Ubls, the enzyme cascade to conjugate Ubls is similar to that of Ub (Kerscher, Felberbaum et al. 2006).

1.2 The Ubiquitin cycle

Ub modifications are transient and are attached through a cascade of three enzymes then removed to replenish the free-Ub pool (Fig. 1.3). The three enzymes are an E1-activating enzyme (Fig 1.4 A), an E2 conjugating enzyme (Fig 1.3 B) and an E3 ligating enzyme (Fig 1.3 C) (Hershko, Heller et al. 1983). The E1 enzyme activates the Ub in an ATP-dependent process that produces adenylated-Ub (Fig 1.3 A). The Ub-AMP is is then passed onto an active cysteine (Cys) in the E2 conjugating enzyme by a thioester bond. Once the Ub is bound to the E2 enzyme, termed a charged-E2, an E3 enzyme is recruited and the Ub is covalently bound to a lysine in the substrate through an isopeptide bond (Pickart and Eddins 2004). Ub is then removed by a family of enzymes called Deubiquitinating enzymes (DUBs) (Fig 1.3 D) (Amerik and Hochstrasser 2004).

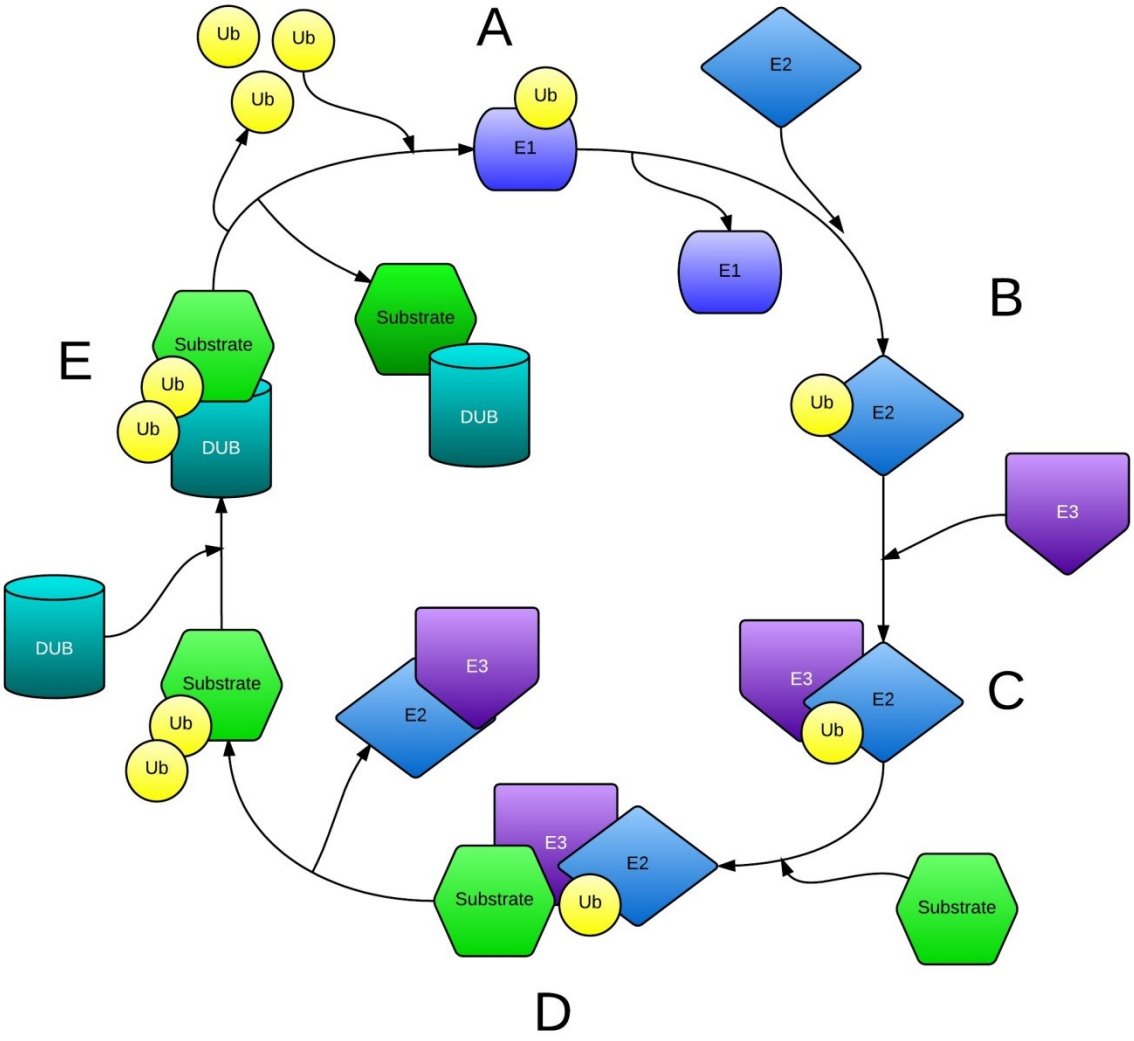

Figure 1.3. Diagram of the Ub ligation cycle. A) Free-Ub is picked up by an E1-activating enzyme and activated in an ATP-dependent reaction. B) Activated Ub is then passed onto an E2-conjugating enzyme onto a catalytic Cys residue. C) The Ub-E2 enzyme complex interacts with an E3-ligase enzyme. D) Depending on the type of E3 ligase, the E3-enzyme binds to the substrate in order to pass the Ub onto a lysine within the substrate. E) Ubiquitinated substrates are then targeted by a deubiquitinating enzyme or the proteasome, which recycles the Ub back into the free-Ub pool.

1.2.1 E1 activating enzymes

Eukaryotic E1 enzymes have arisen from molybdopterin biosynthetic enzyme B (MoeB) and thiamine biosynthesis protein (ThiF) prokaryotic enzymes, which are involved in sulphur transfer (Lake, Wuebbens et al. 2001). However, in eukaryotes the cycle has evolved to transfer Ub. There are two E1s encoded in the human genome, *UBA1* and *UBA6* (Handley-Gearhart, Stephen et al. 1994). Both enzymes contain two active sites and two domains that are required for the transfer of Ub molecules onto E2 enzymes (Haas, Warms et al. 1982).

E1-enzymes contain two active sites that activate then pass on the adenylated-Ub respectively. The E1 binds Ub and adenosine triphosphate with a magnesium ion (ATP-Mg^{2+}) causing an adenylated Ub (Ub-AMP) that is bound to the first active site of the E1 enzyme. The Ub-AMP undergoes nucleophilic attack by the Cys residue from the second active site of the E1 enzyme which forms a thioester bond between the activate Cys residue within the second active site of the E1-enzyme and the C-terminus of Gly-Gly motif of the Ub. The transfer of the Ub moiety from the first active site to second active site causes the release AMP from Ub. The E1 enzyme has to undergo a conformational change to bring the catalytic Cys within the second active site close enough to the Gly-Gly motif of the C-terminus of the Ub which is bound in the first active site. The E2 enzyme interacts with the C-terminal thioester bond of Ub bound in the first active site of the E1-enzyme resulting in the transfer of the Ub onto the Cys residue in the active site of the E2 enzyme via a thioester transfer reaction (Ciechanover, Elias et al. 1980, Schulman and Harper 2009).

1.2.2 E2 Conjugating enzymes

There are around 37 active E2 enzymes known in the human genome that can bind to the E1 enzymes and transfer the Ub (Michelle, Vourc'h et al. 2009). The transfer of Ub requires a core domain that is conserved in the E2 enzymes variants. The active core is formed of four α-helices, with an active Cys residue in the linker region between helix 2 and helix 3, and a β-sheet (Burroughs, Jaffee et al. 2008, van Wijk and Timmers 2010). The E2 can only bind an E1 once the E1 has bound Ub and adopted the closed confirmation, as the E1 in this confirmation has exposed sites that bind to the E2 enzyme via the first α-helix (Lois and Lima 2005, Huang, Hunt et al. 2007, Lee and Schindelin 2008).. The specificity of E2 binding to E1s is likely to be partially determined by sequences flanking the core region (Huang, Miller et al. 2004, Huang, Zhuang et al. 2008).

The E2s themselves can specify which Ub-linkages form (Chen and Pickart 1990, Vannocker and Vierstra 1991, Hofmann and Pickart 1999). This is demonstrated by an E3-ligase that is important in DSB repair, RNF8, which produces both K48-linked Ub or K63-linked Ub chains depending on which E2 is bound. When bound to UbcH8, RNF8 produces K48 linked chains, but when bound to Ubc13 RNF8 is known to specify the K63-linkage (Lok, Sy et al. 2011). Although it is generally accepted that the E3 ligase provides the specificity for the linkage formed.

1.2.3 E3 ligase enzymes

Although E2 enzymes can form chains without E3 enzymes, there are hundreds of Ub-ligases in the human genome that are divided into subfamilies (Semple, Grp et al. 2003). The three families are: Homologous E6-AP C-terminus (HECT) ligases, Really Interesting New Gene (RING) ligases, which also contain the multisubunit Cullin E3

ligases, and the RING-between-RING (RBR) E3 ligases (Fig. 1.4) (Weissman 2001). HECT ligases utilise an active Cys as part of a large C-terminal 350 amino acid domain, which form a thioester intermediate with Ub and pass it on to the substrate (Huibregtse, Scheffner et al. 1995). The HECT E3 ligases, therefore, have enzymatic activity that removes the Ub from the E2 enzyme and then ligates it to the substrate (Bernassola, Karin et al. 2008). The N-lobe of the HECT domain binds to the substrate whereas the C-lobe binds to the Ub. Beyond the HECT domain, the N-terminal portion of HECT ligases proteins acts to provide specificity for the substrate (Huang, Kinnucan et al. 1999). There are approximately 30 HECT ligases in the human genome, but the vast majority of E3 ligases are RING type ligases, with over 600 genes specifying RING-type E3 ligases (Metzger, Hristova et al. 2012).

RING ligases have two zinc ions and act as a docking system to allow transfer of Ub between the E2 enzyme and the substrate (Lorick, Jensen et al. 1999, Ozkan, Yu et al. 2005). RING E3s can be monomeric or dimeric with the dimeric E3s forming either homodimers or heterodimers (Metzger, Hristova et al. 2012). This dimerization occurs either through the RING-domain, which provides the E3-ligase activity, such as with the RNF4 homodimer, or through helical domains, comprised of α-helices around the RING domain, as seen in the BRCA1/BARD1 heterodimer, which is involved in homologous recombination repair (Brzovic, Rajagopal et al. 2001, Linke, Mace et al. 2008, Liew, Sun et al. 2010). The binding of the E2-Ub complex to the RING E3 enzyme causes an allosteric activation of the E3 enzyme, by holding the donor Ub in a stable conformation to allow the selection of the type of linkage as well as placing the thioester bond between the Ub and the E2 into the optimal orientation for nucleophilic attack to transfer the Ub onto the substrate. Substrate recognition is likely to be

mediated by other domains within the RING E3 ligase, including the BRCT domain in BRCA1 (Plechanovova, Jaffray et al. 2012, Berndsen and Wolberger 2014).

There is a subset of the RING ligases that are known as the Cullin ligase family. Cullins are multiple protein complexes which contain a RING-box protein that has the E3 ligase activity, a substrate adaptor component (such as an F-box protein) which binds to the substrate and a Cullin scaffold protein. In humans there are six Cullin proteins, Cul1, Cul2, Cul3, Cul4A/Cul4B and Cul7. There are a few conserved mechanisms of substrate recognition throughout the Cullins, including the recognition of phosphodegrons, which are short peptide sequences that signal degradation in a phosphorylation dependent manner (Lydeard, Schulman et al. 2013).

The final subtype of E3-ligases is the RBR family. These proteins consist of an N-terminal RING domain with two zinc ions, as seen in the classical RING E3-ligase structures. A C-terminal RING domain which only contains one zinc ion. While the N-terminal RING domain is essential for Ub-ligase activity, the C-terminal ligase appears to be dispensable in some cases and less well conserved than its N-terminal counterpart (Eisenhaber, Chumak et al. 2007). The mechanism of Ub-transfer between E2 and substrate of the RBR ligases combines that seen in the RING and HECT E3-ligases. The believed mechanism of action is that the N-terminal RING facilitates the movement of Ub from the E2 and passes this onto another domain of the RBR, including the C-terminal RING. The Ub can then be transferred from the E3 onto the substrate (Wenzel and Klevit 2012).

Once the Ub has been ligated onto the substrate, the type of modification is important in determining what pathway is signalled.

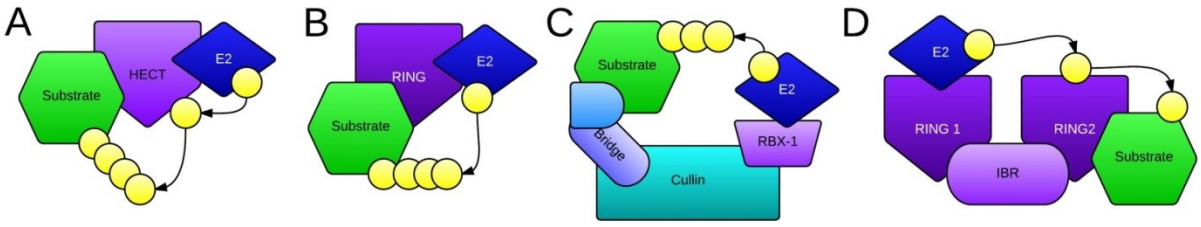

Figure 1.4. Representation of the different families of E3 ligases. There are three main groups of E3-ligases active in the cell. **A)** The HECT type of E3 ligase interacts with the E2-conjugating enzyme and the Ub molecule is passed onto the E3-enzyme before being passed onto the substrate. **B)** The RING type E3 ligases act as a scaffold to bring the E2 and substrate into proximity with each other in order for the E2 to directly pass the Ub onto the substrate. **C)** The Cullin E3-ligases are multi-subunit complexes. The E3-ligase activity comes from a RING-box protein. Cullins also contain a Cullin scaffold protein, a substrate adaptor protein and the substrate. D) Ring-inbetween-Ring ligase composed of two RING domains and an the "in-between-ring" (IBR) domain.

1.3 Mono-Ub

The addition of a single Ub molecule to a substrate is an important regulatory mark in cells. Histones are known to be mono-ubiquitinated in many cellular processes, with UV damage causing the mono-ubiquitination of H2A around the lesion. This modification occurs as part of the removal of damaged bases during nucleotide excision repair (NER) (Bergink, Salomons et al. 2006). Further to this the H2AX variant has been shown to be modified by mono-Ub after damage in order to regulate the chromatin architecture around the break (Ikura, Tashiro et al. 2007). Within the DNA damage response, the mono-Ub of FANCD2 and FANCI are important modification in the removal of intrastrand crosslinks (ICLs) (Garcia-Higuera, Taniguchi et al. 2001, Smogorzewska, Matsuoka et al. 2007, Kitao and Takata 2011). The Fanconi Anemia Core complex contains the FANCL protein that is the active component of an E3-ligase which mono-ubiquitinates the FANCD2-FANCI heterodimer (Meetei, de Winter et al. 2003, Yuan, El Hokayem et al. 2009). This allows recruitment of the heterodimer to the

ICL in order for repair to continue with the interaction with DNA enhancing the mono-Ub signal (Kitao and Takata 2011, Longerich, Kwon et al. 2014).

The proliferating cell nuclear antigen (PCNA) is known to be mono-ubiquitinated and there is debate as to whether this ubiquitination is required for translesion synthesis (TLS), to allow replication fork bypass of damaged template DNA during replication (Kannouche, Wing et al. 2004, Watanabe, Tateishi et al. 2004, Hendel, Krijger et al. 2011).

Substrates can also be mono-ubiquitinated at several sites leading to multi-mono-Ub. Similarly to mono-Ub, the multi-mono-ubiquitination of receptor tyrosine kinases (RTKs) signals endocytosis of this receptor and subsequent degradation within a lysosome (Haglund, Sigismund et al. 2003). Importantly for cell cycle control and DNA damage, mouse double minute 2 homolog (Mdm2) multi-mono-ubiquitinates the cell cycle and tumour suppressor protein, Protein 53 (p53), which is hypothesised to expose the nuclear export sequence. Once in the cytoplasm, p53 can be poly-ubiquitinated and then degraded (Lai, Ferry et al. 2001). However, the signal for proteasomal degradation requires a poly-Ub chain of at least four Ub molecules (Piotrowski, Beal et al. 1997).

1.4 Poly-Ub

The process that is signalled by poly-Ub depends, in part, on the structure of the Ub chains (Komander 2009). Poly-Ub can form as a single linkage through one of the conserved lysines, a single chain of multiple linkages or branched structures (Peng, Schwartz et al. 2003, Kim, Kim et al. 2007). The most well studied chains are K48-chains which are known to signal the degradation of substrates by the proteasome and are the most abundant chain type within the cell (Chau, Tobias et al. 1989, Peng, Schwartz et al. 2003, Xu, Duong et al. 2009). The K48-chains form a closed, tight

structure where the Ub molecules not only interact via the isopeptide bond but also via other interfaces. This closed confirmation means the Ile44 hydrophobic patch of Ub, which interacts with many Ub binding domains (UBDs) is no longer accessible, therefore, there is flexibility in the structure that opens the confirmation to allow for binding (Varadan, Walker et al. 2002, Fushman and Walker 2010). Other closed conformation chains include K6 and K11 (Fig. 1.2) (Fushman and Walker 2010).

The open confirmation of K63-linked chain structure is mimicked by the linear Ub chains (Fig. 1.2). In K63-chains, the only contact between the Ub moietys is via the isopeptide bond between the Ub molecules (Weeks, Grasty et al. 2009). The lysine bond in K63-linked chains is inherently more flexible than the methinonine bond of linear chains meaning K63-chains can be recognised by a wider range of Ub-binding proteins than linear chains (reviewed in Chen and Sun 2009, Komander, Reyes-Turcu et al. 2009, reviewed in Trempe 2011). K63-linkages are biologically relevant in reaction to DSBs (reviewed in Chen 2005, Kolas, Chapman et al. 2007, Zhao, Sonoda et al. 2007, reviewed in Chen and Sun 2009).

The remaining linkages are less well studied. It is known that K6 chains can be made by the E3-ligase Breast cancer 1, early onset (BRCA1) after DNA damage, as well as another E3 enzyme Ring1b-Bmi1 (Nishikawa, Ooka et al. 2003, Wu-Baer, Lagrazon et al. 2003, Ben-Saadon, Zaaroor et al. 2006). The E3-ligase Parkin also has been shown to auto-ubiquitinate itself with K6-chains, which need to be removed in order for parkin to relocate to the mitochondria (Durcan, Tang et al. 2014).

K27-chains are present on Inhibitor of Kappa Light polypeptide gene enhancer in B-cells, kinase gamma (IKKγ) and recruit Rhomboid domain containing 3 (Rhbdd3), which itself is ubiquitinated with K27-chains. These chains recruit the deubiquitinating

enzyme A20, which inhibits the activation of NF-κB by cleaving the K63-linked chains that stimulate interleukin 6 (IL-6) (Liu, Han et al. 2014). Therefore K27-chains may have a role in suppressing auto-immune responses. Ring1B is also known to auto-ubiquitinate itself with K27-chains (de Bie, Zaaroor-Regev et al. 2010).

The other closed confirmation linkage are K11-chains, they are the second most abundant chain in yeast although this is not observed within higher eukaryotes (Xu, Duong et al. 2009, Dammer, Na et al. 2011). They act as another signal for degradation by the proteasome that is distinct from the K48-chain signal and may affect the processing of the substrates. One ligase known to produce K11-chains is the APC complex along with UbcH10 or UBE2S E2-activating enzymes. By targeting cell-cycle regulators, such as cyclinB1, Aurora A and Plk1, for degradation by the proteasome, K11-chains regulate the cell cycle (Williamson, Wickliffe et al. 2009, Song and Rape 2010, Wu, Merbl et al. 2010, reviewed in Bremm and Komander 2011).

The K29 and K33 lysine residues are close together within the ubiquitin structure (Xu, Duong et al. 2009). The role of chains formed through these lysines *in vivo* is not understood, although recent work has demonstrated a role for K33-linked chains for protein trafficking within the *trans*-Golgi network (TGN) (Yuan, Lee et al. 2014). Two members of the HECT family of E3-ligases can form K29 and K33 chains, these are UBE3C and AREL1, with both these poly-Ub chains form open structures (Michel, Elliott et al. 2015).

Poly-Ub does not just form as a single linkage type, chains of multiple linkages can form, as well as branched structures that allow for a multitude of signalling pathways to be signalled (Kim, Kim et al. 2007). This is demonstrated by the formation of mixed K63 and linear chains in the activation of IκB kinase (IKK) complex (Emmerich,

Ordureau et al 2013). Further to this the branched structure of some poly-Ub chains is believed to inhibit degradation of substrates by the proteasome and, therefore a proteasome subunit s5a inhibits the formation of branched chains (Kim, Kim et al. 2009). Conversely, recent work has demonstrated that mixed chains formed by the anaphase-promoting complex (APC) Ub-ligase are a more potent signal for degradation than the homogenous K11-chains (Meyer and Rape 2014). Therefore the role of branched chains in the cell is under debate.

The complexity and variety of the Ub-chains allow for many different signals to form through this one modification. Poly-Ub chains are known to be a major signalling mechanism for DSB repair (Bekker-Jensen and Mailand 2011).

1.5 Deubiquitinating enzymes (DUBs)

There are believed to be 79 active DUBs in the human genome (reviewed in Nijman, Luna-Vargas et al. 2005). DUBs are required to hydrolyse the bonds between either the Ub moieties or Ub and the substrate, in order to remove the Ub modification and recycle it back into free Ub that can be utilised by the cell. This is carried out by a nucleophilic attack on the isopeptide bond formed between the C-terminal end of Ub and the acceptor lysine. There are five subsets of DUBs, Ub specific peptidases (USPs), Ub C-terminal hydrolases (UCHs), Ovarian tumour proteases (OTUs), Jab1/MPN domain-associated metalloproteases (JAMM) and Machado-Joseph disease protein domain proteases (MJDs) (Fig. 1.5) (reviewed in Nijman, Luna-Vargas et al. 2005, reviewed in Komander, Clague et al. 2009). The USPs, UCHs, MJDs and OTUs all use an active Cys to break the isopeptide bond (Cys-DUBs), whereas the JAMM-type DUBs use a zinc ion (Zn^{2+}) in order to break the isopeptide bond. The catalytic triad of the USP, UCH, MJD and OTU type DUBs, works by the Cys residue attacking the C-

terminal end of the Ub molecule with the help of the His residue that is part of the catalytic triad. The third residue of the catalytic triad, Asn/Asp, polarises the His residue and stabilises it in the correct position allowing effective breakage of the bond (Fig. 1.5) (Komander and Barford 2008, reviewed in Komander, Clague et al. 2009). The binding of Ub to the DUB causes the extension of the isopeptide bond within the active site (Drag, Mikolajczyk et al. 2008, reviewed in Komander, Clague et al. 2009).

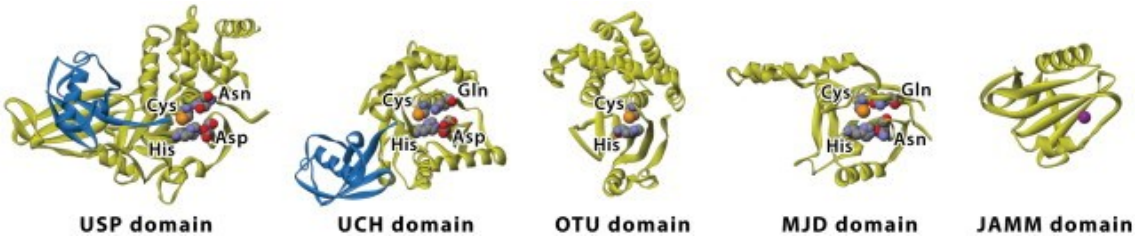

Figure 1.5. Protein structure of the five families of DUB enzymes. The yellow ribbons represent the protein structure of the DUB domain, the blue ribbons represent a Ub molecule. There are five families of DUBs: USP, UCH, OTU, MJD and JAMM-type. The first four types of DUBs utilise a catalytic triad consisting of a Cys, His and Asn/Asp residue, which carry out nucleophilic attack on the isopeptide bond between Ubs or Ub and substrate. The final form of DUB is a JAMM-type DUB which uses a zinc ion to break the Ub bond. Reprinted from (Nijman, Luna-Vargas et al. 2005) with permission from Elsevier.

Of the Cys-DUBs, the USPs are the major group, with the majority of DUBs in the human genome falling into this family (Semple, Grp et al. 2003, reviewed in Nijman, Luna-Vargas et al. 2005). The structural domains of the USPs are correlated with the shape of the hand, with a palm, thumb and the finger domain which bind the Ub molecules. The boundary between the fingers and palm is the region where the isopeptide bond is actually broken (Hu, Li et al. 2002). The active domain containing the Cys, His and Asn/Asp varies in size being 300-800 residues long, with USP16 and USP30 lacking the active Asn/Asp. Despite lacking the Asn/Asp, USP16 and USP30 remain active and have evolved a separate mechanism to stabilise the active His

(reviewed in Nijman, Luna-Vargas et al. 2005). Binding of Ub causes a conformational change within USPs which causes the residues of the catalytic triad to move into proximity with each other to allow the cleavage of the isopeptide bond (Hu, Li et al. 2005). The USP family of DUBs are the most promiscuous of the DUBs being able to cleave most linkage types (Faesen, Luna-Vargas et al. 2011).

The OTU family makes up the next largest group of DUBs, with 14 members, and is subdivided into four subfamilies, OTUBs, OTUDs, OTULINs, and A20-like DUBs (reviewed in Komander, Clague et al. 2009). Each subfamily is distinguished by their catalytic domain, with the OTUDs having the smallest catalytic domain. The OTU family was named due to their involvement in the formation of the ovaries in *Drosophila melanogaster*, but the first of this family found to have DUB activity was Otubain (OTUB1) (Goodrich, Clouse et al. 2004). The Ub-binding site of this family has diverged, with yeast OTU domain-containing protein 1 (Otu1) DUB utilising a large loop to bind Ub, whereas OTUB1, Otubain2 (OTUB2) and A20 use disordered apo-structures. Further to this difference, the distal Ub-binding site in Otu1 is precluded by a helical domain. Although this family utilise the active Cys and His they do not require the Asn/Asp to be active and instead have evolved a hydrogen binding network which stabilises the His residue, as seen in OTUB2 (Nanao, Tcherniuk et al. 2004). The OTU-family exists in both active and inactive forms, based on a conformational change, with the active form having the Cys residue nearer to the His residue of the catalytic triad (Edelmann, Iphofer et al. 2009). The OTU family has been shown to be very specific to the linkages it cleaves with six members only cleaving one linkage type, including the K48-specific DUB OTUB1 which is implicated in regulating Ub and DNA breaks (Nakada, Tai et al. 2010, Mevissen, Hospenthal et al. 2013). Four of the OTU family cleaving two linkage types, including A20 which cleaves K11 and K48 , as well as

OTUD3 which can cleave both K6 and K11 . The OTU family members display four mechanisms that make them linkage specific, with only one mechanism dependent solely on the OTU catalytic domain. Two of these mechanisms require the proximal Ub to be bound in a certain way, either by an UBD or S1' ubiquitin binding sequence to specify which linkage can be cleaved. The final mechanism allows recognition of poly-Ub chains by using an S2 sequence to provide the specificity (Mevissen, Hospenthal et al. 2013).

The smallest groups of Cys-dependent DUBs are the MJD and UCH families; both contain only four family members. The UCH family has a conserved active domain that has a loop that restricts access to the active site, as the loop straddles the C-terminus of Ub (Johnston, Larsen et al. 1997). Originally this restriction was believed to limit substrates to small proteins; however, more recent research has shown a conformational change which allows larger substrates to access this site (Johnston, Larsen et al. 1997, reviewed in Komander, Clague et al. 2009). Apart from cleaving incorrectly linked Ub, the UCH family is believed to be involved in the processing of newly translated Ub (Larsen, Krantz et al. 1998).

Like the UCH family, the MJD family also contains a loop that restricts the active site (reviewed in Komander, Clague et al. 2009). However, the MJD family is more divergent than other Cys-DUBs, having evolved later than the other described DUBs, with no homologues in yeast, although the catalytic triad is still present (reviewed in Nijman, Luna-Vargas et al. 2005).

The final category of DUBs is the JAMM family, which does not use the active Cys but a Zn^{2+} ion. The binding of the Zn^{2+} to a water molecule polarizes this water molecule to create a non-covalent bond between the water and the Ub molecule. This allows protons

to pass from the water molecule to the newly formed intermediate, which breaks the Ub isopeptide bond (Maytal-Kivity, Reis et al. 2002, Tran, Allen et al. 2003). Most JAMM-type DUBs are part of larger complexes, for example Pad1 homolog 1 (POH1) which is a member of the 26S proteasome (Cope, Suh et al. 2002, Yao and Cohen 2002, Dong, Hakimi et al. 2003). JAMM-type DUBs are unlikely to have originally functioned as DUBs, as bacterial homologues do not have DUB activity, but have evolved this function as the Ub system has become more prevalent in eukaryotes (Burns, Baumgart et al. 2005, reviewed in Nijman, Luna-Vargas et al. 2005).

The removal of Ub is important, not only to recycle free-Ub back into the cell, but also to protect substrates from degradation by the proteasome. Removal of poly-Ub chains which signal degradation can stop the substrate being recognised by the proteasome, thereby stabilising substrates.

1.5.1 Deubiquitinating enzyme specificity

The extensive Ub system within higher eukaryotes suggests there is little functional redundancy between DUBs. The increase in E3-ligases and the complexity of Ub signalling is correlated with a corresponding increase in DUBs, to add further regulation to the Ub system (Semple, Grp et al. 2003). DUB specificity depends on a number of factors, such as whether the modification is mono- or poly-Ub. Poly-Ub chains can also be distinguished based on linkage type, as previously discussed with the specificity of DUBs such as the OUT family (Section 1.5) (Mevissen, Hospenthal et al. 2013). Further to the Ub modification, some DUBs show specificity to the substrate through sequences around the DUB active domain, which has been demonstrated by the CYLD DUB, specifying its interaction with NEMO (Saito, Kigawa et al. 2004, reviewed in Nijman, Luna-Vargas et al. 2005). Many DUBs, particularly JAMM-type DUBs, are part of

larger complexes, with BRCC36 as part of the BRCA1-A complex, indictative of its role in DDR, along with POH1 being part of the proteasome (Dong, Hakimi et al. 2003, Nabhan and Ribeiro 2006). The interactions of the other complex components can limit the substrates accessible to the DUB, increasing substrate specificity. The proteasome, although targeting most proteins within the cell, due to the structure of the proteasome components only Ub-chains of four moieties or more can be recognised (Thrower, Hoffman et al. 2000). The cellular localisation of the DUB will also act to restrict the available substrates, providing further specificity of DUBs to substrates. Therefore, there are many criteria which act to make DUBs specific to certain substrates, adding increased regulation to the Ub system in higher eukaryotes (reviewed in Nijman, Luna-Vargas et al. 2005).

1.6 Evolution of the Ub conjugation enzymes

The Ub protein is highly conserved within eukaryotes, along with the enzymatic cycle that attaches and removes the Ub. However, the number and function of the enzymes involved varies between species.

The Ub-cycle is seen in yeast, which are ancient organism, although there are fewer enzymes, with only 68 E3 enzymes and 20 DUBs present. There has been an exponential increase in the E3 and DUB enzymes throughout evolution with 442 E3s and 78 DUBs in mice. Consistent with this increase, there is also an increase in the number of E2 enzymes, although not to the same extent as the E3s and DUBs. Conversely there is a decrease in E1 enzymes with eight enzymes in yeast which is reduced to two in humans (Semple, Grp et al. 2003).

It is therefore implied by Semple *et al*. that the rise in Ub enzymes suggests an increase in the use of Ub as a signalling mechanism and tighter regulation on the pathways

involved. The anomaly in this expansion is in *Caenorhabitis elegans* which has a higher number of E3 enzymes than would be expected. It is not known what caused this increase and it appears to be an isolated incident (Fig. 1.6). The increase in the number of E3s suggest specificity in substrate selection and distinct pathways for the enzymes (Semple, Grp et al. 2003).

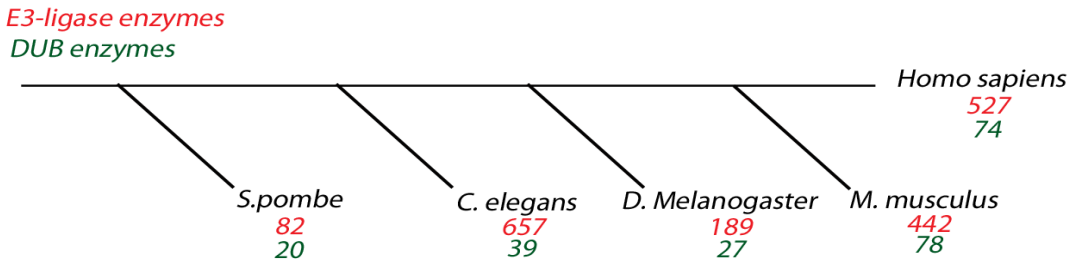

Figure 1.6. Schematic of the increase in Ub E3-ligase and DUB enzymes in different species. Data from (Semple, Grp et al. 2003). Throughout evolution there is an increase in the number of E3 and DUB enzymes. C.elegans is anomalous with an unexplained increase in both E3-ligases and DUBs. The increase in the numbers of both enzymes is believed to be linked.

1.7 Ubiquitin binding domains

Along with the expansion of Ub system, cells have developed more ways of recognising Ub, with 20 different families of Ub binding domains (UBDs) (reviewed in Husnjak and reviewed in Dikic 2012, reviewed in Scott, Oldham et al. 2014). The classical recognition of Ub is through an α-helical domain, which interacts with the hydrophobic patch around the Ile44 residue of Ub (reviewed in Dikic, Wakatsuki et al. 2009). The binding domains that interact with the Ile44 patch include UIMs (Ub-interacting motif), MIU (motif interacting with Ub), DUIM (double-sided Ub-interacting motif), UBA (Ub-associated), CUE (coupling of ubiquitin conjugation to endoplasmic reticulum

degradation), GAT (GGA and TOM), VHS (Vps27/Hrs/STAM) and UBAN (Ubiquitin binding in ABIN and NEMO domain) binding domains. The number and layout of the α-helices varies between the binding domains, with the largest family, the UIMs, only having one, whereas the VHS domains have eight helices (Fig. 1.7) (reviewed in Hicke, Schubert et al. 2005).

Ub binding domains can also use a Zinc ion in order to bind Ub.. There are four UBD families that use the zinc ion which are the UBZ (Ub-binding zinc finger), NZF (Npl4 zinc finger), ZnF (Zinc finger) UBD and the A20 domain. With many DNA damage response and replication proteins possessing Zinc UBDs, including WRNIP, the helicase interacting partner, the translesion synthesis polymerase κ, as well as the E3 ligase important in modifying PCNA, Rad18 (reviewed in Husnjak and Dikic 2012).

A smaller family of UBDs are the Pleckstrin homology (PH) fold family, including GLUE (GRAM-like Ub binding in EAP45) and PRU (pleckstrin-like receptor for Ub) domains. The PH domains have a distinct β-sandwich fold, although the mechanism of binding Ub of the PRU and GLUE domains is different. (reviewed in Dikic, Wakatsuki et al. 2009).

A further set of UBDs include UEV (ubiquitin-conjugating enzyme E2 variant) and UBC (Ub-conjugating enzyme-related) domains (reviewed in Husnjak and Dikic 2012). Although the structure of the UEV domains are conserved between this family of binding-domains, the mechanism of Ub binding has diverged between proteins (Pornillos, Alam et al. 2002).

There are also some unclassified UBDs including the binding domains of some JAMM-type DUBs and the PFU and UBM (Ub-binding-motif) domains (reviewed in Husnjak and Dikic 2012). The JAMM type DUBs, as previously stated (Chapter 1 Section 1.5),

did not originally function as DUBs and, therefore, the UBD evolved from a domain not originally used to bind Ub (Burns, Baumgart et al. 2005).

The diversity of the UBDs demonstrates not only the requirement for different domains to bind specific Ub modifications but, as seen with the JAMM domains, that as the Ub system has become more important in higher eukaryotes there have evolved more mechanisms for recognising Ub.

The recognition of Ub-modifications has been shown to be important in many pathways in eukaryotic cells including the repair of DSBs.

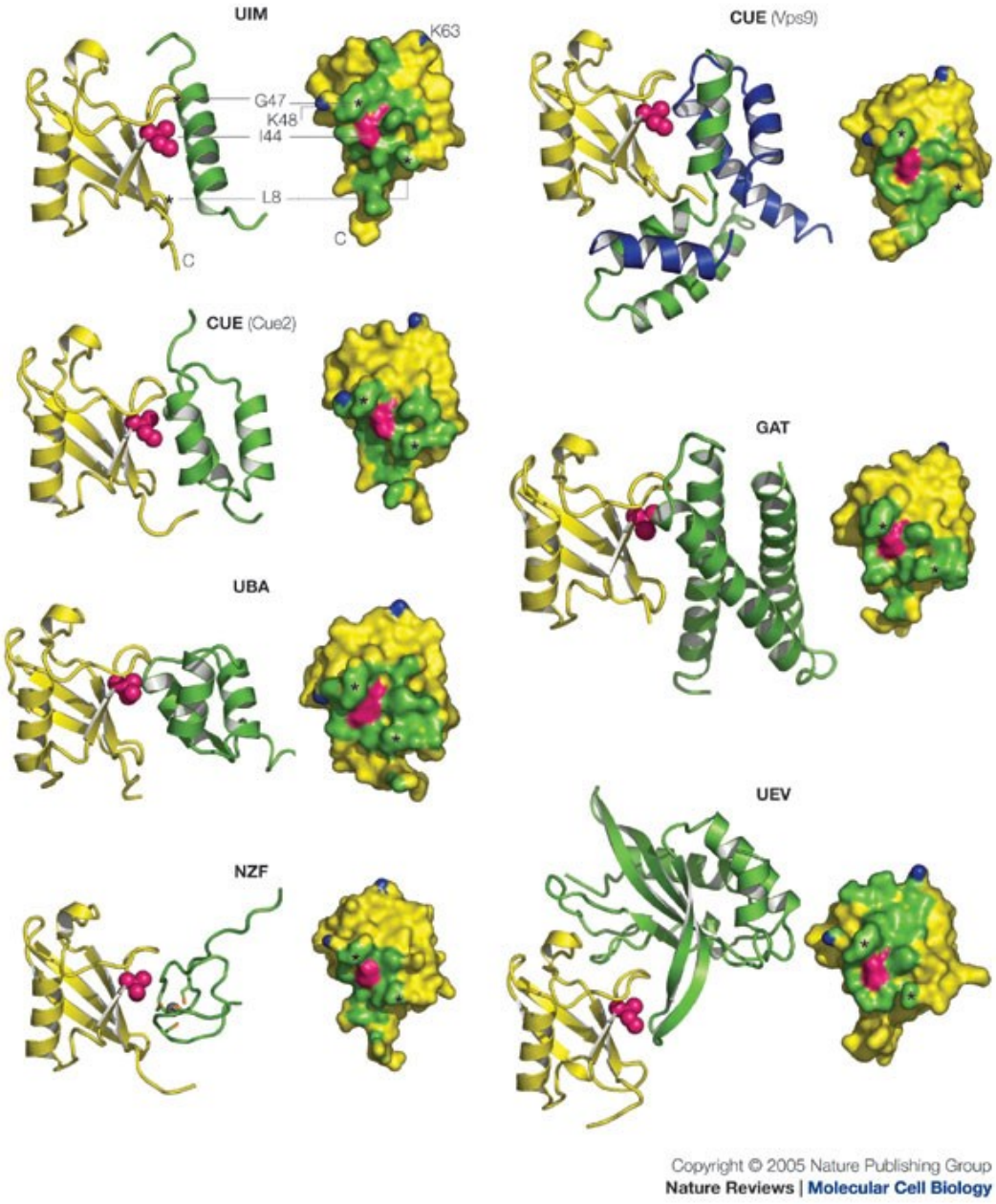

Figure 1.7. Protein structure of the Ub-binding domains. The yellow ribbon represents the protein structure of Ub, the pink dots are the Ile44 residue, the green ribbon represents the Ub-binding domains (UBD). Many UBDs bind Ub through a helical domain, as seen with the UIM, CUE,UBA and GAT domains. The number of helices varies between the different binding motifs. Domains such as NZF bind Ub through a Zinc ion rather than a helix, however the Ile44 residue is still contacted. The final domain pictured, UEV,is a mix of α-helices and β-sheets and resembles the active domain of the E2-conjugating enzyme. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology (Hicke, Schubert et al. 2005).

1.8 The Double Strand Break Response

DNA can be damaged in a variety of ways, including damage caused by endogenous and exogenous agents that cause different types of lesions, which require diverse repair mechanisms (reviewed in Sancar, Lindsey-Boltz et al. 2004). The different pathways vary from enzymatic removal of modifications to bases, such as the removal of aberrant methyl groups on the O^6-methylguanine DNA, to the removal of entire sections of damaged DNA. This includes nucleotide excision repair (NER), base excision repair (BER) or removal of bases from both strands of DNA for interstrand crosslink repair (ICL), combining both NER and Homologous Recombination (HR) to repair the lesion (Koike, Maki et al. 1990, Huang, Svoboda et al. 1992, Kubota, Nash et al. 1996, reviewed in Noll, Mason et al. 2006). The regulation of all these repair responses requires Ub modifications to efficiently signal repair.

DSBs are lethal to cells if they remain unrepaired, therefore there are two pathways that can repair them (Huang, Clarkin et al. 1996, Takata, Sasaki et al. 1998). Non-homologous end joining (NHEJ) works throughout the whole cell cycle and ligates the broken ends together; however this process is error prone as base pairs might be deleted during end processing (Rothkamm, Kruger et al. 2003, reviewed in Betermier, Bertrand et al. 2014). On the other hand HR only occurs during S and G2 phase of the cell cycle, when there is a sister chromatid that acts as a template for repair (Kadyk and Hartwell 1992, Rothkamm, Kruger et al. 2003). If repair fails, cells are destined for apoptosis;, unless another fault arises leading to diseases including cancer, due to the accumulation of mutations (Lips and Kaina 2001, reviewed in Jackson 2002).

1.8.1 Ubiquitin and the Early Double Strand Break response

When DNA is damaged, early sensors are the first to respond. Poly-ADP-ribose polymerase (PARP1) is the first protein that is rapidly recruited to sites of DSBs, inducing poly-ADP-ribose (PAR) chains at the break. The PARP1 accumulation causes efficient recruitment of the Mre11/NBS1/Rad50 (MRN) complex, which is recruited quickly to breaks and acts to tether the broken DNA ends together through the Rad50 subunit (de Jager, van Noort et al. 2001). The recruitment of MRN by PARP1 is probably due to an interaction between PARP1 and Mre11, and also through the binding of a putative PAR-binding domain within Mre11 that binds to PAR molecules (Haince, McDonald et al. 2008). Once MRN is at the sites of damage it can dissociate the inactive ATM dimer into an active kinase (Fig. 1.8). The C-terminal end of the MRN subunit Nbs1, has a conserved motif that recruits ATM to the DSB (You, Chahwan et al. 2005). The TIP60 histone acetyl transferase complex participates in ATM activation by acetylating K3106 of ATM, along with MRN (Sun, Xu et al. 2007). Once activated ATM phosphorylates downstream targets of the DSB response starting a cascade (Lee and Paull 2005).

Activated ATM phosphorylates the H2AX histone variant to form γH2AX which acts as a signal to the cell to recruit the mediators of the damage response (Burma, Chen et al. 2001). The γH2AX chromatin mark appears as rapidly as a minute after the damage has occurred and then spreads 1-2 Mbp either side of the break site spreading its maximum distance between 10-20 mins after damage (Rogakou, Pilch et al. 1998, Rogakou, Boon et al. 1999). This chromatin mark, although not necessary for the initial opening of chromatin structure, is believed to be the modification that allows recruitment of repair proteins potentially by maintaining the decondensed state of chromatin until the break can be repaired (Kruhlak, Celeste et al. 2006). This γH2AX mark acts as a recruitment

signal for mediators of DNA repair such as Mediator of DNA-damage checkpoint 1 (MDC1) which is also phosphorylated by ATM in response to damage (Fig. 1.8). The phosphorylated MDC1 binds to phospho-group on the modified H2AX and acts as platform to bring in further repair proteins (Spycher, Miller et al. 2008, Stucki, Clapperton et al. 2008).

The E3-ligase RNF8 is one such protein recruited to phosphorylated-MDC1 through its forkhead-associated (FHA) domain, however the interaction is not solely dependent on phosphorylation of MDC1 but also on the demethylation of MDC1 by JMJD1C (Kolas, Chapman et al. 2007, Mailand, Bekker-Jensen et al. 2007). JMJD1C is a histone methyl-transferase that regulates chromatin, impacting on the transcription and the DSB response (Watanabe, Watanabe et al. 2013). The methyl-transferase is stabilised upon damage induction due to its interaction with RNF8 and this causes the ubiquitination at sites of damage (Watanabe, Watanabe et al. 2013). RNF8 is recruited to breaks prior to another E3-ligase, RNF168, which is recruited to breaks through the two MUI domains within RNF168 which binds to poly-Ub chains that form at damage sites (Doil, Mailand et al. 2009, Pinato, Scandiuzzi et al. 2009, Stewart, Panier et al. 2009). The kinetics of RNF8 and RNF168 recruitment to break sites varies, with RNF8 being recruited faster and shuttled on and off the sites of damage rapidly, while the movement of RNF168 is much slower. There are also fewer than half the number of RNF8 molecules at sites of damage than RNF168 molecules, suggesting that the limiting factor at break sites is RNF8 recruitment (Mok, Cheng et al. 2014). Both RNF8 and RNF168 interact with the E2 enzyme, Ubc13, and another E3-ligase, HECT and RLD domain containing E3 protein ligase 2 (HERC2), which promotes the interaction of RNF8 with Ubc13 and stabilises RNF168. HERC2 is phosphorylated on Thr4827 in order to bind the RNF8 FHA domain (Bekker-Jensen, Rendtlew Danielsen et al. 2010). To interact with both

MDC1 and HERC, it is necessary for RNF8 to oligomerize as more than one FHA domain is needed (Bekker-Jensen, Rendtlew Danielsen et al. 2010). Loss of HERC2 causes defects in the DDR, with failure to recruit Tumour protein P53 binding protein 1 (53BP1), a chromatin associated gene involved in promoting NHEJ repair, the BRCA1-A complex, a complex formed of the E3 ligase BRCA1, Rap80 (receptor-associated protein 80), two DUBs BRCC36 and BRCC45, Merit40 (Mediator of Rap80 interactions and targeting 40 kd), and RNF168. Further to this, RNF8 increases it's binding to another E2 enzyme, UbcH8, therefore causing RNF8 to produce K48-linked chains rather than K63-linked chains (Bekker-Jensen, Rendtlew Danielsen et al. 2010).

There is much debate surrounding the role of RNF8 and RNF168 at DSBs. There is evidence that despite RNF8 being recruited first it appears that RNF168 can prime mono-Ub on γH2AX K13/15, which can then be extended by RNF8 to create K63-linked chains (Mattiroli, Vissers et al. 2012). More recently, it has been shown that RNF8 is responsible for the K63-chains on the histone variant H1, independently of RNF168. This suggests that RNF8 acts to ubiquitinate substrates at the break which then recruits RNF168 to the damage (Mattiroli, Vissers et al. 2012). RNF8 is known to make both K48-linked and K63-linked chains depending on which E2 interacts with RNF8, with UbcH8 creating K48-chains and Ubc13 creating K63-linking chains. Both types of chains form at sites of damage through these separate ligase activities, meaning RNF8 can act to regulate different processes at the damage site (Lok, Sy et al. 2011, Meerang, Ritz et al. 2011, reviewed in Mallette and Richard 2012). The Ub chains are known to recruit the BRCA1-A complex through the Rap80 protein which has tandem SUMO interacting motif (SIM)-UIM-UIM domains and binds to SUMO and the K63-linked chains that form at damage (Hu, Paul et al. 2012). The Ub chains are also important in the recruitment of 53BP1, although through indirect interactions. The

Tudor domain of 53BP1 binds to the methylated histone variant H4 at lysine residue 20 (H4K20me2) (Fig. 1.8), but this mark is unavailable to 53BP1 unless the trimethylation-specific demethylase Jumonji domain 2 (JMJD2) is ubiquitinated with K63-linked chains which acts as a signal for its removal from chromatin (Botuyan, Lee et al. 2006, Mallette, Mattiroli et al. 2012). Two isoforms of JMJD2, JMJD2A and JMJD2B, have been identified as blocking 53BP1 from accessing histones, as well as the chromatin remodeller, Lethal (3) malignant brain tumour like 1 (L3MBTL1), which also blocks 53BP1 from accessing the chromatin. L3MBTL1 is marked by K48-chains and is extracted by Valosin containing protein (VCP) to allow 53BP1 recruitment (Acs, Luijsterburg et al. 2011, Mallette, Mattiroli et al. 2012). The K48-linked chains appear quickly after damage but are just as rapidly cleared (Feng and Chen 2012). The roles of these chains are under dispute as RNF8 mutants that cannot make K48-chains do not inhibit repair (Lok, Sy et al. 2011). However, there is a lot of evidence showing that Ub-chains formed at damage are needed to recruit many DDR proteins.

Interestingly an RNF168 paralogue, RNF169, is also recruited to DSBs by RNF168. Although RNF169 is an E3-ligase, its enzymatic activity does not appear to act at DSBs, instead RNF169 acts to antagonise the recruitment of 53BP1 and BRCA1-A by binding to and blocking the Ub-chains. RNF169 thereby acts as a negative regulator of repair, stopping excessive recruitment of repair proteins to the break site (Poulsen, Lukas et al. 2012).

To allow the repair proteins access to the DSB, the chromatin structure must be remodelled to an open conformation. Histone Ub modifications are a known mechanism of changing chromatin structure. An E3-ligase complex Ring1b/Bmi1 is known to mono-ubiquitinate histone H2A on K118/K119. Unlike other DDR proteins, the

Ring1b/Bmi1 complex is normally found on chromatin and does not require damage to be recruited. However, after damage, Bmi1 localises to the break site by an interaction with Nbs1 and is stabilized (Ismail, Andrin et al. 2010, Wei, Ojo et al. 2014). The mono-ubiquitination of K118/K119 is dependent on the phosphorylation of H2AX by ATM and a related kinase, Ataxia telangiectasia and Rad3-related (ATR). Without recruitment of Ring1B/Bmi1, other DDR proteins such as 53BP1 and BRCA1 cannot be recruited to DSBs and cells display increased radiosensitivity (Ismail, Andrin et al. 2010).

The relaxation of chromatin can also be attributed to the mono-ubiquitination of histone H2B at K120 by another E3-ligase complex, RNF20/40. RNF20 has been shown to interact with Nbs1 of the MRN complex and have a role in resection (Nakamura, Kato et al. 2011). The mono-ubiquitination of H2B by this E3-ligase complex, promotes the methylation of different lysine residues on histone H3, H3K4 and H3K79. Further to this, the mono-ubiquitination of H2B recruits chromatin remodellers such as SNF2h (Sucrose Non-fermenting 2 homolog) and the FACT complex (facilitates chromatin transcription) (Zhu, Zheng et al. 2005, Nakamura, Kato et al. 2011). All together these actions allow efficient recruitment of BRCA1 and the strand invasion protein, Rad51, to breaks; however 53BP1 is recruited independently of RNF20-mono-Ub (Nakamura, Kato et al. 2011). The requirement of RNF20 suggests the RNF20/40 complex has an important role in modulating chromatin around DSBs and therefore plays a role in regulating HR.

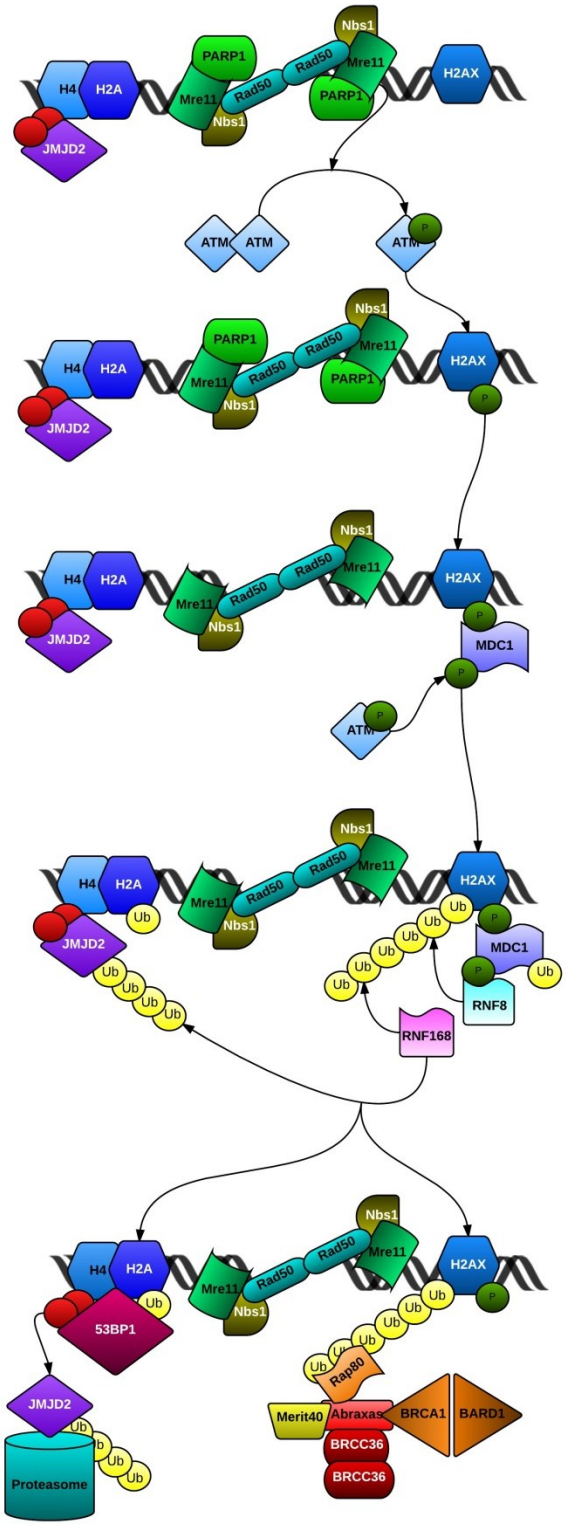

Figure 1.8. Depiction of the early response to a DSB. On damage, early sensor proteins, PARP and MRN are recruited to the break, the Rad50 component of the MRN complex tethers the broken ends together. The MRN complex activates the ATM kinase. ATM phosphorylates histone H2AX which recruits MDC1, which itself is phosphorylated by ATM. Phosphorylated MDC1 recruits the E3-ligase RNF8, which ubiquitinates proteins around the break. The ubiquitination recruits another E3-ligase RNF168 which amplifies the Ub signal. The Ub modification recruits the BRCA1-A complex through Rap80 and opens up histone marks for the binding of 53BP1.

1.8.2 Homologous recombination

Homologous recombination (HR) occurs during the S and G2 phases of the cell cycle when there is a sister chromatid available, which act as a template for the synthesis of the missing bases in the break ensuring an error free method of repair (Kadyk and Hartwell 1992, Rothkamm, Kruger et al. 2003).

An important step that commits the pathway to HR is the resection of the break, which is initially started by the Mre11 nuclease as part of the MRN complex (Taylor, Cecillon et al. 2010). Resection is continued, in part, by phosphorylated CtIP (CtBP-interacting protein) which binds the BRCT domain of BRCA1, forming the BRCA1-C complex (Wong, Ormonde et al. 1998). The interaction of BRCA1:BARD1 with CtIP is cell-cycle regulated so that it only occurs during S and G2 phase (Yun and Hiom 2009, Escribano-Diaz, Orthwein et al. 2013). However, it has been demonstrated that the interaction of BRCA1 and CtIP is not necessary for resection of DSBs, with CtIP potentially interacting directly with DNA (Nakamura, Kogame et al. 2010, Peterson, Li et al. 2011, Reczek, Szabolcs et al. 2013, Polato, Callen et al. 2014). The recruitment of CtIP to the DSB requires the dimerization of CtIP. Although BRCA1 is not required for CtIP resection, the interaction does appear to accelerate resection by removing RIF1 and 53BP1 from DNA ends (Cruz-García, López-Saavedra et al. 2014). Whether this removal of 53BP1 and RIF1 requires the E3 ligase activity is not fully understood.

The combined resection of MRN and BRCA1-C remove between 50-100bp around the break site leaving a 3’ single stranded overhang (Mimitou and Symington 2008, Buis, Stoneham et al. 2012). The resection of the break is inhibited by factors that promote NHEJ, this has namely been 53BP1, which, along with other factors, is believed to protect the broken ends of the DNA and, therefore, the BRCA1-C complex must

remove these factors from the ends of the DNA before resection can occur (Bunting, Callen et al. 2010, Chapman, Barral et al. 2013, Zimmermann, Lottersberger et al. 2013). However, 53BP1 is recruited to breaks even if HR is set to occur and it is now believed to have a role in opening the heterochromatin in order to allow repair factors to access the DSB (Kakarougkas, Ismail et al. 2013). 53BP1 also interacts with Replication protein A (RPA) on the single stranded overhang, although the function of this interaction is not well understood (Yoo, Kim et al. 2005).

Once CtIP and MRN have carried out the initial resection, the 3' overhang is extended further by the helicase BLM (Bloom syndrome protein) and the nucleases Dna2 (DNA replication helicase/nuclease 2) or Exo1 (exonuclease 1) (Cejka, Cannavo et al. 2010). The single stranded DNA (ssDNA) is coated by RPA, which protects the DNA, removes secondary structures and recruits repair factors to the break (Sugiyama, Zaitseva *et al*. 1997). RPA must be replaced by Rad51 for homology searching and subsequent repair (reviewed in Krejci, Altmannova et al. 2012). There are five paralogues of Rad51 which exist in two complexes. The first complex contains Rad51B/Rad51C/Rad51D/XRCC2 (BCDX2), whereas the second complex contains only Rad51C and XRCC3 (CX3). These paralogue complexes can bind DNA and have weak ATPase activity but have distinct roles in HR. The BCDX2 complex works before the formation of the Rad51 filament, unlike the CX3 complex which acts downstream of Rad51 recruitment (Chun, Buechelmaier et al. 2013).

In order for the displacement of RPA to occur, Rad51 requires further factors for loading, which include BRCA2, an interactor of BRCA2, DSS1 (deleted in split hand/split foot 1), and Rad52 (Sugiyama and Kowalczykowski 2002, Gudmundsdottir, Lord et al. 2004, Wang and Haber 2004, Jensen, Carreira et al. 2010). The interaction of

DSS1 with BRCA2 stabilises the latter and improves its function (Li, Zou et al. 2006). Rad51 binds to the BRC repeats within BRCA2, with six Rad51 molecules binding to one BRCA2 molecule (Jensen, Carreira *et al*. 2010). BRCA2 itself interacts with the coiled coil domain of BRCA1 via Partner and localizer of BRCA2 (PALB2), recruiting BRCA2 to the DSB (Zhang, Ma et al. 2009). PALB2 has also been shown to have a physical interaction with Rad51 and can aid in the displacement of RPA alone or in conjunction with BRCA2 (Buisson, Dion-Cote et al. 2010). Once formed, the Rad51 filament can invade the template DNA and search for homologous sequences. In order to find these sequences, which are usually around 100 bp long, the Rad51 helical filament stretches out the ssDNA and then invades the double stranded DNA (dsDNA) of the template DNA creating a D-loop (Ira and Haber 2002, Filippo, Sung et al. 2008). Rad51 physically interacts with the template DNA and allows the extension of the 3' overhang by the filament dissociation, leaving the 3' hydroxyl group accessible. The DNA is extended until it has filled in the break and the ssDNA is recaptured at the other side creating a double Holliday junction (HJ) (Filippo, Sung et al. 2008). The BLM helicase with Topo IIIα performs double HJ dissolution as the preferred method of resolving this structure, as it avoids sister chromatid exchange (Wu and Hickson 2003). This works by BLM and TopoIIIα moving each HJ towards each other until they converge, the resulting structure can then be unwound without the need for endonuclease activity (Plank, Wu et al. 2006). However, resolvase enzymes can cut the four-way DNA junction in one of two ways. One way is where the sister chromatids remain as they were originally with no genetic information being exchanged between sister chromatids. The second way is where a cross-over of genetic information occurs and there is a switch between the chromatids. How the junction is resolved depends on the resolvase involved, for example cleavage of the HJ by Mus81/Mms4 resolvase

complex causes cross-over of genetic information between sister chromatids (reviewed in Hartlerode and Scully 2009). The use of the sister chromatids allows faithful and error-free repair of the DSB. Despite this, HR is not the most prominent form of repair as it only occurs during S and G2 phases of the cell cycle, unlike NHEJ which occurs throughout the cell cycle (Saleh-Gohari and Helleday 2004).

1.8.3 Non-homologous end-joining

The most prevalent form of repair, NHEJ, is an error-prone method of repair that occurs throughout the whole of the cell cycle, unlike HR. NHEJ does not use a template to ensure that genetic information is not lost, instead the repair factors specific for NHEJ act to ligate the broken DNA ends (reviewed in Valerie and Povirk 2003). Microhomology is known to be used occasionally in NHEJ but only a few base pairs are matched and only act to guide the breaks to ligate in the correct place but NHEJ can also act without using microhomology (reviewed in Lieber, Lu et al. 2008).

NHEJ repair is an important pathway for the immune system, used in V(D)J recombination, giving rise to different immunoglobulin chain genes to provide a wide variety of antibodies (reviewed in Malu, Malshetty et al. 2012).

There are two NHEJ pathways, canonical and alternative. During canonical-NHEJ (C-NHEJ), the first proteins recruited to the break, which drive repair towards NHEJ, are the Ku70/80 heterodimer. The Ku70/80 heterodimer has high affinity to duplex DNA structure and, therefore, binds the ends of the DNA and blocks resection (Walker, Corpina et al. 2001, Sun, Lee et al. 2012). Ku70 and Ku80 form a ring around the DNA double helix, although they do not come into physical contact with any of the bases of the DNA. Once in a complex with DNA, the Ku70/80 complex also brings in the DNA-dependent protein kinases (DNA-PKs) in to the break site (Gottlieb and Jackson 1993,

Gell and Jackson 1999). DNA-PKs then can act as a bridge to tether the two broken ends of the DNA together (reviewed in Weterings and van Gent 2004). The binding of DNA-PKs makes the Ku70/80 heterodimer translocate along the DNA, dependent on the p460 kinase subunit of DNA-PKcs, in order to accommodate this large kinase (Calsou, Frit et al. 1999). The Ku70/80 heterodimer is stabilised on DNA ends by the interaction of Paralogue of XRCC4 and XLF (PAXX) (Ochi, Blackford et al. 2015). The Ku:DNA:DNA-PKs complex then phosphorylates NHEJ factors, most importantly itself. This autophosphorylation of DNA-PKs within the complex acts to recruit nucleases. The most well-known nuclease is Artemis, although others that have been implicated including Flap structure-specific endonuclease 1 (FEN1) (Wu, Wilson et al. 1999, Goodarzi, Yu et al. 2006). Also recruited to the Ku:DNA complex are two polymerases, pol μ and λ, which bind via their BRCT domains, with pol μ acting as the main polymerase that fills in the gap between the broken ends (Mahajan, McElhinny et al. 2002). However, as there is no template DNA required, the polymerases have greater flexibility in order to allow for synthesis (Ramadan, Shevelev et al. 2004). Finally, XLF:XRCC4 is recruited to ligate the DNA ends back together. In order to allow this to happen XRCC4 interacts with polynucleotide kinase/phosphatase (PNKP) which restores the 5'phosphate group and the 3' hydroxyl group onto the broken ends of the DNA, making them compatible for re-ligation (Jilani, Ramotar et al. 1999, Koch, Agyei et al. 2004). XLF:XRRC4 is known to be able to ligate across gaps, so although the damage has been repaired, the lack of template means incorrect information can be incorporated into the DNA or information lost (Gu, Lu et al. 2007). However, DSBs can be repaired at any point in the cell cycle and at a much quicker rate than repair of DSBs by HR (Mao, Bozzella et al. 2008, Mao, Bozzella et al. 2008).

The distinct pathway of alternative-NHEJ (Alt-NHEJ) is a Ku-independent process, but it can be restricted by Ku (Bennardo, Cheng et al. 2008). Similarly to C-NHEJ, Alt-NHEJ simply ligates the broken ends of the DNA back together. However, unlike C-NHEJ, Alt-NHEJ uses regions of microhomology, in a process that is slower than C-NHEJ (Guirouilh-Barbat, Huck et al. 2004, Han and Yu 2008). Alt-NHEJ is considered to be a back-up repair pathway for when C-NHEJ and HR fail, therefore, it follows that early response proteins such as PARP1, MRN and CtIP are also required for Alt-NHEJ (Wang, Wu et al. 2006, Bennardo, Cheng et al. 2008, Truong, Li et al. 2013). The broken ends are resected back until short regions (5-20bp long) of homology are reached (reviewed in McVey and Lee 2008). These regions anneal, creating 3' flaps which are then removed. The ends can then be ligated back together using Ligase 1 (Lig1) or Ligase 3 (Lig3) (reviewed in Simsek and Jasin 2011).

The repair of damage is a tightly regulated process so that the process does not occur aberrantly and to ensure efficient repair.

1.8.4 DUBs in Double Strand Break Repair

Ub is well documented in signalling the recruitment of proteins to the sites of DNA damage in order to facilitate repair; however, it must be removed by DUBs once DNA is repaired (reviewed in Bekker-Jensen and Mailand 2011). Recent screens have shown that there are many different DUBs that can have a detrimental effect on the DNA damage response (Nishi, Wijnhoven et al. 2014). The large number of different DUBs that effect DNA repair show the importance of Ub as a signalling molecule in this pathway and also demonstrates the tight regulation required for this process (reviewed in Panier and Durocher 2013).

One of the first DUBs known to have a role in DNA repair was Ub-specific protease 1 (USP1), which targets both the mono-Ub of FANCD2 (Fanconi anemia complementation group D2) and PCNA (Nijman, Huang et al. 2005, Huang, Nijman et al. 2006). USP1 needs its cofactor UAF1 (USP1 associated factor 1) in order to have strong DUB activity, as on its own it has very weak DUB activity (Cohn, Kowal et al. 2007). USP1 and mono-Ub FANCD2 are both cell-cycle regulated and co-localise on the chromatin (Garcia-Higuera, Taniguchi et al. 2001, Nijman, Huang et al. 2005). The mono-ubiquitinated form of FANCD2 is recruited to DNA damage and interacts with BRCA1 and Rad51 in order to repair interstrand crosslinks (ICLs) (Taniguchi, Garcia-Higuera et al. 2002). USP1/UAF1 is known to promote HR in cells by promoting the disassembly of Rad51 foci (Murai, Yang et al. 2011).

USP1 also removes the mono-Ub mark from PCNA, which is important for TLS during replication (Hendel, Krijger et al. 2011). The USP1/UAF1 complex is recruited to mono-Ub-PCNA via the Enhanced level of genomic instability 1 (ELG1) protein (Lee, Yang et al. 2010). The loss of USP1 in transgenic mice and DT40 chicken cells causes genomic instability, supporting the hypothesis that USP1 is required for effective repair of DNA by HR (Kim, Parmar et al. 2009, Murai, Yang et al. 2011(Oestergaard, Langevin et al. 2007)). As USP1 protein levels are cell cycle regulated, when levels are low (in G1 phase) there is an increase in the amount of mono-Ub-PCNA if the cell is stressed by UV (Cotto-Rios, Jones et al. 2011). USP1/UAF1 has an important role in ICL repair by also removing the FANCD2- or PCNA-mono-Ub. This action could be through releasing the FAN1 (FANCD2/FANCI-associated nuclease 1) and SNM1A (DNA cross-link repair 1A) nucleases from the FANCD2 complex (reviewed in Huang and D'Andrea 2010).

USP7 is well studied due to its role in regulating p53 and therefore the G2/M checkpoint. The levels of p53 are usually kept low in the cell by the Mdm2 E3 ligase targeting p53 for degradation. USP7 has been shown to be able to deubiquitinate p53 *in vitro* and *in vivo* (Li, Chen et al. 2002). Conversely, USP7 also stabilises MDM2 and, therefore, this pathway must be highly dynamic to allow correct regulation of the p53 pathway (Li, Brooks et al. 2004). Upon DNA damage, ATM phosphorylates p53 on Ser15 which drives the phosphorylation on Thr18, as well as indirectly causing the phosphorylation of Mdm2 through the c-Abl (mammalian Abelson murine leukaemia viral oncogene homolog 1) kinase (Baskaran, Wood et al. 1997, reviewed in Canman, Lim et al. 1998, Dumaz, Milne et al. 1999, Goldberg, Vogt Sionov et al. 2002). Thr18 phosphorylation is believed to reduce the affinity of Mdm2 for p53, reducing ubiquitination of p53 and, therefore, reducing the degradation of p53 (Dumaz, Milne et al. 1999, Schon, Friedler et al. 2002). Phosphorylation of Mdm2 upon DNA damage is thought to cause destabilisation of Mdm2 and the subsequent activation of p53 (reviewed in Alarcon-Vargas and Ronai 2002). The binding partner of Mdm2, MdmX, is also deubiquitinated by USP7 in normal cells but upon DNA damage the interaction of USP7 and MdmX is reduced resulting in a decrease in MdmX levels and again a stabilised p53 (Meulmeester, Maurice et al. 2005).

Further to its role in stabilising p53, USP7 has been implicated in Deubiquitinating Forkhead box O4 (FOXO4), a transcription factor that is important in regulating cell-cycle progression. The removal of the mono-Ub from FOXO4 is necessary for controlling the transcriptional response to oxidative stress and base-excision repair after oxidative damage (van der Horst, de Vries-Smits et al. 2006).

A DUB known to be associated with DNA damage repair is BRCC36, a JAMM-type DUB that is in complex with BRCA1, BRCA2 and Rad51. The aberrant expression of BRCC36 is seen in many breast cancers suggesting an important role in the DNA damage response (Dong, Hakimi et al. 2003). Depletion of BRCC36 sensitises cells to ionising radiation. This sensitivity may be due to BRCC36 preventing the phosphorylation of BRCA1 and stopping the recruitment of BRCA1 to damage foci (Chen, Arciero et al. 2006). BRCC36, therefore, seems to have a role in activating BRCA1, and, although BRCC36 is a DUB, it is implicated in increasing the E3 ligase activity of the BRCA1:BARD1 heterodimer (Dong, Hakimi et al. 2003). BRCC36 is specific to K63-linked Ub and is known to antagonise the RNF8-Ubc13 chains formed at DSBs, this is thought to allow Ub remodelling by BRCA1 at the break sites (Shao, Lilli et al. 2009). Further to the role of BRCC36 at DSBs, a new target for BRCC36 DUB activity is the ICL repair protein FANCG (Fanconi anemia complementation group G). The poly-ubiquitination of FANCG occurs upon DNA damage and is required to recruit FANCG to chromatin and facilitate HR by permitting the loading of Rad51 after crosslink repair (Zhu, Yan et al. 2014).

The proteasome subunit UCHL5 is involved in resection at break sites, promoting HR. By stabilising the Nuclear factor related to Kappa B (NFRKB) component of the chromatin remodelling complex, INO80 complex, UCHL5 indirectly causes the recruitment of the exonuclease Exo1 to the DSB. Exo1 can then resect the DNA around the break site to allow repair by HR (Nishi, Wijnhoven et al. 2014).

Another DUB, USP44, has been implicated in restraining the RNF8/RNF168 Ub signal formed at DSBs, which are required for repair protein recruitment. USP44 is recruited to damage caused by lasers, which allow highly precise DNA damage at only small

regions of the genome. USP44 has also been shown to displace RNF168 from the damage sites. siRNAs to USP44 caused a mild but significant increase in conjugated Ub and 53BP1 at DSBs, correlating with the fact that the RNF8/RNF168 mediated modification is targeted by a number of DUBs as loss of USP44 does not completely deregulate the recruitment of these repair factors (Mosbech, Lukas et al. 2013).

USP3 has been shown to also target H2A and H2B Ub and, when USP3 is overexpressed it can specifically inhibit the formation of irradiation induced foci (IRIF) of 53BP1, Rap80 and even RNF168 whilst not affecting RNF8 recruitment (Nicassio, Corrado et al. 2007, Doil, Mailand et al. 2009).

OTUB1, a K48-specific DUB (Edelmann, Iphofer et al. 2009), acts to inhibit the formation of RNF8/RNF168 induced Ub-chains rather than degrading chains. OTUB1 also interacts with the E2 enzyme Ubc13 and inhibits the formation of the K63-linked chains made by RNF168. OTUB1, therefore, acts to restrict the Ub signal at DSBs without using its enzymatic activity. OTUB1 knockdown resulted in more persistent 53BP1 foci whereas overexpression of OTUB1 suppresses the formation of 53BP1 foci. However, early responders such as MDC1 as well as RNF8 and RNF168 are still recruited to sites of damage, meaning it is likely that it is only K63-chains downstream of RNF8 that are restricted by OTUB1 (Nakada, Tai et al. 2010).

A highly related DUB, OTUB2, acts to modulate the repair choice of cells, but unlike OTUB1, OTUB2 acts on RNF8-dependent Ub chains on L3MBTL1. OTUB2 constitutively binds to L3MBTL1, probably in order to stop aberrant ubiquitination and removal; however, when damage occurs the Ub signal exceeds the removal of chains and, therefore, L3MBTL1 is removed from chromatin. OTUB2 can also act to block the formation of damage induced K63-chains. By slowing down the Ub signal formed at

damage, OTUB2 regulates which mode of repair the cell utilises. When OTUB2 is lost from cells, the ubqiuitination of DSBs is rapid, recruiting 53BP1 and Rap80 to the break sites and restricting resection of the break, effectively blocking HR repair. Therefore, OTUB2 acts to slow the repair processes in order to allow the most effective method of DNA repair to be used (Kato, Nakajima et al. 2014).

USP5 also works on HR, rather than NHEJ, by rapidly removing the Ub-chains from sites of damage. By interacting with Rad18, USP5 is recruited to damage and, upon knockdown, Rad51 foci are much more persistent, suggesting a role in regulating Rad51 in HR repair (Nakajima, Lan et al. 2014).

Another DUB, BAP1 has been shown to be phosphorylated upon DNA damage, resulting in recruitment of HR factors to the DSB. The detailed mechanism of BAP1 in recruitment of HR proteins is unknown but a few theories exist. BAP1 has been shown to interact with the BRCA1/BARD1 heterodimer, although it may be that this interaction is a transient (Jensen, Proctor et al. 1998, Nishikawa, Wu et al. 2009). The depletion of BAP1 decreases the BRCA1 foci upon damage, as well as affecting other HR protein recruitment like Rad51. The loss of recruitment of HR proteins upon BAP1 depletion means HR repair is restricted (Yu, Pak et al. 2014).

An important effector of HR is the BRCA2 protein which has been shown to be ubiquitinated. USP11 has been shown to interact with and deubiquitinated BRCA2. Depletion of USP11 causes sensitivity to DNA damaging agents, suggesting a role in the repair of DNA. However the role USP11 plays in repair is still undetermined as the increase in ubiquitination of BRCA2 on Mitomycin C (MMC) treatment is not regulated by USP11 (Schoenfeld, Apgar et al. 2004).

The requirement of DUBs for correct repair of DNA correlates with the fact that in many different cancers DUBs are seen to be misregulated (reviewed in Hussain, Zhang et al. 2009).

1.9 DNA Replication

Efficient repair of DSBs is required for genome stability. Another cellular process which must be highly regulated in order to maintain genome stability is DNA replication. DNA damage within cells can be caused by endogenous sources, and one source of these errors is DNA replication (reviewed in Mazouzi, Velimezi et al. 2014). Replication is the process by which DNA is duplicated before the cell divides into two daughter cells. Faithful replication must occur in order for correct transfer of genetic information. There are three stages of replication; initiation, elongation and termination, with each stage being tightly regulated (reviewed in Masai, Matsumoto et al. 2010).

1.9.1 DNA Replication Initiation

The first step in DNA replication occurs in the G1-phase of cell cycle where the origins of replication are licensed (Dimitrova, Prokhorova et al. 2002). The origins are distributed throughout the eukaryotic genome, with mammals having between 30,000-50,000 replication origins, although what defines a site as an origin is unknown (Huberman and Riggs 1966, reviewed in Gilbert 2001). Origin licensing is the recruitment of the inactive replicative helicases by the origin recognition complex (ORC), Chromatin licensing and DNA replication factor 1(Cdt1) and Cell division cycle 6 (Cdc6) (reviewed in Nishitani and Lygerou 2002). First, the six subunit ORC complex binds to the origin of replication (Bell, Kobayashi et al. 1993, Li and Herskowitz 1993); it can bind to dsDNA or to ssDNA of 80 or more bases with high affinity (Lee, Makhov et al. 2000, Li and Stillman 2012). ORC binds to chromatin with the Cdc6 AAA+

(ATPases Associated with diverse cellular Activities) ATPase and is associated with the ATP binding subunit of the ORC complex—Orc1p (Klemm, Austin et al. 1997, Wang, Feng et al. 1999). Both these proteins bind ATP and the hydrolysis of ATP is required for ORC assembly although not by the Orc1p (Siddiqui and Stillman 2007). Cdc6 protein (Cdc6p) is cell cycle regulated, with high levels being present in G1 when it is required for replication licensing (Hateboer, Wobst et al. 1998, Mailand and Diffley 2005). It is phosphorylated during early S-phase by cyclin-dependent kinase 1 (Cdk1) to give an inactive form of Cdc6p, which stops aberrant replication licensing during S-phase (Elsasser, Chi et al. 1999, Diffley 2004). Once ORC and Cdc6p are bound to the DNA, Cdt1 and the MCM helicase hexamer, comprised of the MCM subunits 2-7, are recruited to the origin (Fernandez-Cid, Riera et al. 2013).

Cdt1 binds to the ORC-Cdc6p complex in order to load two MCM 2-7 hexamers. Cdt1 first binds to the MCM hexamer before it can bind to the ORC-Cdc6p complex (Tanaka and Diffley 2002, Kawasaki, Kim et al. 2006). The C-terminal portion of Cdt1 binds to the MCM hexamer but it is the N-terminus of Cdt1 which is necessary to correctly load the MCM subunits onto the origin (Yanagi, Mizuno et al. 2003, Teer and Dutta 2008, You and Masai 2008, Takara and Bell 2011, Wu, Wang et al. 2012). Two Cdt1 molecules are required at the ORC-Cdc6 complex to load the two MCM2-7 hexamers (Evrin, Clarke et al. 2009, Remus, Beuron et al. 2009, Takara and Bell 2011). The ORC6 subunit has two independent Cdt1 binding sites that allow loading of the MCM hexamers in a head-to-head orientation. CDK1 can act to phosphorylate one of the Cdt1 binding sites within ORC6 and therefore inhibit the loading of the replicative helicases but also competes with Cdt1 for binding to the Orc subunit (Chen and Bell 2011). Therefore it is only when CDK1 levels are low, during G1, that the MCM 2-7 hexamers can be loaded onto origins (reviewed in Bashir and Pagano 2005, Wheeler, Lents et al.

2008). Once both Cdt1 proteins and the two MCM hexamers are bound to the ORC-Cdc6 complex, further ATP hydrolysis by Cdc6 facilitates the release of the two Cdt1 molecules (Randell, Bowers et al. 2006). As the cell progresses into S-phase the pre-replication complex (pre-RC) is activated by two kinases, Cdc7 and CDK (reviewed in Bell and Dutta 2002, Tsuji, Ficarro et al. 2006, Thomson, Gillespie et al. 2010), which recruit Cdc45 to the pre-RC and subsequently the GINS complex (Go, Ichi, Nii and San) (Jares and Blow 2000, reviewed in Sclafani and Holzen 2007, Bruck and Kaplan 2015). The four GINS proteins (Sld5, Psf1, Psf2 and Psf3) and Cdc45 physically interact with the loaded MCM hexamers and form the active helicase (Kubota, Takase et al. 2003, Moyer, Lewis et al. 2006). The loading of the GINS complex and Cdc45 are mutually dependent. The GINS complex is therefore required for replication initiation (Takayama, Kamimura et al. 2003).

The MCM hexamer is initially bound to dsDNA, however, the DNA at the break must be opened and the MCM complex reloaded onto the ssDNA (Costa, Ilves et al. 2011, Bruck and Kaplan 2015). How the origin DNA denatures is under investigation, but the process in bacteria has been studied. The DnaA AAA+ ATPase enzyme in bacteria binds to the DNA and undergoes a conformational change on ATP hydrolysis (Fuller, Funnell et al. 1984, Erzberger, Mott et al. 2006, Duderstadt, Chuang et al. 2011). A filament of DnaA molecules forms a right handed helix on one strand of the origin DNA and as each monomer of DnaA is added, they destabilise the double helix structure (Erzberger, Mott et al. 2006). In bacteria this region of DNA is termed the DNA unwinding element (DUE), which is an AT-rich region allowing it to be denatured more easily (Kowalski and Eddy 1989). However it has been shown that the ORC complex does not melt the origin DNA (Lee, Makhov et al. 2000, reviewed in Diffley 2011). The next most likely candidate for origin unwinding is the MCM complex which, as it

changes from the inactive double hexamer to the active separated hexamer; disrupts the DNA to allow single strand binding (reviewed in Lei and Tye 2001, reviewed in Costa and Onesti 2008, Sun, Fernandez-Cid et al. 2014). The MCM complex binds the GINS complex and Cdc45 to create a CMG (Cdc45/MCM/GINS) active complex bound to ssDNA (Gambus, Jones et al. 2006, Moyer, Lewis et al. 2006). The CMG complex binds the DNA polymerase α (pol α) but only once RPA has also been recruited to the origin by Cdc7 (Adachi and Laemmli 1994, Mimura and Takisawa 1998, Tanaka and Nasmyth 1998, Aparicio, Stout et al. 1999, Walter and Newport 2000). The origin is then unwound, usually to around 150 bp and is coated and supercoiled by RPA (Fig 1.9) (Walter and Newport 2000). The start of DNA synthesis requires the interaction between pol α and DNA primase which creates the RNA primers that DNA synthesis initiates from (Conaway and Lehman 1982, Dornreiter, Hoss et al. 1990, Harrington and Perrino 1995, Waga and Stillman 1998).

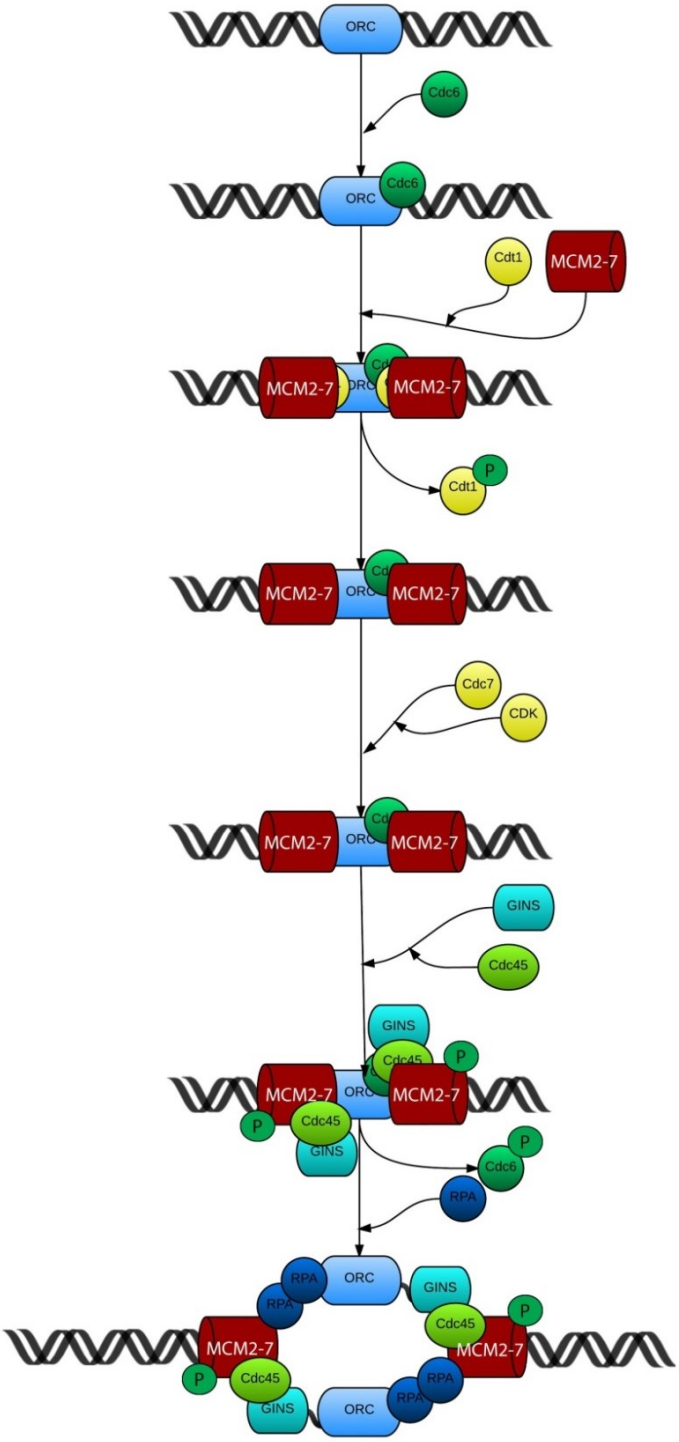

Figure 1.9. Representation of the initiation of replication. The origin of replication complex binds to the origin DNA and binds the AAA+ ATPase, Cdc6. This complex then recruits two Cdt1 and MCM complexes, which are brought into in a head-to-head formation, ATP hydrolysis occurs and Cdt1 is released. The pre-replication complex (pre-RC) is then activated by two kinases, Cdc7 and CDK. Once activated the GINS complex and Cdc45 are recruited and Cdc6 is released. The origin DNA is denatured through an unknown mechanism and the ssDNA is coated in RPA.

1.9.2 Replication elongation

Pol α is not used for elongation of DNA as both strands of DNA need to be duplicated, there are two polymerases associated with the CMG helicase, pol ε and pol δ, the leading and lagging polymerases, respectively (Tsurimoto and Stillman 1989, Weinberg and Kelly 1989, Waga and Stillman 1998, Fukui, Yamauchi et al. 2004). Each polymerase requires an RNA primer sequence created by a primase that is recruited to the fork; however, unlike the leading strand—that can be synthesised in one continuous stretch— the lagging strand is synthesised in short 1000-2000 bp fragments in prokaryotes, and 100-200 bp sections in eukaryotes, separated by RNA primers (Ogawa and Okazaki 1980, Kornberg 1988). The primers must be removed by Ribonuclease H (RNAseH) and FEN1, then the gaps filled in by DNA polymerase I (Stein and Hausen 1969, Okazaki, Arisawa et al. 1971, Champoux, Gilboa et al. 1984, Ishimi, Claude et al. 1988, Goulian, Richards et al. 1990, Harrington and Lieber 1994, Turchi, Huang et al. 1994, Waga and Stillman 1994, Allen, Simcha et al. 2011). The fragments are then ligated back together by DNA ligase I (Henderson, Arlett et al. 1985, Waga and Stillman 1994, Levin, Bai et al. 1997). As the replication fork progresses newly available ssDNA, mainly on the lagging strand, is coated in RPA (Wold 1997).

The replication fork recruits many factors to allow efficient elongation, including AND1, Timeless, Tipin and Claspin, which regulate the polymerases (Zhu, Ukomadu et al. 2007, Errico, Cosentino et al. 2009, Sercin and Kemp 2011, Aria, De Felice et al. 2013). These proteins form a complex which protects the replication fork (Fig 1.10) (Katou, Kanoh et al. 2003, Noguchi, Noguchi et al. 2003, Gotter, Suppa et al. 2007).

Timeless and Tipin are brought into the replication fork by RPA and make up the fork protection complex (FPC) (Noguchi, Noguchi et al. 2004, Ali, Shin et al. 2010, Leman

and Noguchi 2012, Witosch, Wolf et al. 2014). They bind to and mutually stabilise each other and cause the localisation of the complex to the nucleus (Gotter 2003). The fact that these proteins are evolutionarily conserved demonstrates their importance in replication (Gotter, Suppa et al. 2007). They are important as members of the inter-S-phase checkpoint, which stabilises stalled replication forks, by activating Checkpoint kinase 1 (Chk1) in response to perturbed replication (Yoshizawa-Sugata and Masai 2007, Kemp, Akan et al. 2010), and also regulates the replication fork throughout (reviewed in Leman and Noguchi 2012). By tethering the CMG helicases and the DNA polymerases together and actively affecting the enzymatic activity of both, the FPC links the DNA unwinding activity to the DNA duplication activity (Aria, De Felice et al. 2013, Cho, Kang et al. 2013). The FPC inhibits the action of the CMG complex in order to slow down the unwinding of the DNA, as well as stimulating the activity of the polymerases (Cho, Kang et al. 2013). Both Timeless and Tipin can interact directly with the MCM hexamer, with Tipin showing the highest affinity to the MCM7 subunit whereas timeless interacts with either MCM 4, 6 or 7 (Cho, Kang et al. 2013; Errico, Costanzo et al. 2007). The inhibitory role of the FPC on the CMG complex might not be required throughout normal replication. However, when the fork is stalled, either in response to drugs like HU, or due to hard to replicate DNA regions such as common fragile sites or secondary structures, this role may be important for fork stability. By linking unwinding and replication at the fork, the FPC stops accumulation of ssDNA that would otherwise cause activation of the ATR-CHK1 checkpoint pathway (Cho, Kang et al. 2013). The requirement of the FPC is demonstrated by the loss of Timeless and Tipin causing increased ssDNA and increased genomic instability in cells (Chou and Elledge 2006, Smith, Fu et al. 2009, Urtishak, Smith et al. 2009).

As the DNA is unwound there is an increase in the torsional stress ahead of the fork. This stress is relieved by breakage and ligation reactions which are mediated by Top1 and Top2 topoisomerases, which break the DNA backbone to allow removal of the DNA supercoils which arise as the replication fork opens up the double helix; the DNA breaks are then repaired (Bermejo, Doksani et al. 2007).

The DNA can therefore be duplicated until the replication fork collides with another replication fork travelling in the other direction to signal termination of replication (reviewed in Leman and Noguchi 2013).

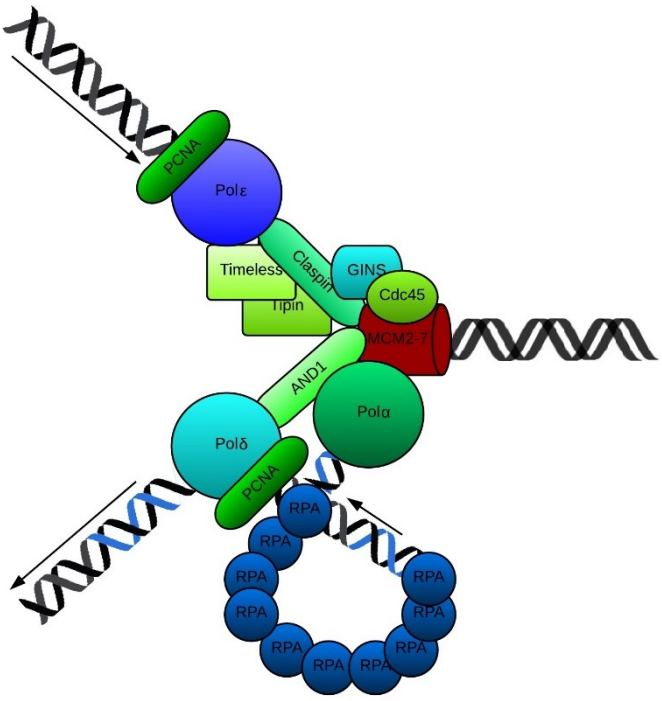

Figure 1.10. Schematic of the replication fork. The dsDNA is opened by the MCM2-7 helicase in conjunction with the GINS complex and cdc45. Pol α lays down the RNA primer for DNA replication. The Pol ε is the leading strand polymerase and the lagging strand polymerase is Pol δ which interact to the helicases via a complex of proteins. These proteins include Claspin, Timeless and Tipin and the And1 protein. The polymerases also interact with the sliding clamp, Proliferating cell nuclear antigen (PCNA). Any ssDNA on the lagging strand is coated by RPA.

1.9.3 Replication Termination

Replication termination is poorly understood, however the CMG complex must be removed from chromatin and the DNA resolved once replication is complete. The mechanism by which CMG is removed must be tightly regulated to stop aberrant removal of this complex when replication has not finished. How the DNA that is covered by the CMG complex is replicated upon termination is also not characterised.

Recently one of the mechanisms for removal of the CMG complex has come to light. The MCM7 subunit of the helicase hexamer is poly-ubiquitinated, seemingly by a cullin-E3 ligase, with K48-linked chains. These chains signal to VCP, which can then extract the helicase directly off the chromatin. However, what signals the MCM7 subunit to be modified solely at the end of replication is unknown (Maric, Maculins et al. 2014, Moreno, Bailey et al. 2014). Once the CMG has been removed the DNA is decatenated by Topoisomerase II, which allows the resolution of the two copies of the DNA (Baxter and Diffley 2008, Cuvier, Stanojcic et al. 2008).

Interestingly, it has been shown that the BRCA1 E3-ligase is important for unloading the MCM replicative helicases when they collide with an ICL. BRCA1 is recruited quickly to stalled forks in order to unload the helicase. Although the Ub-system is important for removal of helicases both at ICL stalled forks and replication termination, BRCA1 is not involved in termination (Cuvier, Stanojcic et al. 2008). However, both events require a Ub-signal in order to remove the helicases (Cuvier, Stanojcic et al. 2008, Maric, Maculins et al. 2014, Moreno, Bailey et al. 2014).

However, the inter-S-phase checkpoint will act to stop the removal of the replication fork components to allow the cell time to bypass or repair a lesion that may be stalling the fork.

1.9.4 Stalled Replication forks

The process of replication can be perturbed by many factors, these include chromatin bound proteins that the replication fork cannot bypass, or obstacles to the polymerase or helicase (reviewed in Mazouzi, Velimezi et al. 2014). Once a lesion has been reached by the fork, it is stalled and stabilised on the chromatin to stop the removal of the replication helicases unnecessarily as these cannot be reloaded during S-phase (Lopes, Cotta-Ramusino et al. 2001, Tercero and Diffley 2001, Lucca, Vanoli et al. 2004, Nishiyama, Frappier et al. 2011). Once forks are stalled the inter-S-phase checkpoint is activated with the ATR kinase as a major regulator of this checkpoint (Cobb, Bjergbaek et al. 2003). ATR is recruited to replication forks by its binding partner ATRIP (ATR interacting partner) which in turn is bound to the RPA which is coating the ssDNA at the fork (Zou and Elledge 2003, Ball, Myers et al. 2005, Namiki and Zou 2006, Ball, Ehrhardt et al. 2007). In order to activate the ATR at the replication fork, the RAD9-RAD1-HUS1 (9-1-1) complex must also be loaded onto the DNA (Delacroix, Wagner et al. 2007). (St Onge, Besley et al. 2003, Delacroix, Wagner et al. 2007, Yan and Michael 2009, Takeishi, Ohashi et al. 2010). The loading of the 9-1-1 is an ATP and damage-dependent reaction and forms at the replication fork when the polymerase and the MCM helicase activities are separated creating stretches of RPA (Bermudez, Lindsey-Boltz et al. 2003, Zou, Liu et al. 2003). The 9-1-1 complex can only be loaded onto the DNA by a damage-specific clamp loader that recognises the template-primer border adjacent to the RPA stretch (Bermudez, Lindsey-Boltz et al. 2003, Zou, Liu et al. 2003). The clamp loader, Rad17-replication factor C (RFC), is brought to forks by polymerase α (You, Kong et al. 2002). Once the RFC is localised at the fork, the 9-1-1 is recruited by an early damage sensor, DNA topoisomerase 2-binding protein 1 (TOPBP1), which binds to RAD9 (radiation-sensitive 9) via the casein kinase 2 (CK2)

phosphorylation mark at the amino acid residue, Ser387. TOPBP1 then interacts directly with ATRIP and can activate ATR (Mordes, Glick et al. 2008). How TOPBP1 activates ATR is still unknown, but once activated ATR then phosphorylates the downstream CHK1 kinase causing activation of the checkpoint (Guo, Kumagai et al. 2000). By having independent recruitment of both the ATR-ATRIP complex and 9-1-1-TOPBP1 complex the S-phase checkpoint is only activated when absolutely necessary.

The activation of CHK1 by ATR requires the binding of CHK1 to Claspin, this binding acts to bring CHK1 into the vicinity of ATR in a damage dependent manner (Kumagai and Dunphy 2000, Chini and Chen 2003). Upon damage, Claspin is phosphorylated at two sites due to ATR activation, although ATR is not necessarily directly phosphorylating Claspin (Chini and Chen 2003, Jeong, Kumagai et al. 2003, Kumagai and Dunphy 2003, Kim, Kakusho et al. 2008). CHK1 is phosphorylated by ATR at Ser317 and Ser-345 and this modification is maintained by the interaction of Claspin with the 9-1-1 complex (Zhao and Piwnica-Worms 2001, Wang, Zou et al. 2006). Once phosphorylated CHK1 dissociates from the replication fork in order to target downstream effectors to initiate the checkpoint, including inhibiting the activation of dormant origins (Smits, Reaper et al. 2006, reviewed in Cimprich and Cortez 2008).

The checkpoint then causes replication to slow down by means of fewer origins being fired (Tercero and Diffley 2001, Merrick, Jackson et al. 2004). The CHK1 activity signals to the cell that replication has been blocked and allows the damage to be repaired before further replication occurs (Feijoo, Hall-Jackson et al. 2001, reviewed in Willis and Rhind 2009). Apart from the global effect of signalling damage, ATR acts to stabilise the fork so that replication can be restarted once the block has been repaired or bypassed (reviewed in Petermann and Helleday 2010). ATR phosphorylates many of

the components at the replication fork including the RFC, the MCM complex and the polymerases (Brush, Morrow et al. 1996, Bao, Tibbetts et al. 2001, Cortez, Glick et al. 2004, Yoo, Shevchenko et al. 2004, Matsuoka, Ballif et al. 2007). Although it is unknown how most of these modifications stabilise the fork, the phosphorylation of MCM2 is known to bind polo-like kinase 1 (PLK1) (Trenz, Errico et al. 2008). PLK1 acts locally to modify the chromatin and locally fire more origins (Fig 1.11). The ATR-CHK1 checkpoint globally reduces origin firing but locally stimulates more origins to fire. So if the fork cannot be restarted the DNA around the stalled fork can be completely replicated, while slowing replication under conditions of replication stress (reviewed in Cimprich and Cortez 2008, Trenz, Errico et al. 2008).

The protein kinase Wee1 was first discovered in fission yeast and is integral to the G2/M checkpoint that prevents cells with DNA damage entering mitosis (Fantes 1979, Russell and Nurse 1987, reviewed in Donzelli and Draetta 2003). By phosphorylating the conserved Tyr15 amino acid of Cdk1/Cdc2 the activity of this kinase is blocked and therefore the checkpoint is maintained until the cell is ready to enter mitosis, then the phosphatase Cdc25C removes these inhibitory marks (Featherstone and Russell 1991, Lundgren, Walworth et al. 1991, Parker, Athertonfessler et al. 1992). If ATR is activated during S-phase and subsequently Chk1 is activated, Wee1 and Cdc25C are in turn phosphorylated by Chk1 (Lee, Kumagai et al. 2001). The activation of Wee1 by this phosphorylation causes checkpoint activation so that the DNA can be repaired before entry into mitosis (Russell and Nurse 1987, reviewed in Donzelli and Draetta 2003). Further to this, Wee1 has been implicated in protecting stalled forks during replication stress. It has been shown that Wee1 depletion slows fork speeds and creates Mus81-dependent DSBs that indicate stalled replication fork intermediates which are cleaved by the Mus81 endonuclease. Co-depletion of Mus81 and Wee1 decreases

genomic instability and allows progression through S-phase and restores entry to M-phase (Dominguez-Kelly, Martin et al. 2011).

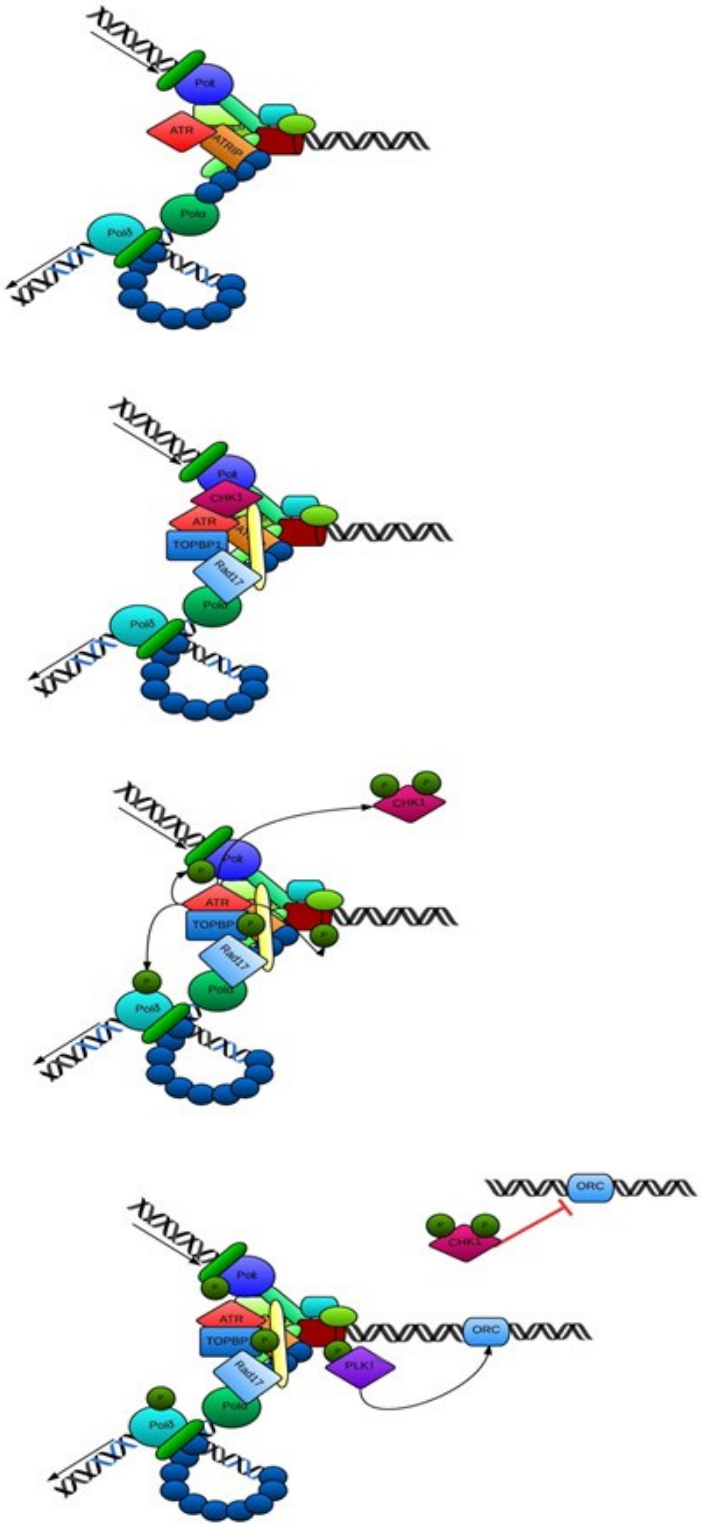

Figure1. 11. Representation of the ATR checkpoint once the replication fork has stalled. Once the helicase and the polymerase become uncoupled, ssDNA is exposed which is coated by RPA. This recruits ATR and its binding partner ATRIP. This brings in TOPBP1 and the clamp loader Rad17, which loads the 9-1-1 complex onto the stalled fork. ATR can then phosphorylates many components at the stalled fork including CHK1. CHK1 acts globally to stop origin firing, the PLK1 kinase acts locally to fire forks to complete the replication of that region of DNA.

Replication can be restarted by various different pathways once the block has been removed. To ensure replication is completed, the eukaryotic genome has dormant origins of replication that are not activated during normal replication. When replication is perturbed the cell can activate and fire these dormant origins, through PLK1 as previously discussed. Therefore allowing replication to proceed from another origin in order to ensure the whole genome is duplicated. Other methods of completing replication include re-priming the fork downstream of the lesion, by introducing a new RNA primer after the lesion to allow replication to proceed from this new primer, re-modelling the fork to allow repair or replicating across the barrier, termed translesion synthesis (TLS) (Fig 1.12) (reviewed in Zeman and Cimprich 2014).

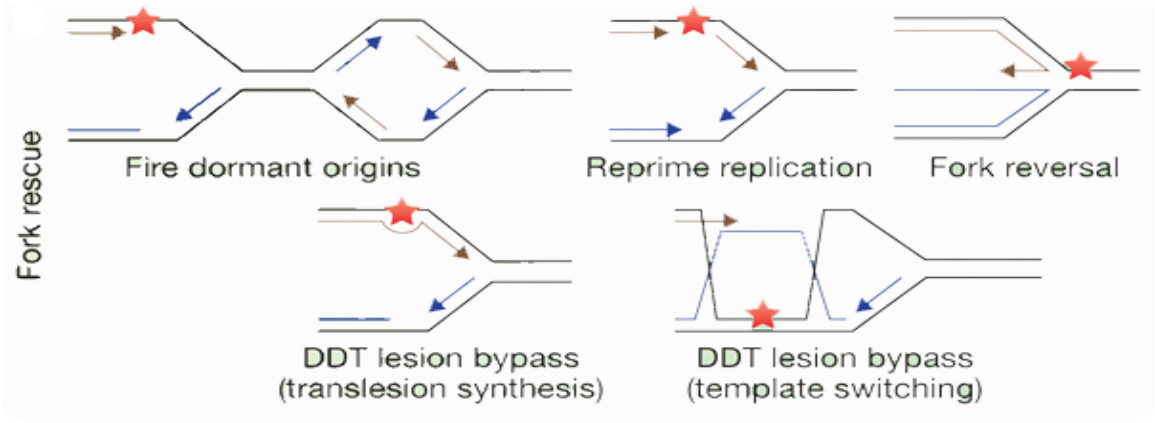

Figure 1.12. Representation of the different mechanisms of replication fork restart after fork stalling. The different mechanisms for rescue include firing of dormant origins in the proximity to the stalled fork, in order to complete the duplication event. Another mechanism involves the production of a new RNA primer after the lesion for replication to be reprimed from, the ssDNA gap can then be filled in. Further to this, the stalled fork can be restructured by helicases including, BLM, WRN and SMARCAL1. The fork can be reversed into a chicken-foot like structure and replication can occur using the newly replicated strand (blue) as a template for the stalled strand, this allows repair at the stalled fork. Lesions can be bypassed by switching polymerases to a more flexible versions. Replication can then occur across the lesion. The final mechanism for rescue involves the creation of a Holliday-junction structure. In this pathway, the newly replicated strand (blue) on one side of the fork is used as the template for the stalled strand (brown) so that replication can continue bypassing the lesion. This repair occurs behind the stalled fork. Reprinted by permission from Macmillan Publishers Ltd: Nature Cell Biology (Zeman and Cimprich 2014).

TLS allows the fork to carry on replicating across a damaged site; this process uses polymerases with low fidelity. However, the ability to bypass the damage means there is a greater chance of mutagenesis (reviewed in Waters, Minesinger et al. 2009). In mammalian TLS there are five known polymerases that can be recruited to the fork, pol η, κ, ζ, ι and Rev1, which are members of the Y-family of polymerases, each one with higher efficiency for specific lesions (except pol ζ) although there is some redundancy between polymerases (reviewed in Ohmori, Friedberg et al. 2001, reviewed in Prakash, Johnson et al. 2005). The main signal for the swap in the polymerases appears to be the ubiquitination of PCNA. The sliding clamp is known to be modified on Lys 164 by Rad6/Rad18 to give a mono-Ub mark at stalled forks (Fig 1.13 A) (Hoege, Pfander et al. 2002, Stelter and Ulrich 2003). The Y-family of polymerases contain a PCNA interacting peptide (PIP) and UBDs that would recruit them to the modified but not the unmodified form of PCNA, specifically bringing the polymerases to the damaged DNA (Fig 1.13 B) (Burnouf, Olieric et al. 2004, Kannouche, Wing et al. 2004, Vidal, Kannouche et al. 2004, Bienko, Green et al. 2005, Guo, Tang et al. 2006). Rev1 is a slight exception as, although it contains a UBD, it does not contain a PIP box but instead uses its BRCT domain to interact with PCNA (Pustovalova, Maciejewski et al. 2013). Pol η has been shown to interact with mono-Ub PCNA and, along with Pol κ, requires Rad18 to form foci at the stalled forks (Kannouche, Wing et al. 2004, Watanabe, Tateishi et al. 2004, Bi, Barkley et al. 2006).

Despite this, TLS does not seem to be purely dependent on PCNA ubiquitination, with more recent research demonstrating that TLS can occur without PCNA-mono-Ub, although at a lower efficiency (Hendel, Krijger et al. 2011). Further to this, HU, which causes stalled forks through the reduction in nucleotides, causes mono-ubiquitination of PCNA despite the fact TLS would not be sufficient to restart the stalled replication fork.

So although PCNA-mono-Ub plays a role in TLS, it is not the full story (Brown, Niimi et al. 2009). The mono-Ub mark may act to change many protein-protein interactions on PCNA, not just causing a polymerase switch (reviewed in Kirchmaier 2011).

PCNA can also be poly-ubiquitinated at the same lysine by Mms2-Ubc13-Rad5 which, rather than stimulating TLS, promotes template switching to allow restart of the replication fork, although the mechanisms for this is not understood (Fig 1.13 B) (Branzei, Seki et al. 2004, Blastyak, Pinter et al. 2007). Two models are currently proposed, one which has been observed in bacteria and more recently in eukaryotic cells and involves the reversal of the replication fork into a chicken-foot structure. The reversal of the stalled helicases causes a single strand of DNA to be dissociated from the replication fork. The ssDNA can then be used as a primer to allow synthesis of the DNA which has been obstructed by the lesion. This model allows repair to occur at the stalled fork (Fig 1.13 C).

The second model would permit repair to happen behind the fork. The DNA synthesised prior to the lesion anneals to the newly synthesised DNA from the other strand. This forms a HJ, allowing synthesis across the lesion using the newly synthesised strand as a template. Both models may coexist and be utilised depending on whether repair occurs at the fork or behind the fork (Fig 1.13 D) (reviewed in Atkinson and McGlynn 2009, reviewed in Branzei 2011, De Septenville, Duigou et al. 2012, Manosas, Perumal et al. 2012).

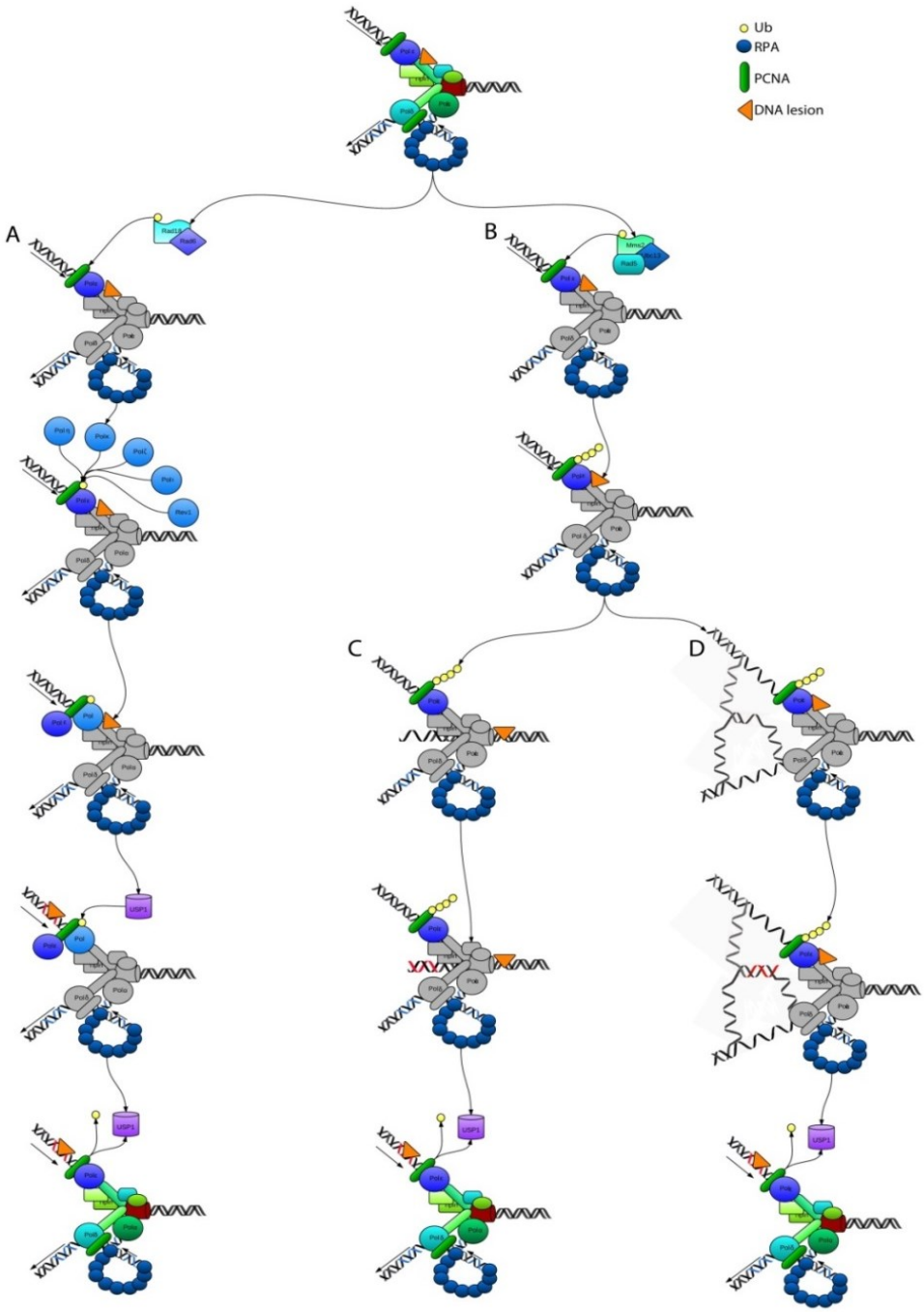

Figure 1.13.Depiction of PCNA ubiquitination and its role in translesion synthesis. PCNA is ubiquitinated when the replication fork is stalled. **A)** Mono-Ub of PCNA is associated with polymerase switching, bringing in a translesion polymerases to the replication fork. The Y-polymerases allow replication across the lesion as they are less stringent. **B)** Poly-Ub of PCNA has two hypothesised models for allowing lesion bypass. **C)** The first pathway involves fork reversal into a chicken-foot structure. This then allows one of the newly synthesised DNA strands to be used for the extension of the other strand that was blocked by the lesion. **D)** The second mechanism is template switching, where one of the newly synthesised strands is again used as a template for the extension of the other strand; however, the fork is not reversed. The HJ created is resolved and replication can continue.

Another enzyme that can carry out TLS is an archaic primase and polymerase, called PrimPol (Iyer, Koonin et al. 2005). As well as carrying out TLS, PrimPol can re-prime the fork to allow replication to continue downstream of the lesion. PrimPol introduces a new RNA primer after the damage in order for replication to proceed from that point (Fig 1.14). PrimPol travels with the fork and may aid in unperturbed replication but recruitment is increased upon damage (Bianchi, Rudd et al. 2013, Garcia-Gomez, Reyes et al. 2013, Helleday 2013, Mouron, Rodriguez-Acebes et al. 2013).

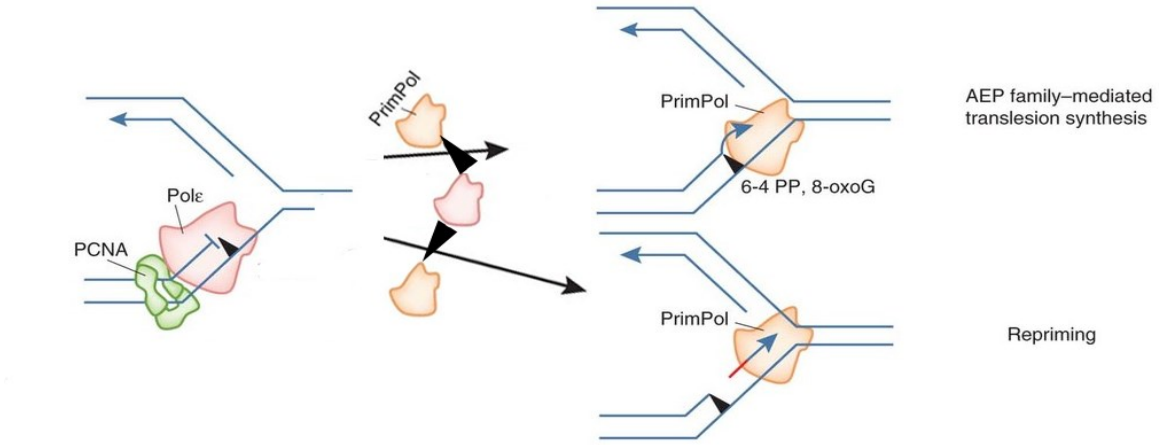

Figure 1.14. The action of Primpol to restart stalled replication forks. Primpol has two modes of action to allow replication to restart, either as a primase or as a polymerase. The polymerase action allows DNA synthesis to occur across the lesion, as with TLS. The primase action produces another RNA primer to re-initiate replication from the new primer, after the damage. Replication can then continue from the new primer and the gap left across the lesion repaired subsequently. Reprinted by permission from Macmillan Publishers Ltd: Nature Structural and Molecular Biology (Helleday 2013).

Another pathway to restart replication uses helicases, which remodel and stabilise the fork and can restart the fork up to 14 hrs after stalling (Fig 1.15) (reviewed in Petermann and Helleday 2010). The RecQ helicase family are involved in replication fork restart by driving an ATP dependent translocation of the replication fork in the 3'

to 5' direction. A well characterised member of the RecQ helicase family is the BLM helicase which is known to be able to restart replication forks. The three helicase activities that BLM possesses include 3'-5' DNA unwinding, HJ migration and ssDNA annealing activity, which may all be used to restart replication (Karow, Chakraverty et al. 1997, Cheok, Wu et al. 2005, Plank, Wu et al. 2006, Cejka and Kowalczykowski 2010, reviewed in Swuec and Costa 2014). BLM *in vitro* has been shown to be able to regress the fork into the chicken-foot structure, BLM may also be responsible for the reversion back to a replication fork structure (Ralf, Hickson et al. 2006, Machwe, Karale et al. 2011). If the chicken foot is not regressed, and instead is converted into a HJ, BLM can dissolve the double HJ structures as previously described (Section 1.8.2). The BLM protein has been shown to specifically interact with Holliday junctions and promote branch migration to converge the junctions and the resulting structure can be dissolved (Plank, Wu et al. 2006, Cejka and Kowalczykowski 2010). The action of BLM to restructure forks allows replication restart which therefore maintains genome stability. This is apparent in Bloom syndrome patients who lack the Bloom helicase and consequently have increased sister chromatid exchanges (SCE), a mark of genomic instability (Chaganti, Schonber.S et al. 1974, McDaniel and Schultz 1992, German 1993, reviewed in Amor-Gueret 2006).

Another RecQ helicase implicated in replication restart is the WRN helicase (Werners syndrome ATP-dependent helicase) (Gray, Shen et al. 1997). WRN is phosphorylated in an ATR-dependent reaction but recruited to chromatin in a separate mechanism (Pichierri, Rosselli et al. 2003). The N-terminal region of the WRN protein interacts with Rad1 of the 9-1-1 complex and brings the WRN helicase to the stalled fork (Pichierri, Nicolai et al. 2012). Once recruited, the WRN helicase makes different protein-protein interactions to regulate its activity, including interacting with Mre11

(Franchitto and Pichierri 2004). These protein interactions are probably mediated through the helicase-and-ribonuclease D-C terminal (HRDC) domain which forms a hydrophobic pocket (Kitano, Yoshihara et al. 2007). The RecQ C-terminal domain (RQC) of WRN forms a winged-helix domain that binds duplexed blunt ended DNA and is the domain responsible for unwinding DNA, using the β-wing, a hairpin structure made of winged-helix motif, to break the dsDNA open (Kitano, Kim et al. 2010). The WRN protein is highly modified, including modifications such as acetylation and ubiquitination (Blander, Zalle et al. 2002, Ianari, Gallo et al. 2004, Li, Wang et al. 2010). The loss of WRN protein causes an increase in the amount of DSBs in the cell, as the stalled replication forks are cleaved by Mus81 (p6 ethyl methansulfonate, UV sensitive) in response to the loss of fork restructuring upon stalling (Franchitto, Pirzio et al. 2008, Murfuni, Nicolai et al. 2013). WRN also interacts with its partner, WRNIP1, which is also heavily modified, and interacts with the TLS polymerase, pol η to allow replication fork restart (Bish, Fregoso et al. 2008, Crosetto, Bienko et al. 2008, Yoshimura, Kobayashi et al. 2014). The way the WRN helicase promotes restart is still under investigation although as it is in the same family of helicases as BLM, the mechanism of restart may be related to the way BLM restarts stalled replication forks (Sidorova, Kehrli et al. 2013, reviewed in Kitano 2014).

DNA translocases also have a role in stalled fork stabilisation, one such translocase is the SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1 (SMARCAL1) (Bansbach, Betous et al. 2009, Postow, Woo et al. 2009). SMARCAL1 is present at the replication fork during normal S-phase (Betous, Mason et al. 2012); however, the recruitment is amplified at stalled forks through an interaction with RPA and can then bind branched DNA structures to allow fork restructuring (Bansbach, Betous et al. 2009, Ciccia, Bredemeyer et al. 2009,

Yusufzai, Kong et al. 2009, Betous, Couch et al. 2013). SMARCAL1 is phosphorylated by ATR in order to negatively regulate its helicase activity (Bansbach, Betous et al. 2009). However, this phosphorylation mark actually reduces the enzymatic activity of SMARCAL1, suggesting ATR phosphorylation occurs after SMARCAL1 has regressed the fork and then blocks further activity that would cause aberrant fork structures (Couch, Bansbach et al. 2013). Further to this, both siRNA and overexpression of SMARCAL1 cause an increase in damage markers during replication, suggesting its activity must be highly regulated (Bansbach, Betous et al. 2009). SMARCAL1 has been shown to anneal DNA in order to reverse the fork into a chicken foot structure, as well as migrate the fork (Yusufzai and Kadonaga 2008, Yusufzai, Kong et al. 2009). The orientation of RPA binding either activates or represses SMARCAL1 activity and, therefore, acts as a mechanism to selectively work on stalled forks and not replication intermediates, such as lagging-strand gaps that are bound by RPA (Bhat, Betous et al. 2015). Surprisingly SMARCAL1 can also migrate and restructure HJ despite the lack of ssDNA and therefore RPA (Betous, Mason et al. 2012).

The enzymatic activities of the proteins described above have similar functions. SMARCAL1 and WRN have been shown to purify together through RPA and to co-localise at stalled replication forks (Ciccia, Bredemeyer et al. 2009, Betous, Glick et al. 2013). Although they both have similar activities and both prevent Mus81 cleavage of stalled forks, they act independently to restart replication forks although some overlap of pathways is possible (Franchitto, Pirzio et al. 2008, Betous, Glick et al. 2013).

In humans, there is another protein that is highly related to SMARCAL1 called ZRANB3 (zinc-finger, RAN-binding domain containing 3), which is also a member of the SWI/SNF translocases (Yusufzai and Kadonaga 2010). Unlike SMARCAL1,

ZRANB3 does not interact with RPA, but instead ZRANB3 does contain a PIP box and binds to poly-Ub-PCNA which is dependent on replication stress being sensed (Yusufzai and Kadonaga 2010, Ciccia, Nimonkar et al. 2012). The interaction of ZRANB3 appears to be dependent on PCNA, K63-linked Ub and branched DNA structures in order for it to only be recruited to stalled forks (Ciccia, Nimonkar et al. 2012, Weston, Peeters et al. 2012). ZRANB3 can also regress forks and dissolve D-loops, DNA structures where the two DNA strands cannot re-anneal due to a third DNA strand binding one of the strands. Further to this ZRANB3 has been shown to interact with TLS polymerases, so may contribute to the TLS pathway to resolves stalled replication forks. Loss of ZRANB3 results in increased SCEs suggesting that it may act to co-ordinate template crossovers during TLS (Ciccia, Nimonkar et al. 2012).

Another DNA translocase, Fanconi anemia complementation group M (FANCM), can also resolve stalled forks and is intimately involved in ATR signalling at stalled forks (Collis, Ciccia et al. 2008, Gari, Decaillet et al. 2008, Schwab, Blackford et al. 2010). FANCM is a component of the replication fork and acts to regulate fork progression during normal replication. Forks lacking FANCM travel more rapidly and are inherently less stable than when FANCM is present, with an increase in forks stalling and collapsing, leading to increased genome instability (Luke-Glaser, Luke et al. 2010). Alongside its role in stabilising forks during unperturbed replication, FANCM has a role in restarting replication forks if they have stalled. The helicase can migrate HJ, dissolve D-loops and reverse forks, as with the previously described translocases, in an ATP-dependent manner (Gari, Decaillet et al. 2008). FANCM and its binding partner, Fanconi anemia-associated protein 24 (FAAP24) bind to branched or ssDNA and also the histone-fold complex, MHF, which itself binds dsDNA. Together these complexes bind to forks and can reverse them with higher affinity than just FANCM alone, these

complexes also associate with components of the BLM-helicase-complex, suggesting overlapping functions (Yan, Delannoy et al. 2010). In cells that are lacking FANCM, there is also deficient ATR signalling during the inter-S-phase checkpoint, with decreased Chk1 phosphorylation (Collis, Ciccia et al. 2008, Luke-Glaser, Luke et al. 2010).

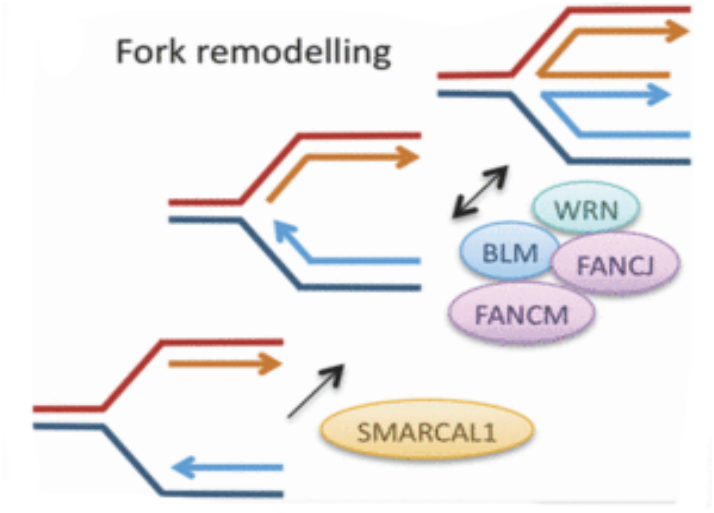

Figure 1.15. The action of helicases to restructure stalled replication forks. Many helicases have been implicated in the restart of replication forks. These include SMARCAL1, WRN, BLM and FANCM. Their main mode of action is to restructure the fork so that lesions can be bypassed. Helicase can both reverse the fork into the chicken-foot structure, so that replication can occur from another template and also restructure the fork back into the fork structure from the chicken-foot intermediate (Jones and Petermann 2012).

If the fork can still not be restarted, it then collapses into a DSB in order to be repaired to enable replication to be completed.

1.9.5 Collapsed Replication fork

If the replication fork is stalled for an extended period of time then it undergoes fork collapse, the removal of the replication components from the fork and a subsequent DSB formation (Saintigny, Delacote et al. 2001, Hanada, Budzowska et al. 2007, Petermann, Orta et al. 2010, Jones, Kotsantis et al. 2014). The collapse of forks is

inhibited by ATR signalling and, therefore, after an extended period of time the ATR signalling must no longer be able to stabilise the fork. Why the forks eventually collapse in a checkpoint proficient cell is still unclear (Lopes, Cotta-Ramusino et al. 2001, Tercero and Diffley 2001, reviewed in Cimprich and Cortez 2008, reviewed in Branzei and Foiani 2010). Several replication factors have been shown to be lost from chromatin and targeted for degradation, such as Claspin. Claspin is first phosphorylated by PLK1 which recruits the β-transducin repeat containing E3-Ub ligase protein (β-TRCP), an F-box protein member of the Cullin1 Ub-ligase. The K48-linked chains are then sensed by the proteasome and the modified components removed (Mailand, Bekker-Jensen et al. 2006, Mamely, van Vugt et al. 2006, Peschiaroli, Dorrello et al. 2006). The fork structure can then be cleaved by endonuclease complexes, including Mus81/Eme2 or Slx1/Slx4, into a DSB that can then be repaired by HR (Hanada, Budzowska et al. 2007).

If ATR signalling is absent, there is a process to collapse the forks which is regulated by Ring Finger protein 4 (RNF4) Ub E3-ligase, which may target components of the replication fork which are known to be degraded upon prolonged stalling, including Claspin (Ragland, Patel et al. 2013). RNF4 is recruited to the vicinity of its substrates via binding the SUMO modification via its SIM domains (Galanty, Belotserkovskaya et al. 2012, Yin, Seifert et al. 2012). In yeast, forks undergoing collapse are poly-SUMOylated in a Mec1 (ATR)-independent mechanism, demonstrated by the loss of Mec1 signalling in yeast causing increased SUMOylation at the fork (Branzei, Sollier et al. 2006, Cremona, Sarangi et al. 2012). As well as RNF4 targeting and collapsing stalled replication forks, the Aurora A kinase in complex with polo-like kinase 1 (AURKA/PLK1) also works in concert with RNF4 to collapse stalled forks into DSB by suppressing replication fork restart. Further to collapsing the fork, the action of RNF4

and PLK1 in ATR-deficient cells is believed to modify the inter-S-phase checkpoint (Yoo, Kumagai et al. 2004, Mailand, Bekker-Jensen et al. 2006, Mamely, van Vugt et al. 2006, Peschiaroli, Dorrello et al. 2006, Ragland, Patel et al. 2013). In cells where ATR is present, inter-S-phase checkpoint modification may occur but only after ATR has signalling is no longer able to stabilise the fork (Ragland, Patel *et al*. 2013).

Once the fork is no longer stabilised the structures, such as the chicken-foot, are targets for endonucleases (Higgins, Kato et al. 1976), these cleave the stalled fork and cause DSBs (reviewed in Klein and Kreuzer 2002).

One such endonuclease is Mus81 which is a member of the XPF/Mus81(Xenoderma pigmentosum group F) family of endonucleases, which target specific DNA structures (Hanada, Budzowska et al. 2007, Ciccia, McDonald et al. 2008). Mus81 is known to have two interaction partners, essential meiotic structure-specific endonuclease 1 (Eme1) and Eme2, with Eme1 believed to be the main partner for the biochemical activity of Mus81 (Ciccia, Constantinou et al. 2003, Ciccia, Ling et al. 2007). Both interaction partners exhibit very similar actions, acting on 3' flap structures, but they also have distinct substrates, demonstrating a separation of function between the two complexes (Pepe and West 2014). Further to this separation of function, it appears that the interaction of Mus81 with these partners is cell-cycle regulated. Mus81 binds to Eme2 from the onset of S-phase, whereas Eme1 is bound throughout the cell cycle (Pepe and West 2014). The action of Mus81 on stalled forks is well established; however, the effect of the binding partner was never fully investigated, with recent work demonstrating that it is in fact Eme2 that is required for resolution of stalled replication forks (Pepe and West 2014). The Mus81/Eme2 heterodimer is more active as an endonuclease with a broader range of substrates than Mus81/Eme1, preferentially

cutting replication forks and being able to cleave 3' flaps, 5' flaps and D-loops. On the other hand Mus81-Eme1 cuts 3' flaps and HJ. The 3' flap endonuclease activity is evolutionarily conserved, although the Mus81-Eme2 has a second step to processing the 3' flap that cuts opposite the nicked duplex DNA, which results from removing the 3' flap, thereby creating smaller duplex DNA products. These findings show that it is the Mus81-Eme2 heterodimer that is important for cleaving stalled replication forks that can no longer be stabilised or restarted (Pepe and West 2014).

The Rad2/XPG endonuclease member, Gen endonuclease homolog 1 (Gen1), dimerises around branched DNA like HJ (Rass, Compton et al. 2010). If Mus81 is lacking from yeast, Yen1 (yeast homologue of Gen1) overexpression can compensate for this loss, suggesting overlapping functions for these endonucleases (Munoz-Galvan, Tous et al. 2012).

Another endonuclease complex, Slx4/Slx1, is recruited to stalled replication forks; however, the Slx4/Slx1 heterodimer does not have a clear role in resolving stalled replication forks (Roberts, Zaidi et al. 2008, reviewed in Rass 2013). Instead, it is currently accepted that Slx4 acts as a scaffold protein for other proteins required for the repair of the fork, including the Mus81-Eme2 endonuclease (Stoepker, Hain et al. 2011). The endonuclease activity of Slx4-Slx1 is required to resolve HJ during HR repair (Fig1.16) (Fekairi, Scaglione et al. 2009).

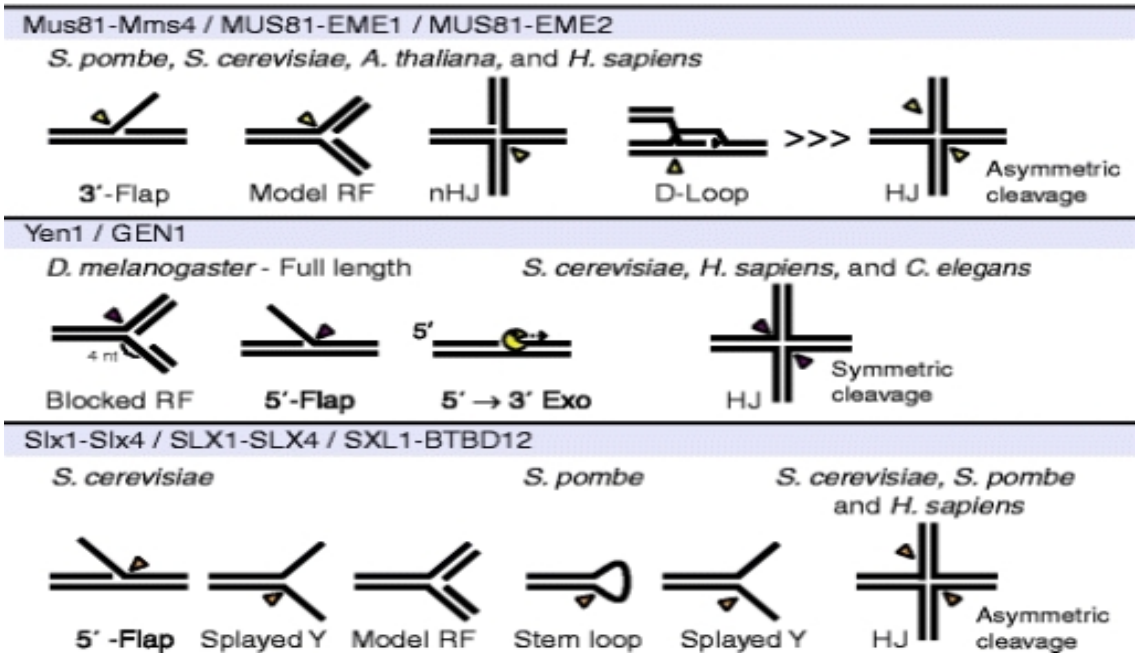

Figure 1.16. The action of endonucleases and the substrates they act on. Mus81-Eme2 works on replication fork structures throughout evolution. Although, other endonucleases could cleave replication forks in the evolutionary past, in humans their preferred substrate is the Holliday junction. Holliday junctions do form as an intermediate of replication fork restart and can be cleaved by Slx1/Slx4 or Gen1 (Schwartz and Heyer 2011) with kind permission from Springer Science and Business Media.

1.9.6 DUBs in Replication

To ensure replication occurs efficiently once per cell cycle mechanisms have evolved to bypass lesions that may block fork progression. This is highly regulated, in part by DUBs. Ub is known to be required for effective replication of DNA, resulting in a requirement for DUBs in replication. They have been studied in the involvement of viral DNA replication within host cells (Si, Gao et al. 2008, Nag and Finley 2012). However the role of DUBs in eukaryotic replication is currently not very well understood but is an active area of research.

The PCNA mono-Ub and poly-Ub marks are important for TLS after damage, the removal of these modifications is caused by USP1; however, the ubiquitinated PCNA

appears to be persistent in the cell after UV damage. The persistent Ub is partly due to the high turnover of USP1 within the cell and the loss of *USP1* mRNA upon UV damage means that there is less USP1 in the cell after UV damage and, therefore, the ubiquitination of PCNA is long lived (Huang, Nijman et al. 2006, Cohn, Kowal et al. 2007, Cotto-Rios, Jones et al. 2011). Although the reduction in USP1 being the cause of the persistent Ub does not stand true for treatment with HU, as USP1 is still present but the PCNA-Ub is also still persistent and it is not understood why USP1 is not deubiquitinating PCNA (Brown, Niimi et al. 2009).

USP3 regulates H2A and H2B ubiquitination and is required for S-phase progression and preventing replication stress, as it regulates the chromatin state of the DNA throughout replication. Consistent with USP3 being required during S-phase, USP3 depletion causes an increase in DNA breaks and an activation of the ATR checkpoint pathway (Nicassio, Corrado et al. 2007). By regulating chromatin, USP3 also regulates the DNA damage response by removing Ub from around the break and activating ATM/ATR (Nicassio, Corrado et al. 2007, Sharma, Zhu et al. 2014).

The role of DUBs in replication is an emerging field and, as Ub is being shown to be important in the replication process, there is a great deal of scope for the regulators of marks like Ub.

Changes to the regulation of replication can therefore give rise to diseases including cancer.

1.9.7 DNA damage and Replication in Cancer

A hallmark of cancer is genetic instability, and various different cancers have mutations in genes that regulate replication (reviewed in Negrini, Gorgoulis et al. 2010, Shlien, Campbell et al. 2015). If replication does not occur faithfully, for example if a non-

proofing polymerase is recruited to the fork, then DNA mutations can occur (reviewed in Waters, Minesinger et al. 2009, Koskiniemi, Hughes et al. 2010). If replication cannot be completed, regions of DNA cannot be separated during mitosis, this can lead to DNA damage and aberrant chromosomal segregation (Chan, Palmai-Pallag et al. 2009, Lukas, Savic et al. 2011). Therefore the regulation of the DNA repair pathway and DNA replication are important to maintain the integrity of genome. One mechanism that regulates these pathways is the ubiquitin proteasome system (UPS) (reviewed in McBride, Iwamoto et al. 2003, reviewed in Branzei and Foiani 2010, Tu, Chen et al. 2012).

Many cancers are known to correlate with mutations within genes important in the DDR. If repair is defective then mutations in cells accumulate, increasing the risk of a cell becoming cancerous (reviewed in Vogelstein, Papadopoulos et al. 2013, Pearl, Schierz et al. 2015). The cell cycle checkpoint protein, p53, is one such protein mutated in many cancers (reviewed in Olivier, Hollstein et al. 2010). Patients with Li-Fraumeni syndrome have early onset cancers including sarcomas, breast cancers and adrenal gland cancers (Li, Fraumeni et al. 1988). Without the loss of p53, the G1/S checkpoint is triggered when damage occurs, and cells undergo apoptosis and do not transform into cancers (reviewed in Vogelstein, Lane et al. 2000). However, when p53 is mutated, the checkpoint is not triggered and cells continue through the cell-cycle with damage and therefore increasing genomic instability, resulting in cancer (Jackson, Post et al. 2011, Gabrielli, Brooks et al. 2012).

Well known repair genes mutated in cancers include the HR components *BRCA1* and *BRCA2* which are correlated with breast and ovarian cancers (Hall, Lee et al. 1990, Narod, Feunteun et al. 1991, Wooster, Neuhausen et al. 1994, Moynahan, Chiu et al.

1999, Yuan, Lee et al. 1999) Further to these mutations, the early response proteins Mre11, Nbs1 and Rad50 have all been linked to hereditary breast cancers (Hsu, Wang et al. 2007).

Further to this mutations within the key DSB signalling protein, ATM, cause Ataxia telangiectasia (A-T) disease with a predisposition to cancer. ATM mutations are also correlated with breast cancers but also several types of leukeamias (Gatti, Berkel et al. 1988, Hecht and Hecht 1990, Savitsky, Barshira et al. 1995).

Deregulation of a few other genes related to the DDR and replication have been linked to leukaemia, including the members of the Fanconi anaemia (FA) complex and BLM (Fanconi 1967, German 1997, Xie, de Winter et al. 2000).

It is becoming apparent that many cancers are related to mutations within repair proteins. Therefore, many new therapies are targeting the repair pathways in order to specifically kill cancerous cells (reviewed in Gullotta, De Marinis et al. 2010). One such treatment is PARP inhibitors in BRCA1 deficient tumours. Damage in cells treated with PARP inhibitors can eventually be repaired by HR; however, in the cancer cells with mutated HR proteins the damage is unrepaired and cancer cells cannot survive. This is termed synthetic lethality (Fig 1.17) (Farmer, McCabe et al. 2005, Lord, McDonald et al. 2008).

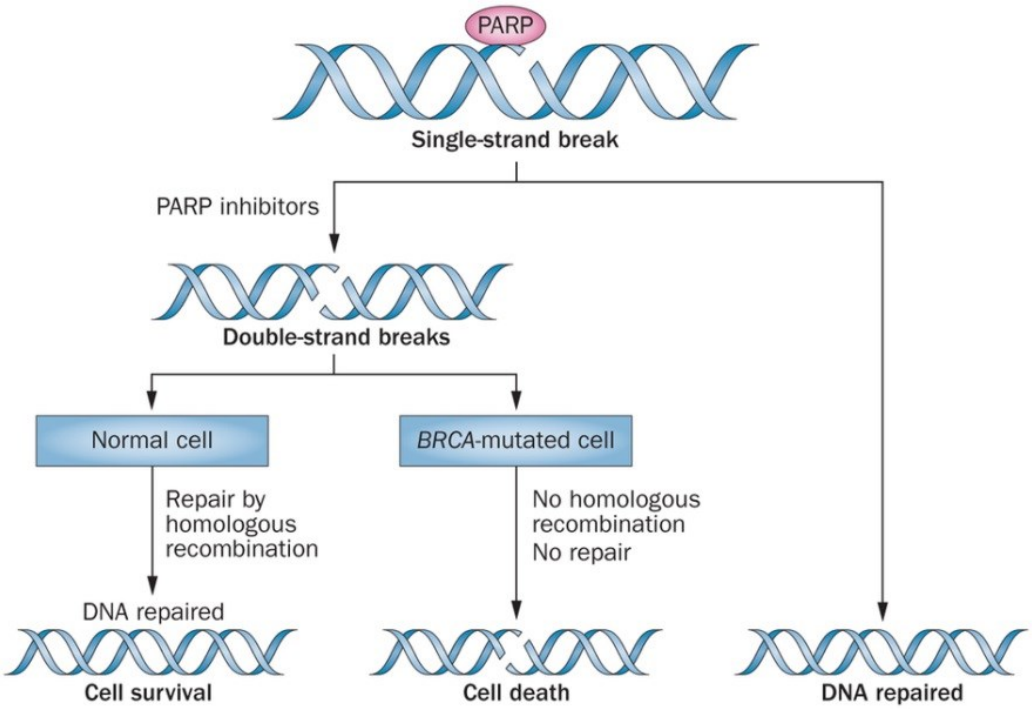

Figure 1.17. Mode of action of PARP inhibitors and synthetic lethality in cancer cells. Single strand breaks are repaired using PARP. When PARP inhibitors are used the single strand break is converted into a double strand break, which in normal cells can be repaired but in cancer cells (where a DDR protein is mutated) the damage cannot be repaired and the cells die. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Clinical Oncology (Sonnenblick, de Azambuja et al. 2015).

1.10 The Proteasome

Although K48 is the canonical signal for proteasomal degradation, all poly-Ub chains, regardless of their confirmation, can be bound by the regulatory particle (19S) of the proteasome (Chau, Tobias et al. 1989, Finley, Sadis et al. 1994). The proteasome is comprised of a proteolytic barrel (20S) made up of two α-rings which flank two β-rings where the proteolysis of the protein occurs. Each ring, both α and β, is formed from seven subunits, with the β subunits, β1, β2 and β5 conferring the proteolytic activity of the proteasome (Loewe, Stock et al. 1995, Groll, Ditzel et al. 1997). The α-rings control the entry of the substrate into the proteolytic core and will only form an open confirmation once activated by the AAA+ ATPases of the 19S particle (Loewe, Stock et

al. 1995, reviewed in Pickart and Cohen 2004, Smith, Chang et al. 2007). The 20S can be bound at either end by the 19S regulatory particle, which is formed of the "base", with six AAA+ ATPases, and the 12 subunit "lid" (Peters, Franke et al. 1994, Glickman, Rubin et al. 1998). These six ATPases are required to form a conformational change which opens a pore within the α-ring, allowing substrate entry (Smith, Chang et al. 2007, Rabl, Smith et al. 2008, Matyskiela, Lander et al. 2013). The energy produced by the ATPases also acts to unfold the substrate by pulling the substrate through the pore (Martin, Baker et al. 2008, Zhang, Wu et al. 2009, Aubin-Tam, Olivares et al. 2011, Maillard, Chistol et al. 2011). Within the 19S particle there are three Ub-binding proteins, adhesion regulating molecule 1 (ADRM1), Proteasome (prosome, macropain) 26S subunit, non-ATPase, 4 (PSMD4/Rpn10) and a DUB, Pad one homolog 1(POH1) (Fig 1.18) (Deveraux, Ustrell et al. 1994, Verma, Aravind et al. 2002, Schreiner, Chen et al. 2008). The distance between POH1 and either PSMD4 or ADRM1 is around 70-80Å meaning only poly-Ub chains of four or more moieties are recognised by the proteasome as the poly-Ub must be bound by one of the Ub-receptors as well as POH1 to allow removal of the chain from the substrate (Riedinger, Boehringer et al. 2010, Lander, Estrin et al. 2012, Schreiber and Peter 2014).

Along with POH1 there are two other DUBs that can associate with the proteasome, Ub carboxyl-terminal hydrolase isozyme L5 (UCHL5) and Ub-specific peptidase 4 (USP4), although they are not core components (Koulich, Li et al. 2008).

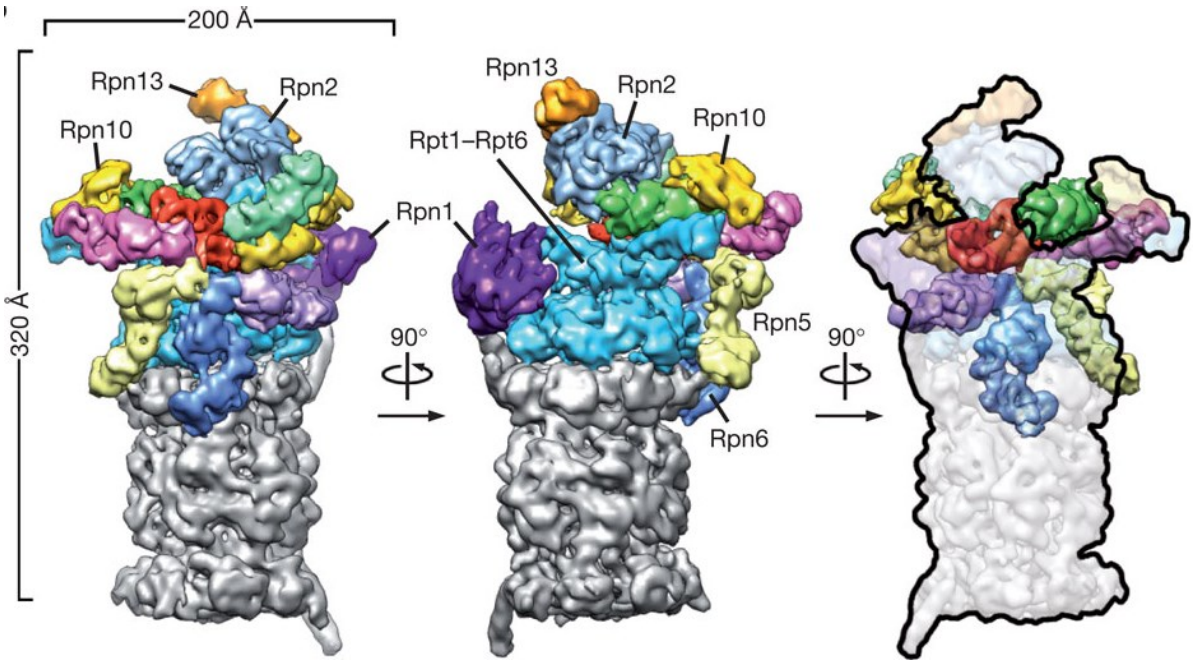

Figure 1.18. Structure of the proteasome. The 26S proteasome is compromised by the 20S core (Grey) and the 19S cap (multi-coloured). The 19S cap is made of the "Base" and the "lid". The base contains ATPases which control the entry of the substrate into the 20S core. The "lid" is important in substrate recognition with three Ub binding subunits, Rpn13/ADRM1 (Orange), Rpn10/PSMD4 (Gold) and the DUB POH1/Rpn11/PSMD14 (Green). The 20S core region is comprised of four rings, two α-ring, made of seven subunits, and two β-rings also formed from seven subunits each. The β-rings are important for the degradation of the targeted proteins. Reprinted by permission from Macmillan Publishers Ltd: Nature (Lander, Estrin et al. 2012), copyright (2012)

1.10.1 The Ubiquitin-Proteasome system (UPS)

The recognition of Ub by the "lid" of the proteasome is an important regulatory mechanism for a variety of important cellular processes. These processes include DNA damage, with the proteasome being recruited to sites of damage, regulating the Ub-chains formed at DNA breaks as well as helping the recruitment and clearance of repair proteins (reviewed in McBride, Iwamoto et al. 2003, Jacquemont and Taniguchi 2007, Shi, Ma et al. 2008). The Ub-Proteasome system (UPS) is also linked to transcriptional regulation through many mechanisms including modulating protein levels within the

cell (reviewed in Muratani and Tansey 2003). An important role of UPS is in cell cycle control, maintaining the correct balance of p53 for either progression through the G1/S checkpoint or causing a block in order to allow DNA repair (reviewed in Bassermann, Eichner et al. 2014, reviewed in Pant and Lozano 2014). In the immune response, the proteasome has roles both proteolytic and non-proteolytic to activate the NF-κB protein (Palombella, Rando et al. 1994, reviewed in Chen 2005). Faults within the UPS have also been shown to cause neurodegeneration, highlighting the important role it has within regulating many cellular processes (reviewed in Ciechanover and Brundin 2003).

In order to determine which components of the UPS were members of the DNA repair pathways a screen of DUBs was performed.

1.13 siRNA screen of DUBs

A screen has previously been carried out by a former PhD student, Laura Butler, reverse-transcribing 103 predicted DUBs into HeLa cells. In order to synchronise the cells in the same phase of the cell cycle, cells were then serum starved for 24 hours, then released into normal media for 24hours in order to allow cells to progress through the cell cycle. In order to induce DSBs cells were treated with 16 hours 3mM HU before fixing. Changes in the levels of conjugated-Ub were tested using the FK2 antobody conjugated to horseradish peroxidase, with luminescence measured on a plate reader. This screen showed that siRNA against the proteasome associated DUB, POH1 had significantly increased conjugated-Ub after release from HU. On the other hand siRNA against a little known DUB, USP50, appeared to show a decreased FK2 signal.

1.14 Summary

There is a large volume of research demonstrating the importance of Ub in a variety of cellular processes, particularly DNA damage and DNA replication. Therefore, it follows

that the regulation of the Ub by DUBs is also critical for cells to maintain correct cellular functions (reviewed in Amerik and Hochstrasser 2004).

Ub in the DDR is a highly researched topic with emerging evidence of the tight regulation of the poly-Ub chains that form at DSBs. There is evidence of K48-, K63- and potentially K6-linked chains that form at the break sites (Morris and Solomon 2004, Polanowska, Martin et al. 2006, Sobhian, Shao et al. 2007, Doil, Mailand et al. 2009, Stewart 2009, Mallette and Richard 2012, reviewed in Brown and Jackson 2015). On the other hand, Ub is implicated in efficient replication but the field is still in its infancy and there is a lot of scope for investigation (Hendel, Krijger et al. 2011, Maric, Maculins et al. 2014, Moreno, Bailey et al. 2014).

The proteasome, as an integral part of Ub processing, is known to be important for the DSB response (reviewed in McBride, Iwamoto et al. 2003, Jacquemont and Taniguchi 2007, Shi, Ma et al. 2008, Finley 2009). DUBs are also important for Ub processing within the cell and act to disassemble Ub-chains and can protect substrates from degradation (reviewed in Guterman and Glickman 2004). The large number of DUBs within the cell means that there is potential for drugs to be targeted against specific DUBs to target specific pathways (Semple, Grp et al. 2003, reviewed in Lim and Baek 2013).

Therefore understanding the role of the proteasome and DUBs in the cell will increase the understanding of Ub-signalling in important processes such as DSB repair and replication. This opens up a range of potential drug targets for cancer therapies.

1.14 Aims

1. To continue the characterisation of the proteasome and POH1 at sites of damage, finishing work from a previous PhD student.

2. To determine whether USP50 is an active DUB

3. To characterise USP50 and its DDR role within cells

2. Materials and Methods

2.1 Molecular Biology

2.1.1 Bacterial transformations

1-50 ng of plasmid DNA was transferred into pre-chilled eppendorfs with 10µl of *Escherichia Coli* DH5α (Bioline). The bacteria were incubated on ice for 20 mins before being heat shocked at 42°C for 30 seconds. Bacteria were transferred back onto ice for a further 2 mins prior to 200µl Luria Bertani (LB) being added. Cells were incubated at 37°C for 45 mins then plated onto LB agar plates containing either Ampicillin (50 µg/ml) or Kanamycin (50 µg/ml) depending on the antibiotic resistance within the transformed plasmid. Plates were incubated at 37°C overnight.

2.1.2 Plasmid DNA preparation

Single transformed bacterial colonies were selected from plates and grown up in overnight starter cultures at 37°C at 200 rpm. 10 ml starter cultures were required for Minipreps and 300 ml starter cultures were grown for Maxipreps. Bacteria cultures were spun down in a centrifuge at 3000 rpm for 30 mins to pellet the bacteria. The supernatant was discarded. The DNA extraction was then performed using the Genejet from ThermoFisher Miniprep or Maxiprep kits following the manufacturer's protocol.

2.1.3 RNA extraction

Cells were plated and then RNA extracted using the Bioline RNA extraction kit using the manufacturer's instructions

2.1.4 cDNA synthesis

RNA was converted into cDNA using the Bioline cDNA synthesis kit. Controls were performed without the reverse transcriptase.

2.1.5 Quantification of Nucleic Acids

DNA and RNA were quantified using the Labtech Spectrophotometer using ND-1000 software. Blank measurements were made using the resuspension solution of the nucleic acid, either H_2O or 1x TE buffer. 1 µl of sample was loaded onto the pedestal and the concentration of nucleic acid measured. The programme used depended on the sample (DNA-50 or RNA-40).

2.1.6 Polymerase Chain Reaction

PCR was used to amplify DNA fragments, including amplification of cDNA and cloning DNA. PCR reactions were also used to introduce selected mutations into plasmids. Standard reactions were carried out using Pfu polymerase from Thermo and dNTPs from Bioline as outlined in Table 2.1 and Table 2.2.

Constitutent	Stock Concentration	Final Concentration	Volume in 50 µl
Pfu buffer	10 x	1 x	5 µl
Pfu enzyme	2.5 U/µl	2.5U	1 µl
dNTPs	10 mM	400 µM	2 µl
Forward Primer	20 µM	0.8 µM	2 µl
Reverse Primer	20 µM	0.8 µM	2 µl
DNA	Variable	1 µg	-
H_2O	-	-	Up to 50 µl

Table 2.1. Standard PCR reaction mix

Step	Temperature °C	Time	Cycles
Initial denaturing	95	5 minutes	1
Denaturing	95	30 seconds	25-30
Annealing	55	1 minute	25-30
Extension	72	2 minutes	25-30
Final Extension	72	2 minutes	1
Hold	4	Infinite	-

Table 2.2. Standard PCR programme

Extension times vary depending on the length of the template DNA usually calculated as a minute per Kilobase (kb) of DNA. Annealing temperatures were calculated depending on the melting temperature (Tm) of the primers -5°C.

2.1.7 Agarose gel electrophoresis

DNA was visualised on 1% Agarose gels made with 1x TAE buffer and Ethidium Bromide at a concentration of 1:100,000. Gels were loaded into a tank and submerged in 1x TAE buffer. 5 µl of the relevant DNA standards were loaded onto the gel (Thermofisher Hyperladder 1kb or Hyperladder IV). The samples containing QIAGEN loading dye were loaded into each well of the gel and ran at 130 volts. DNA was visualised using UV light on the GeneSnap by Syngene system.

2.1.8 DNA Gel extraction

DNA was visualised on a UV light box and cut out using a scalpel. Samples were weighed in an Eppendorf, and then extracted using the QIAGEN gel extraction kit according to the manufacturer's instructions.

2.1.9 Restriction enzyme digest

DNA was digested using NEB enzymes and the recommended buffers. Standard restriction digests were carried out as outlined (Table 2.3). Reactions were performed at 37°C for an hour.

Constituent	Volume
Enzyme 1	1 µl
Enzyme 2	1 µl
10 x Buffer	1 µl
BSA	1 µl
DNA	1 µg
H_2O	Up to 10 µl

Table 2.3 Standard restriction enzyme mix

2.1.10 DNA ligation

Plasmid and fragment DNA were digested with the same restriction enzymes to leave matching cut ends. The ligation was set up using 3:1 fragment to vector ratio with the vector at 10-50 ng per reaction. Ligations were carried out using 400U T4 DNA ligase with 1µl T4 ligase buffer made up to 10 µl with H_2O. Reactions were carried out at room temperature for 1 hour. Control ligations containing vector only or insert only were performed. All reactions were transformed into *E. Coli* DH5α as in 2.1.1.

2.1.11 Site-Directed Mutagenesis

Site-directed mutagenesis was performed with specifically designed primers (Table 2.5), primers contained mutations surrounded by 10-20 bps either side. PCR reactions were carried out as below. Negative controls were carried out without the primers.

After amplification the reaction was treated with 1µl DpnI and incubated at 37°C for 1 hour to digest the original template DNA. To determine if the amplification was successful, 10µl of the reaction was run on a 1% agarose gel as in 2.1.6. If the amplification had worked 2µl of the DNA was transfected into competent *E.Coli* DH5α as in 2.1.1.

2.1.12 DNA Sequencing

Once DNA had been extracted as in 2.1.2, DNA was quantified as in 2.1.3, then diluted to a concentration of 10 ng/µl in 10 µl sent to Source Biosciences for Sanger sequencing and analysed on SeqMan software.

2.1.13 Cloning USP50 mutants

USP50 was amplified out of the addgene USP50- Flag plasmid vector using specially designed primers containing restriction enzyme sites. The forward primer for the pET28a plasmid contained a BamH1 restriction site. The reverse primer contained the Xho1 restriction enzyme site. The PCR fragments and the pET28a vector were digested with BamH1 and Xho1 restriction enzymes. The digested USP50 fragment was then ligated as in 2.1.10. DNA was run on a gel to check the size of the plasmid, to confirm the fragment had been inserted. Correct plasmids were transfected into bacteria to amplify the plasmid as in 2.1.1. Point mutations were introduced using specifically designed primers as in 2.1.11 and the plasmid DNA sequenced. For a list of primers see Table 2.5.

The pcDNA5/FRT/TO USP50 plasmids were designed and sent to GenScript for synthesis. These plasmids were made siRNA resistant by introducing a series of silent point mutations as shown below.

USP50 siRNA sequence 5 - TAT GAT ACC CTT CCA GTT A

siRNA resistant form - TAT GAC ACA CTA CCA GTT A

Amino Acid - Tyr Asp Thr Leu Pro Val

USP50 siRNA sequence 7 - C TAC CCA GCA TTT ACG

siRNA resistant form - C TAT CCG GCT TTT ACG

Amino Acid - Tyr Pro Ala Phe Thr

2.2. Protein Methods

2.2.1 SDS Polyacrylamide Gel Electrophoresis (SDS PAGE)

Biorad gel casting equipment was used to make polyacrylamide gels. The percentage of acrylamide depended on the protein being studied (see Table 2.4).

Consituent (ml)	Resolving Gel			Stacking Gel
	6%	10%	15%	5%
H_2O	7.9	5.9	3.4	2.7
30% polyacrylamide mix	3.0	5.0	7.5	0.67
1.5 M Tris pH 8.8	3.8	3.8	3.8	-
1.0 M Tris pH 6.8	-	-	-	0.5
10% SDS	0.15	0.15	0.15	0.04
10% PS	0.15	0.15	0.15	0.04
TEMED	0.012	0.006	0.006	0.004
Final Volume (ml)	15	15	15	4

Table 2.4. SDS polyacrylamide gel solutions.

The resolving gel was poured between the glass plates and covered in water-saturated isobutanol and allowed to set. The water-saturated isobutanol was removed before the

stacking gel was poured on top. Plastic combs were inserted to form wells. The comb was removed once the stacking gel had polymerised.

Gels were loaded into the Biorad tank and filled with 1x SDS Running buffer. 5µl of ThermoFisher PageRuler Prestained protein ladder was loaded into one of the wells. Protein samples were loaded in 4x SDS loading buffer and sonicated before loading onto the gel then ran at 130 volts.

Gels were then either Western blotted (2.2.2) or stained with Coomasie (2.2.3)

2.2.2 Western Blot

Protein gels were transferred on PVDF immobillon membrane. The membranes were activated in methanol and then stored in water. Transfers were set up in Biorad Trans-blot cassettes, sponges and 3mm filter paper were soaked in transfer buffer. The transfer was built with a sponge and 2 pieces of filter paper either side of the membrane and gel. The cassette was then placed in the tank and submerged in 1 x Transfer buffer and ran at 100 volts for 1 hour.

Once the transfer was complete the membrane was blocked in 5% marvel milk in PBS with 0.1% Tween (PBStw), unless otherwise stated, for a minimum of 30 mins before being transferred into primary antibody overnight at 4°C on a roller (Table 2.7). Blots were then washed 3x 10 mins in PBStw and then transferred into secondary HRP antibodies in 5% marvel milk for a minimum of 1 hr whilst being rocked (Table 2.7). The blots were again washed 3x 10 mins in PBStw. Once washed the membranes were probed with homemade ECL (0.1 M Tris pH 7.8, Lumional, Courmic Acid, H_2O_2), excess removed and placed inside a plastic wallet inside a cassette. Fuji film X-Ray film was placed in the cassette for varying length of time based on the strength of the ECL signal and the film developed inside the Xograph Compact X4 developer.

2.2.3 Coomassie stain

Gels were soaked in Coomassie blue stain (1.25g brilliant blue, 10% Acetic acid, 45% Methanol, 45 % H_2O) for 30 mins at room temperature on a rocker. Gels were then destained using 10% ethanol, 10% Acetic acid and 80% H_2O, overnight.

2.2.4 Protein Expression

Bl21 *E.Coli* were transformed with pET 28a protein expression vector containing the protein of interest. Colonies were picked and grown up in 10 ml starter cultures containing Kan at 37°C overnight at 200 rpm (Table 2.9). Starter cultures were then transferred into 1 litre LB containing Kan and grown for 6 hours at 37°C at 200 rpm. Bacterial expression was induced using 200 mM IPTG and bacteria left to grow overnight at 20°C at 200 rpm.

2.2.5 Protein Isolation

Bacteria were pelleted by centrifuging at 3000 rpm for 30 mins at 4°C. Bacteria were lysed in 2 ml ice cold lysis buffer (50 mM Sodium Phosphate pH7, 300 mM Sodium Chloride, 5% Glycerol, 10 mM beta-Mercaptoethanol) with 1 protease inhibitor tablet and 5 mg/ml Lysozyme per 10ml. Resuspended bacteria were left on ice for 5 mins and then sonicated at 20% intensity for 1 min. Lysed bacteria were spun at 13000 rpm to pellet debris. Supernatant was transferred to a 15 ml falcon tube and made up to 10 ml with lysis buffer and 500 µl of Nickel beads and rotated at 4°C overnight.

Beads were pelleted by pulse centrifugation and washed 3x ice cold wash buffer (50 mM Sodium phosphate pH7, 300 mM Sodium Chloride, 5% Glycerol, 10 mM beta-mercaptoethanol, 50 mM Imidazole) with agitation between each wash. The protein was then eluted using 400 µl ice cold lysis buffer plus 300 mM Imidazole, vortexed and left on ice for 1 hour. The falcon tube was spun at 1000 rpm for 2 mins and supernatant

kept. The supernatant was spun through a filter column at 13000 rpm to remove any remaining beads. Eluted proteins were dialysed in a dialysis column in dialysis buffer (25 mM TRIS-HCl pH7.5, 10% Glycerol, 2 mM DTT, 150 mM KCl) overnight at 4°C. Proteins were checked by Coomassie (2.2.3) then stored at -80°C until required.

2.2.6 Deubiquitinating assay

In order to determine if the purified USP50 enzyme had any Deubiquitinating activity a Deubiquitinating assay was carried out. Proteins were serially diluted across four different concentrations from 10nM to 1.25nM with dialysis buffer. Proteins were then incubated with 50 mM HEPES pH7.5, 10 mM DTT, 0.01% Tween and 0.25 µg/ml poly-Ub. After 10 mins, 4.8% DMSO is added and the reaction is incubated overnight at 28°C shaking at 200 rpm. The reaction is stopped by adding 4x SDS loading buffer and analysed using Western blotting (2.2.2)

2.2.7 Binding Assay

Proteins were expressed as in 2.2.4. Proteins were then isolated as in 2.2.5 but not eluted. Beads were washed 3x ice cold wash buffer and then equilibrated into Tris with BSA (TBSA – 50 mM Tris pH7.5, 0.1% BSA) by carrying out 3 x 1ml washes. Beads were then resuspended in 50 µl TBSA with 0.5µg Ub. Beads were incubated on ice for 30 mins with gentle agitation every 5 mins. Controls were carried out using Ni-beads without bound proteins and a reaction without beads. Beads were pelleted and solution removed, beads were then washed 3x 1ml washes of TBSA. Proteins were eluted by adding 50 µl 4x loading buffer and resolved on an SDS PAGE gel (2.2.1) and analysed by Western blotting (2.2.2).

2.2.8 Flag Immunoprecipitation

Cells with Dox inducible USP50-Flag were plated on a 10 cm plate and Dox treated for 48 hours. Cells were washed with 5 ml ice cold PBS before being scraped in ice cold Nuclear Lysis Buffer (10 mM HEPES pH7.6, 200 mM NaCl, 1.5 mM $MgCl_2$, 10% Glycerol, 0.2 mM EDTA, 1% Triton) for every 10 ml, 1 protease inhibitor tablet, 1 phosphatase tablet, 20 µM MG132 and 1 µl DNase were added. The lysed cells were transferred into a pre-chilled Eppendorf 1.5 ml tubes and rotated at 4°C for 1 hour. The Eppendorf was spun at 1500 rpm at 4°C for 10 mins and the supernatant kept, the pellet discarded. 50 µl of the supernatant was mixed with 20 µl 4x Loading buffer and boiled at 95°C for 5 mins.

For every IP 9 µl Flag-agarose beads were washed out of storage buffer into PBS by doing 3x 1ml PBS washes centrifuging at 3000 rpm between each wash. 91 µl of PBS was added for every 9 µl of agarose beads. Once the beads were resuspended in PBS, 100µl were transferred into a bijou.

Into each bijou 1.5 ml of nuclear lysis buffer and 2 ml of PBS was added, then 500 µl of supernatant added to each bijou. The bijous were rotated overnight at 4°C.

The bijous were spun at 1000 rpm and 3 ml of the supernatant removed, the remaining 1ml including beads was transferred into a pre-chilled Eppendorf. The Eppendorf was centrifuged at 3000 rpm for 1 min and the beads left to settle. The supernatant was then removed before 3x 1 ml PBS-0.02% tween washes. The wash buffer was completely removed before adding 60 µl 2x loading buffer. This was boiled at 95°C for 5 mins and then 10 µl loaded onto an SDS PAGE gel (2.2.1) and analysed by western blotting (2.2.2).

2.3. Cell Biology

2.3.1 Tissue culture

HeLa, U2OS, MCF7, BJ h-Ras and NIH3T3 cells were grown in Dulbeccos Modified Eagle Media (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Pencillin/Streptomycin. Monolayers of cells were cultured in Corning T75 flasks and kept at 37°C and 5% CO_2. When cells reached 80% confluency they were passaged by removing all media, washing the cells with 1x PBS, then adding 2ml 1x Trypsin/EDTA and leaving cells at 37°C until cells have detached. Cells were split 1:10 into new flasks.

Stocks of cells were stored in liquid nitrogen at a concentration of $1x10^6$ cells per ml. In order to freeze cells they were trypsinised, counted using a haemocytometer, pelleted and resuspended in the relevant amount of freezing media (70% DMEM, 20% FCS, 10% DMSO). Cells were cooled at a -1°C/min in the -80°C freezer before being transferred into liquid nitrogen.

2.3.2 Plasmid transfection

DNA plasmids were transfected into cells using the nonliposomal transfection reagent FuGENE 6 at a ratio of 4 µl:1 ng FuGENE:DNA following the manufacturers guidelines.

2.3.3 siRNA transfection

siRNA transfections were carried out using the transfection reagent Dharmafect1 (Dharmacon) following the manufacturer's instructions (Table 2.8)

2.3.4 shRNA transfection

Lentiviral shRNA was purchased from Sigma-Aldrich. Viral particles were transfected as per the manufacturers' protocol. Cells were selected by Puromycin resistance and

clones picked and grown up. Clones were screened by the increase of 53BP1 foci upon shRNA expression. The shRNA sequence was the same as the USP50-7 siRNA sequence (Table 2.6).

2.3.5 Stable cell line creation

HeLa Flip-In cells were plated in 10cm dishes and transfected with pcDNA5/FRT/TO-USP50 constructs along with the Pog44 recombinase at a ratio of 3:1 (as in 2.3.1). Cells were left for 48hrs and then treated with Hygromycin to select cells that had been successfully transfected. Control transfections were carried out without the Pog44 recombinase.

Colonies were selected and expanded.

2.3.6 Immunofluorescent staining

Cells were plated in a 24 well plate on 13 mm circular glass coverslips at a density of 5 x 10^4 cells/ml. Cells were treated as required and then fixed in 4% PFA (unless otherwise stated). Once fixed, cells were permeabilised with 0.2% TritonX in PBS, for 5 mins. Following permeabilisation, cells were blocked using 10% FCS in PBS for a further 5 mins. Cells were incubated with the desired primary antibody at stated concentrations for 1 hr at room temperature in 10% FCS/PBS, unless otherwise stated (Table 2.7). Cells were then washed in FCS/PBS before being incubated for 1 hour with AlexaFluor antibodies at a concentration of 1:2000. Cells were washed in PBS and then fixed for 10 mins in 4% PFA before being washed again in PBS. DNA was stained using Hoescht at 1:20,000 for 5 mins and then washed with PBS before mounting onto Snowcoat slides using Immunomount mounting media (Table 2.8).

2.3.7 EdU staining

HeLa cells were plated on coverslips in a 24 well plate at a density of 2 x 10^4 cells/ml and treated as required. Cells were then incubated with 10 μM final concentration of EdU. Staining was carried it out following Click-iT® EdU Imaging Kits (Table 2.8). When stated in the protocol slides were counter stained with 53BP1 primary antibody in 10% FCS/PBS (Table 2.7) for 1 hour at room temperature. Slides were washed in 10% FCS/PBS before being incubated for 1 hour at room temperature in AlexaFluor 555. Following this, the staining was continued as per the manufacturer's guidelines. Slides were mounted using Immunomount mounting media on Snowcoat slides and stored at 4°C until required.

2.3.8 Fibre Spreading

Fibre spreading uses two thymidine analogues, CldU and IdU, to allow visualisation of fork progression as the analogues are incorporated into newly synthesised DNA.

Cells were seeded at a density of 5x 10^4 cells/well, in 6 well plates and treated as required (siRNA knockdown 2.3.2). Cells were incubated at 37°C with CldU at a final concentration of 25 μM for 20 mins. Cells were washed with PBS and then media and then incubated for a further 20 mins in 250 μM IdU at 37°C (Table 2.8). Cells were washed twice with ice-cold PBS, trypsinised and resuspended in 1ml of PBS and counted. The desired concentration was 50 x 10^4 cells/ml and cell concentrations were adjusted to this and stored on ice.

For each treatment four Snowcoat slides were labelled, 2 μl of the cell sample was placed on the each slide near the label and left for approximately 5 mins to allow to dry slightly. On top of each sample 7 μl of spreading buffer (200 mM Tris pH7.4, 50 mM EDTA, 0.5% SDS) was pipetted and mixed with the sample and incubated for 2 mins.

Slides were gradually tilted to allow the slow spread of the sample down the slide. Once the buffer has reached the bottom, the slides are allowed to dry for 2 mins. Slides were then fixed in a 3:1 ratio of Methanol:Acetic acid for 10 mins and then air dried for 5-10 mins and stored at 4°C till staining.

2.3.9 Fibre Immunostaining

Slides must be kept shielded from light as much as possible throughout this process. Slides were washed 2 x 1ml H_2O and rinsed with 2.5M HCl before denaturing the DNA with 2.5M HCl for 1 hour 15 mins. Slides were rinsed with 2 x PBS followed by 2 x 5 min washes in Blocking solution (PBS, 1% BSA, 0.1% Tween20). Slides were incubated in blocking solution for 30 min-1 hr. Once blocked, 115µl of primary antibodies was added to each slide, Rat αBrdU (AbD Serotec) was at a concentration of 1:1000 and Mouse αBrdU (Becton Dickinson) was used at 1:750, the slides were covered with large coverslips to get even distribution of the antibodies. Antibodies were incubated on the slides for 1 hour (Table 2.7). Slides were washed 3 x PBS and then 3 x Blocking solution for 1 min, 5 mins and 25 mins respectively. Following the washes the slides were incubated with 115µl of secondary antibodies (α Rat AlexaFluor 555 and αMouse AlexaFluor 488) in blocking solution at a concentration of 1:500 again covered with a large coverslip for 1 hour 30 mins. Slides were washed 2 x PBS, then 3 x blocking solution for 1 min, 5 mins and 25 mins respectively, followed by 2 x PBS. Mounting media was applied to the slide and a large coverslip placed over the slide and left to dry. Coverslips were secured with clear nail varnish and stored at -20°C till analysed on the microscope.

2.3.10 Colony survival assays

Colony survival assays were used to determine sensitivity to cells in response to different damaging agents (Table 2.8).

HeLa cells were plated at 2 x 10^5 cells/ml in a 24 well plate and treated as required (e.g. siRNA and drug treatments). Cells were then trypsinised and cells and transferred to a 6 well plate (volume transferred based on plating density experiments), with one 6 well plate per treatment, 3 wells were plated at a 1:2 dilution. Plates were incubated for 14 days at 37°C at 5% CO_2 until colonies formed. Colonies were stained using 0.5% Crystal violet at 50% methanol, washed with PBS and the H_2O and colonies counted.

2.3.11 Double strand break repair assays

Double strand break repair assays were carried out using U2OS cells with a stable DR-GFP for HR repair or EJ5-GFP for NHEJ.

Cells were plated at a density of 3 x 10^6 cells per/well and treated with siRNA as in 2.2.3.

Cells were transfected using RFP plasmid as a transfection control and a Sce-I plasmid to induce the double strand break. After 16hours cells were put into fresh media and left for a further 32 hours. Cells were then trypsinised and transferred into FACs tubes (Bioline) and spun at 1800rpm for 5 mins. Supernatant was removed and cells resuspended in 500µl 4% PFA and left to fix for 30mins on a rocker protected from light. After the cells are fixed they are spun down at 1800 rpm for 5 mins to pellet the cells. Supernatant is removed and the cells are resuspended in PBS and stored at 4°C until analysis is performed. Analysis was carried out on the Cyan4 Flow cytometer using Summit software.

2.3.12 Cell cycle FACs analysis

Cell cycle profiles were analysed using BrdU incorporation into cells and visualised by FACs.

Cells were plated at a density of 3 x 10^6 cells/well of a 6 well plate and treated as required (e.g siRNA). Cells were incubated for 30 mins in 30 µM BrdU then washed in PBS and then trypsinised. The media, PBS wash and trypsin were collected and spun at 1400 rpm for 5 mins. The supernatant was removed, discarded and the cell pellet washed in PBS and spun as before. Supernatant was removed and cell pellet resuspended in 1ml cold PBS before 4ml 100% ethanol added dropwise whilst vortexing. Tubes are stored for up to a month at -20°C until stained.

In order to stain the cells they were spun at 1400rpm for 5 mins at 4°C. All ethanol is removed and the pellet resuspended in 1ml of pepsine (15mg pepsine in 20 ml of 30 mM HCl), then a further 2ml of pepsine added. Cells were incubated at 37°C for 20 mins protected from light, agitated every 5 mins. Cells were spun down again at 1400 rpm for 5 mins and the supernatant discarded. The pellet was resuspended in 1.5 ml 2N HCl and incubated in the dark at room temperature for 20 mins. Cells were spun at 1400 rpm for 5 mins and the supernatant removed, the pellet was resuspended in 1 ml PBS and then a further 4 ml PBS added and spun as before. The pellet was resuspended in 4 ml of Bu buffer (0.5% FCS, 0.5% Tween20, 20 mM HEPES pH8, PBS) and vortexed. Cells were spun as before and resuspended in 250 µl Bu buffer with BrdU antibody (1:50) and incubated for 45 mins at room temperature in the dark. After incubation 5 ml PBS was added to each tube and spun as before, the supernatant was removed and the pellet resuspended in 100 µl Bu buffer with 1:2000 AlexaFluor 488 and incubated in the

dark for 30 mins. 5 ml of PBs was added to each tube, tubes were covered in foil and stored at 4°C overnight.

Tubes were spun down at 1400rpm for 5 mins and the pellet resuspended in 25 µl/ml Propidium Iodide, 50 µg/ml RNase in PBS and incubated at room temperature for 30 mins in foil (Table 2.8). Cells were analysed using Accuri software.

2.3.13 Cell titre Glo assay

The Cell titre Glo assay measures cell viability through the measuring the concentration of ATP produced by metabolically active cells. Lucerifin is oxygenated in the presence of ATP to Oxylucerifin which produces a luminesence signal that correlates to the number of viable cells.

Cells were plated at a density of 1000 cells/well of a 96 well plate with a transparent bottom and opaque sides. Cells were treated as required and then incubated at 37°C, 5% CO_2 until treatment has reached optimal levels but not allowing cells to become confluent. Once cells are ready all media is removed and then 100µl of media added back into each well. The assay was performed following manufacturers guidelines and analysed using a Victor plate reader.

2.5 Microscopy

2.5.1 Zeiss microscopy

Images of immunofluorescent staining were captured on the Zeiss 510 Meta confocal microscope, using three lasers to give excitation at 647, 55 and 488 nM wavelengths. Images at each wavelength were collected sequentially at a resolution of approximately 1024 x 1024 pixels, using the Plan-Apochromat 100x/1.4 Oil objective.

2.5.2 Leica Microscopy

Immunofluorescent staining was imaged using the Leica DM6000B microscope using a HBO lamp with 100W mercury short arc UV bulb light source and four filter cubes, A4, L5, N3 and Y5 to produce excitations at wavelengths 360 488, 555 and 647 nm respectively. Images were captured at each wavelength sequentially using the Plan Apochromat HCX 100x/1.4 Oil objective at a resolution of 1392x1040 pixels.

2.4 Primers sequences

Primer Name	Primer Sequence	Purpose
BGH_Rev	TAGAAGGCACAGTCGAGG	Sequencing
CMV_Fwd	CGCAAATGGGCGGTAGGCGTG	Sequencing
T7_Fwd	TAATACGACTCACTATAGGG	Sequencing
T7_Rev	TATGCTAGTTATTGCTCAG	Sequencing
GAPDH_Fwd	ATTGTCAGCAATGCATCCTG	Control
GAPDH_Rev	ATGGACTGTGGTCATGAGCC	Control
USP50_IR_Fwd	GATGCTCAGGAATTCTTGCGTTGTGTCCTAAATGAAC	Mutagenesis
USP50_IR_Rev	GTTCATTTAGGACACAACGCAAGAATTCCTGAGCATC	Mutagenesis
USP50_Fwd	GGAAGTATATCACCGCTCTGC	Control
USP50_Rev	TGATCTTCTCCGGGAGTAGTGG	Control
pET-F-BamH1	ATGGGTCGCGGATCCTTTACTTCTCAGCCGTCTCTCC	Cloning
Pc5-R-Xho1	ATTCTCGAGCTCGAGCTAGGCCTGGGTGACTGAATTCTTGC	Cloning
USP50_QR_Fwd	CAACAAGATGCTCGGCGATTCTTGATTTG	Mutagenesis
USP50_QR_Rev	CAAATCAAGAATCGCCGAGCATCTTGTTG	Mutagenesis
USP50_N240/241D_Fwd	CAAGACGCACTGACCTGGGACGACGAAATTCACTGCTCC	Mutagenesis
USP50_N240/241D_Rev	GGAGCAGTGAATTTCGTCGTCCCAGGTCAGTGCGTCTTG	Mutagenesis

Table 2.5 Primer sequences and function

2.5 siRNA sequences

siRNA Name	5'-3' Sequence
NTC	Dharmacon ON-TARGETplus SMARTpool D-001810-01-20
Bard1	Sense: UGG UUU AGC CCU CGA AGU AAG [dT][dT]
	Antisense: [Phos] CUU ACU UCG AGG GCU AAA CCA [dT][dT]
BRCA2	Dharmacon On-targetPLUS SMARTpool L-003462-00-0005
53BP1	Dharmacon On-targetPLUS SMARTpool L-003548-00-0005
POH1 F	AGAGUUGGAUGGAAGGUUU
USP50 5	UAUGAUACCCUUCCAGUUA
USP50 6	CAACACAUGCUGCGUGAAU
USP50 7	CUACCCAGCAUUUACGAAA
USP50 8	GGACCUCACUCCUUAUAUU
USP8	Dharmacon On-targetPLUS SMARTpool L-005203-00-0005
WRN	Dharmacon On-targetPLUS SMARTpool L-010378-00-0005
Wee1	Dharmacon On-targetPLUS SMARTpool L-005050-00-0005
SMARCAL1	Dharmacon On-targetPLUS SMARTpool L-013058-00-0005
XPC	Dharmacon On-targetPLUS SMARTpool L-016040-00-0005

Table 2.6 siRNA sequence or catalogue number, all primers are from Dharmacon (Thermo Fisher)

2.6 Antibodies

Antibody	Animal	Procedure	Concentration	Time	Supplier
γH2AX (phospho S139)	Rabbit	IF	1:2000	1 hour	Abcam
γH2AX (phospho S139)	Mouse	IF	1:2000	1 hour	Abcam
γH2AX (phospho S139)-FITC	-	IF	1:200	Overnight 4°C (Dark)	Millipore
53BP1	Mouse	IF	1:1000	1 hour	Abcam
β-actin	Rabbit	WB	1:2000	Overnight 4°C	Abcam
BRCA1 (D9)	Mouse	IF	1:200	Overnight	Santa Cruz
FK2 (M2)	Mouse	IF	1:8000	1 hour on ice	Enzo
Flag	Mouse	WB	1:2000	Overnight 4°C	Sigma
GFP	Mouse	WB	1:5000	Overnight 4°C	Roche
Hexa-Histidine	Mouse	WB	1:1000	Overnight 4°C	Sigma
CENPF	Rabbit	IF	1:1000	1 hour (pre-extract with TritonX)	Abcam
BrdU	Mouse	Fibres	1:750	1.5 hours in the dark	Becton Dickinson
BrdU	Rat	Fibres	1:1000	1.5 hours in the dark	AbD Serotec
BrdU	Mouse	FACs	1:50	45 mins in the dark	Dako

T7-tag	Goat	WB	1:1000	Overnight 4°C	Abcam
Poh1	Rabbit	WB	1:1000	Overnight 4°C	Epitomics
MDC1	Rabbit	IF	1:500	Overnight 4°C	Grant Stewart
Mus81	Mouse	WB	1:250	Overnight 4°C	Santa Cruz
c-Myc (A14)	Rabbit	IF	1:2000	1 hour	Santa Cruz
Myc	Mouse	IF	1:2000	1 hour	Santa Cruz
Sug1 79	Rabbit	IF	1:500	2 hours (pre-extract with YG buffer for 3 mins)	Andy Turnell
Ub (P4D1)	Mouse	WB	1:2000	Overnight 4°C	Santa Cruz
WRN	Rabbit	WB	1:1000	Overnight 4°C	Abcam
Wee1(C20)	Rabbit	WB	1:1000	Overnight 4°C	Santa Cruz
Goat α Rat AlexaFluor 555	Goat	Fibres	1:2000	1.5-2 hours	Life Technologies
Goat α Mouse AlexaFluor 488	Goat	IF	1:2000	1 hour	Life Technologies
Goat α Rabbit AlexaFluor 488	Goat	IF	1:2000	1 hour	Life technologies
Goat α Mouse AlexaFluor 555	Goat	IF	1:2000	1 hour	Life technologies
Goat α Rabbit AlexaFluor 555	Goat	IF	1:2000	1 hour	Life technologies
Donkey α Mouse	Donkey	IF	1:2000	1 hour	Life

AlexaFluor 488					technologies
Donkey α Rabbit AlexaFluor555	Donkey	IF	1:2000	1 hour	Life technologies
Rabbit α Mouse HRP	Rabbit	WB	1:5000	1 hour	Dako
Swine α Rabbit HRP	Swine	WB	1:5000	1 hour	Dako

Table 2.7 Antibodies including species raised in, dilution, conditions and protocols

2.7 Drug treatments and Inhibitors

Name	Activity	Storage	Final Concentration	Supplier
Aphidicolin	Inhibits polymerases α	1mg/ml we DMSO at -20°C	1 µg/ml	Sigma
Camptothecin	Inhibits topoisomerase I causing DSBs	10mM in DMSO at -20°C	10 µM	Sigma
Cisplatin	Crosslinks DNA	1mM made fresh in 0.9% Saline	0.5 nM-25 nM	Sigma
Hydroxyurea	Ribonucleotide reductase, creating stalled and collapsed replication forks	1M stock in H2O at -20°C	3 mM	Sigma
MG132	Inhibits proteasome	10mM in DMSO at -80°C	5 µM	Viva
VE-821	Inhibits ATR kinase	1mM in DMSO at -80°C	5µM	Grant Stewart
Propidium Iodide solution	Fluorescent DNA stain	1 mg/ml at 4°C	25 µg/ml	Sigma

Hoescht	Fluorescent DNA stain	10 mg/ml in PBS at -20°C	500 ng/ml	Sigma
CldU	Thymidine analogue, incorporates into DNA	2.5 mM in DMEM at -20°C	25 μM	Sigma
IdU	Thymidine analogue, incorporates into DNA	2.5mM in DMEM at -20°C	250 μM	Sigma
EdU	Thymidine analogue, incorporates into DNA	10 mM in DMSO at -20°C	10 μM	Thermo Fisher

Table 2.8 Cell treatment and stains

2.8 Buffers

PBS

1 Tablet (Sigma) in 200 ml H_2O

LB Broth

10g LB Broth powder (Sigma) in 500 ml H_2O

LB agar

1 Capsule LB agar (Thermo Fisher) in 500ml H_2O

50x TAE Buffer

2M Tris base, 17.5% Acetic Acid (Glacial), 10% 0.5M EDTA pH 8

10x Tris-EDTA (TE) pH 8

100 mM Tris-HCl pH 8, 1mM EDTA

Tris-BSA (TBSA)

50 mM Tris- HCl pH 7.5, 0.1% BSA

4x SDS Loading Buffer

0.25M Tris pH 6.8, 8% SDS, 40% Glycerol, 6 M Urea, 10% β-Mercaptoethanol

1x SDS Running Buffer

10% 10x Tris/Glycine/SDS in 90% H_2O

1x Transfer Buffer

10% 10x Tris/Glycine, 10% Methanol, 80% H_2

Crystal Violet stain

0.5% Crystal Violet, 50% Methanol, 49.5% H_2O

Coomasie Stain

1.25g Brilliant Blue, 10% Acetic Acid (Glacial), 45% Methanol, 45% H_2O

De-stain

10% Acetic Acid (Glacial), 10% Methanol, 80% H_2O

Protein Lysis Buffer

50 mM Sodium Phosphate pH 7, 300 mM Sodium Chloride, 5% Glycerol, 10 mM β-Mercaptoethanol

Protein Wash Buffer

50 mM Sodium Phosphate pH 7, 300 mM Sodium Chloride, 5% Glycerol, 10 mM β-Mercaptoethanol, 50 mM Imidazole

Protein Dialysis Buffer

25 mM Tris-HCl pH 7.5, 10% Glycerol, 2 mM DTT, 150 mM Potassium Chloride

De-Ubiquitinating assay Buffer

50 mM HEPES pH 7.5, 10 mM DTT, 0.01% Tween 20, 4.8% DMSO

Flag Immunoprecipitation Nuclear Lysis Buffer

10 mM HEPES pH 7.6, 200 mM Sodium Chloride, 1.5 mM Magnesium Chloride, 10% glycerol, 0.2 mM EDTA, 1 % Triton

Fibre Spreading Buffer

200 mM Tris pH 7.4, 50 mM EDTA, 0.5% SDS

Fibre Slide Fixative

75% Methanol, 25% Acetic Acid (Glacial)

YG pre-extraction Buffer

20mM HEPES pH7.5, 20mM NaCl, 1mM DTT, 5mM $MgCl_2$, 0.5% NP40

2.9 Antibiotics

2.9.1 Tissue Culture Antibiotics

100x Penicillin/Streptomycin (Invitrogen) was kept in 5 ml aliquots at -20°C and used at a working concentration of 1x. Stock concentration 10,000 U.

2.9.2 Bacterial Antibiotics

All antibiotics were stored in 1 ml aliquots at -20°C

Antibiotic	Stock Concentration	Working Concentration
Ampicillin	50 mg/ml in H_2O	50 µg/ml
Kanamycin	10 mg/ml in H_2O	10 µg/ml

Table 2.9 Antibiotics and working concentrations

3. The Proteasome and its constituent DUB, POH1, regulates the Ubiquitin chains at sites of Double Strand Breaks

3.1 Introduction

Ub conjugates are an important signalling mechanism in the double strand breaks (DSB) response (Mailand, Bekker-Jensen et al. 2007, Wang and Elledge 2007, Shi, Ma et al. 2008, Fradet-Turcotte, Canny et al. 2013). In this chapter the role of different Ub-linkages and Ub-processing by the proteasome and its associated DUB POH1 was investigated.

The formation of K48-, K63-, K6- and K27-Ub have all been linked to correct repair of DNA breaks by regulating the kinetics of proteins at the DSB (Morris and Solomon 2004, Polanowska, Martin et al. 2006, Sobhian, Shao et al. 2007, Doil, Mailand et al. 2009, Stewart, Panier et al. 2009, Gatti, Pinato et al. 2012, Mallette and Richard 2012). Many repair proteins have been demonstrated to be regulated by Ub-linkages with Ub required for recruitment or clearance from around the DSB. This includes the early sensor, MDC1, which requires Ub in order to be cleared from breaks (Shi, Ma et al. 2008). Conversely the recruitment of 53BP1 and BRCA1 to DSB also requires the formation of poly-Ub chains (Sobhian, Shao et al. 2007, Acs, Luijsterburg et al. 2011, Mallette, Mattiroli et al. 2012, Mallette and Richard 2012, Fradet-Turcotte, Canny et al. 2013).

In order for repair of DSBs to progress efficiently, the Ub-linkages that are formed must also be regulated. Many DUBs have already been implicated in the regulation of these chains and consequently the repair of DSBs (Chapter 1 Section 1.8.4).

In addition to the DUBs, the proteasome has also been implicated in the repair of DSBs. The proteasome is not only required for the degradation of substrates, potentially clearing proteins from DSBs, but also the processing of Ub back into the cell to

replenish the free-Ub pool in order to allow formation of new Ub-modifications upon sensing of DSBs (Eytan, Armon et al. 1993, Hanna, Leggett et al. 2003, Krogan, Lam et al. 2004, Jacquemont and Taniguchi 2007, Shi, Ma et al. 2008).

Therefore the role of the proteasome and its Ub-processing activities were investigated in conjunction with the role of proteasome associated DUB-POH1 at DSBs. Further to this the importance of the individual lysine residues within Ub and therefore potential poly-Ub chain type, upon the kinetics of repair proteins was also studied.

3.2 The role of the proteasome at DSBs is not limited to protein degradation

Shi *et al*. have demonstrated that proteasome inhibition causes persistent MDC1 foci coupled with loss of BRCA1 foci. To determine whether the defect in the protein clearance was due to a loss of degradation or loss of free-Ub due to inhibited Ub-processing, the effect of proteasome inhibition on MDC1 and BRCA1 was tested.

HeLa cells were plated on coverslips and transfected with Myc-Ub or Myc-LacZ, treated with MG132 for an hour before 2 Gy IR then allowed to recover for 4 hours in MG132 before fixing and staining. The transfection of cells with Myc-Ub allows the distinction between the proteolytic activity of the proteasome and the effect of Ub-starvation brought about by proteasome inhibition. As an early sensor protein of the DDR, MDC1 has been shown to be undergoing clearance from the breaks by 4 hours after damage (Shi, Ma et al. 2008). In MG132 treated cells which were transfected with Myc-LacZ, MDC1 foci were persistent at 4 hours supporting the data from Shi *et al*. Conversely, MG132-treated cells transfected with Myc-tagged Ub had reduced numbers of MDC1 foci, at levels seen in untreated cells (Fig 3.1 A and B). These results suggest that the proteasome is required for the clearance of MDC1. However since transfection with Myc-Ub can reduce MDC1 foci numbers back to untreated levels the

overexpression of Ub is sufficient to allow clearance in MG132 treated cells. Therefore it is likely to be the Ub-processing activity of the proteasome that is required for MDC1 clearance rather than proteolytic degradation.

This suggests that maintenance of the free-Ub pool by the proteasome is required for MDC1 clearance, implying the clearance of MDC1 requires formation of *de novo* Ub modifications upon damage.

The observation that MDC1 clearance requires Ub to be available in the cell supports the idea that Ub modifications are important for MDC1 removal from chromatin. To try and assess if a specific Ub-linkage type was required for this clearance, Ub mutants were transfected into cells. As poly-Ub chains form through the seven conserved lysines, mutations that change one of those lysines to an arginine (K→R) limit which types of chains can form. Three mutated Ub constructs were used, where the K63, K48 or K6 residues were converted into arginine (K63R, K48R and K6R). These were transfected into the cells before MG132 treatment and damage. Cells were fixed 4 hours post IR and stained for MDC1. Interestingly the introduction of Myc-Ub-K63R, Myc-Ub-K48R or Myc-Ub-K6R abrogated the clearance of MDC1 with Ub-Myc. The K63R mutant had the most dramatic MDC1 retention but all three mutants used had a significant increase in MDC1 foci when compared to Wild-type Myc-Ub transfection (Fig 3.1 B). So although *de novo* Ub-conjugates are required for MDC1 clearance, there did not appear to be one single lysine residue that was required for MDC1 clearance, rather several variants reduced foci loss. It is therefore possible that either mixed linkages or multiple chains are needed for MDC1 clearance.

The clearance of MDC1 was suggested to be a prerequisite to BRCA1 recruitment as proteasome inhibited cells can neither clear MDC1 nor recruit BRCA1 (Shi, Ma et al.

2008). As MDC1 clearance can be restored by Myc-Ub overexpression in cells treated with proteasome inhibitors, it was considered that BRCA1 recruitment may also be restored by Myc-Ub transfection. As with the previous experiment, cells were transfected with Myc-LacZ or Myc-Ub and treated with MG132 before being damaged with 2 Gy IR. One hour later cells were fixed and stained for BRCA1.

Unlike MDC1 foci, there was no rescue of the defect seen upon MG132 treatment. BRCA1 foci were unable to form regardless of the addition of Ub back into the cell (Fig 3.1 C). Therefore it is likely BRCA1 recruitment requires the proteolytic activity of the proteasome in order to be recruited to DSBs. Degradation of an upstream repair protein is a possibility. Although MDC1 is a candidate, the clearance by Myc-Ub would, in theory, allow partial recruitment of BRCA1 in these cells. This demonstrates that the recruitment of BRCA1 requires the proteolytic activity of the proteasome, although the target for degradation is currently unknown. However the role of the proteasome to aid repair is not solely due to its proteolytic function but also relies on the maintenance of free-Ub in the cell.

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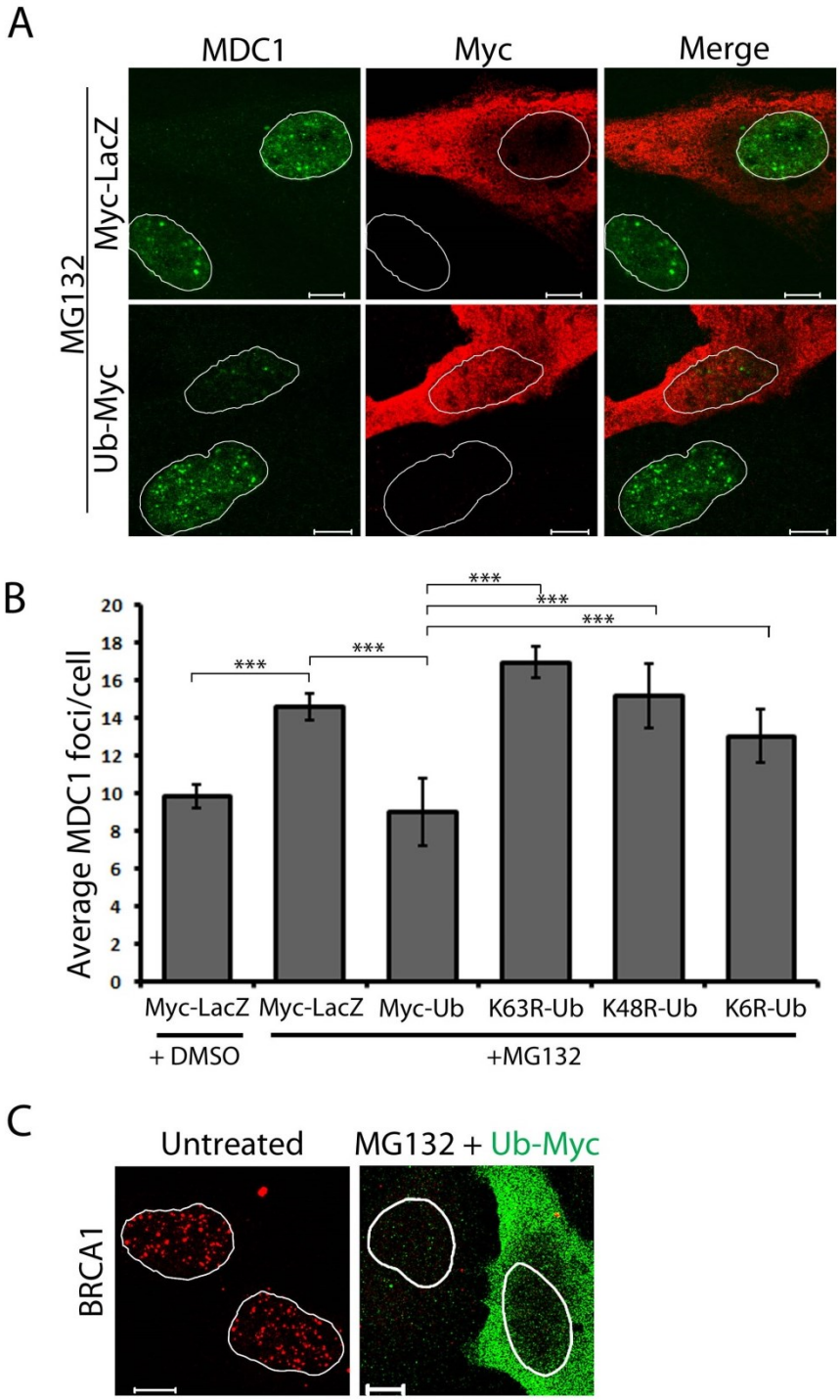


Figure 3.1. Replenishment of the free-Ub pool can partially restore MDC1 clearance but is not sufficient for BRCA1 recruitment upon proteasome inhibition. HeLa cells were transfected with Myc-LacZ or Ub-Myc for 24 hrs before MG132 treatment. Cells were left for 1 hour and treated with 2 Gy IR. **A**) Confocal microscopy images of MDC1 foci after treatment and fixed 4 hours after IR. **B**) Quantification of MDC1 foci in MG132 treated cells expressing either Myc-LacZ or Ub-Myc or Ub-Mutants, K63R, K48R and K6R.Ub-Myc transfected and treated as described (n=100, 2 repeats) *** represents significance $p < 0.001$. **C**) Confocal images depicting representative images of BRCA1 foci after treatment and fixed 1 hour after IR. White line represents DNA from Hoescht staining. Scale bar = 10µm

3.3 53BP1 recruitment can be restored by overexpressing Ub in proteasome inhibited cells

Similarly to BRCA1, 53BP1 recruitment to DNA damage sites is lost after proteasome inhibition although whether this is due to the proteolytic activity or Ub-starvation, due to loss of free-Ub pool, is not known (Jacquemont and Taniguchi 2007). Ub modifications at the break site are required for 53BP1 recruitment, although 53BP1 is not known to directly bind to poly-Ub chains. Instead the ubiquitination and subsequent degradation of JMJD2A is believed to open up the H4K20me2 mark that can therefore be bound by the Tudour domain of 53BP1 (Mallette, Mattiroli et al. 2012, Mallette and Richard 2012). Therefore whether the recruitment of 53BP1 was dependent on the proteolytic activity of the proteasome was investigated.

Cells were plated, transfected with Myc-LacZ or Myc-Ub and then treated with MG132 before being damaged with 2 Gy IR and fixed 1 hour later. Cells were stained for 53BP1 to determine how proteasome inhibition affected foci formation. As expected MG132 treatment abrogated 53BP1 foci formation. Interestingly, the recruitment of 53BP1 could be brought back to levels seen in untreated cells by the introduction of Ub-Myc (Fig 3.2 A and B). Consequently, the recruitment of 53BP1 was not dependent on the proteolytic activity of the proteasome; instead the availability of Ub was the factor inhibiting recruitment. These data suggest that the proteasome is important for 53BP1 localisation to DSBs but through its role in maintaining the free-Ub and allowing de novo Ub-modifications to form rather than the proteolytic role.

To try and distinguish if a specific linkage was involved in the recruitment of 53BP1, Ub K→R mutations were introduced into cells. The experiment was carried out as above but as

well as Myc-Ub and Myc-LacZ, K63R-Ub, K48R-Ub, K27R-Ub or K6R-Ub was also transfected into cells. The rescue of 53BP1 foci upon WT-Myc-Ub transfection was not observed when cells were transfected with K63R-Ub. Cells transfected with K63R-Ub had as few 53BP1 foci as cells transfected with Myc-LacZ, demonstrating a requirement for K63 residue in 53BP1 localisation. Intriguingly, the K6R-Ub and K27R mutants showed a partial rescue of 53BP1 foci, suggesting the K6 and K27 residues may also promote the recruitment of 53BP1 but are not the most integral residues. The K48R mutant rescued 53BP1 foci back to levels comparable with Myc-Ub (Fig 3.2 B). From these experiments it would seem that K63-linked poly-Ub chains or mixed chains, containing the K63 , K27 and K6 residues, may act to bring 53BP1 to DSBs.

This was further supported by a reciprocal experiment where Ub mutants which only contained one lysine residue, such as K63O, K48O, K6O, K27O and K63-K6O-Ub were transfected into cells. As expected the K6O, K48O and K27O-Ub mutants could not rescue 53BP1 foci as all these mutants lacked the K63 residue which has been shown to be required for 53BP1 recruitment. The K63O mutation only had a partial rescue of 53BP1 foci, suggesting K63O is not enough for the complete rescue of foci localisation. Interestingly, the K63-K6O mutant had the greatest effect on rescuing foci at DSBs and in conjunction with the data from the K63R and K6R mutations gives a strong indication that K63 and K6 linkages are important for 53BP1 recruitment (Fig 3.2 C). However, the K6-K63O-Ub transfection did not have as many 53BP1 foci as seen in cells with WT-Myc-Ub, suggesting that the K6 and K63 residues are important but not completely sufficient for 53BP1 foci formation. There may be another lysine residue required, potentially the K27 residue, or the lysine mutations may be causing an unknown defect in 53BP1 foci recruitment.

These data suggest a model where 53BP1 foci formation is not dependent on the proteolytic activity of the proteasome but instead requires the maintenance of the free-Ub pool by the proteasome in order to form poly-Ub chains, potentially of mixed K6-K63 linkages.

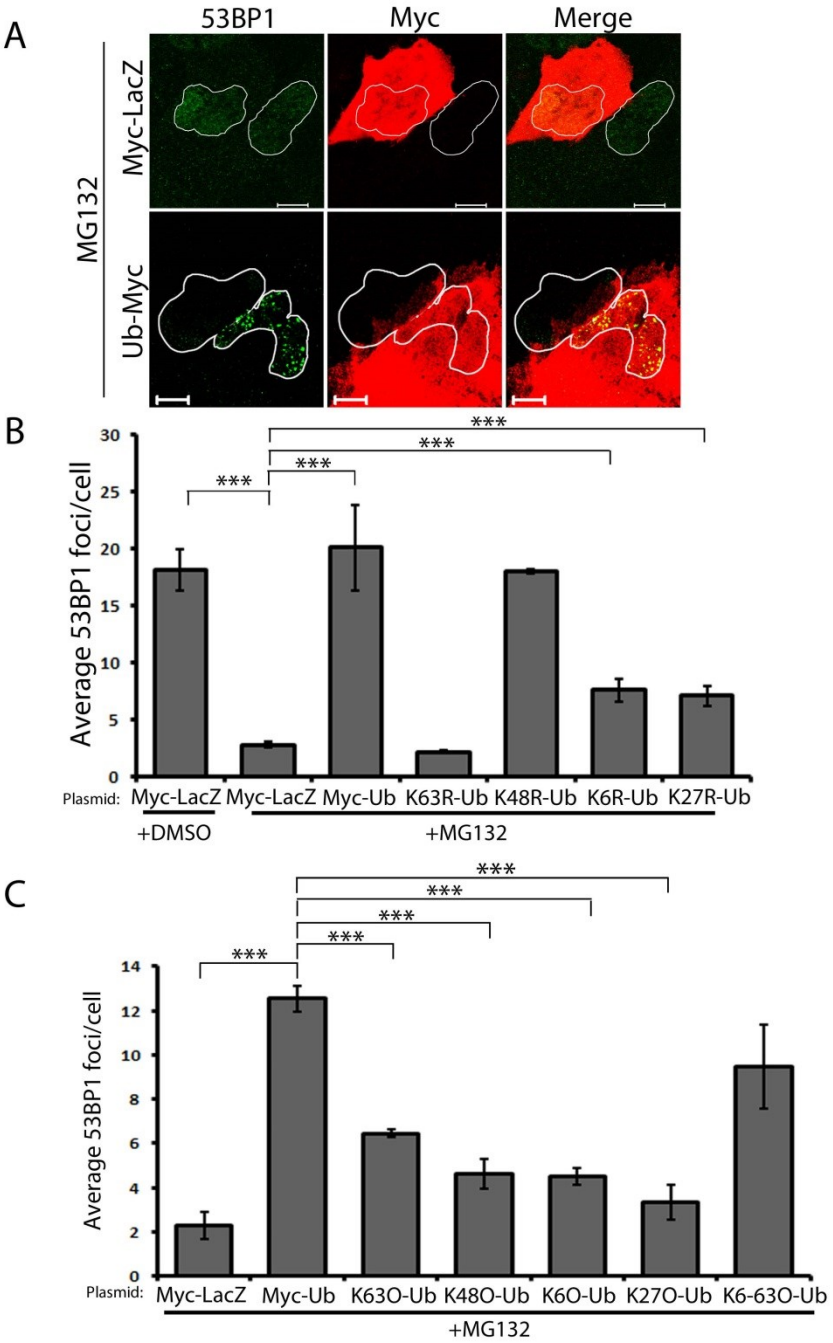


Figure 3.2 53BP1 foci recruitment requires poly-Ub chains, likely through K63 and K6 linkages. HeLa cells were plated on coverslips and transfected with Myc-LacZ and Ub-Myc. 24 hrs post transfection cells were treated with 5µM MG132 for 1 hr. Cells were subjected to 2 Gy IR allowed to recover for 1 hour, fixed in 4% PFA the stained for 53BP1 foci **A**) Confocal microscopy of 53BP1 in Myc-LacZ and Myc-Ub transfected cells **B)** Quantification of 53BP1 foci in Myc-LacZ, Myc-Ub and Ub K → R mutant transfected cells (75 cells per treatment, 3 repeats) **C**) Quantification of 53BP1 foci in Myc-LacZ, Myc-Ub and Ub mutants containing only specified lysine residues transfected cells (75 cells per treatment, 3 repeats).White line shows the outline of DNA from Hoescht staining. Scale bar = 10µM. Error bars = Standard Error. T-test show significance at $p < 0.001$.

3.4 Damage-dependent ubiquitin chains are restricted by the proteasome-associated DUB POH1

The previous experiment highlights an important role for K63-linked Ub at DSBs. RNF8 and RNF168 are known to form Ub-chains at sites of damage, with RNF168 being important for creating K63-linked chains on H2A and H2AX (Doil, Mailand et al. 2009, Stewart, Panier et al. 2009, Lok, Sy et al. 2011). However, whether the proteasome actually acts on the Ub-conjugates at DSBs is not known. The 19S lid of the proteasome contains a JAMM-type DUB POH1 which cleaves Ub chains *en bloc* from substrates, cleaving the isopeptide bond between the substrate and the proximal Ub. This protects the Ub from degradation by detaching the Ub modification before it enters the catalytic core of the proteasome or in fact saving the modified substrate from degradation by removing the signal before it is committed to degradation (Yao and Cohen 2002). Additionally, POH1 is a K63-specific DUB that can cleave the isopeptide linkages between Ub moieties of these chains rather than just between the substrate and proximal Ub (Cooper, Cutcliffe et al. 2009, Patterson-Fortin, Shao et al. 2010).

The following work was a collaborative effort carried out by members of the Morris lab. Laura Butler carried out a screen of 103 putative DUBs in the human genome, studying the levels of conjugated Ub (FK2) after release from HU, a damaging agent. Pools of 4 siRNAs against each DUB were transfected into cells plated onto 96 well plates. The cells were serum starved for 24 hours in order to synchronise the cells into the same phase of the cell cycle, damaged with 3 mM HU for 24 hours before being released into fresh media for 16 hours. Once fixed cells were probed with FK2-HRP antibody and the luminescence measured. The pool of siRNA towards POH1 significantly increased luminescence suggesting an increase of

Ub-conjugates after release from damage, thereby POH1 may be acting to restrict conjugated Ub that forms after damage (Fig 3.3 A and B).

As POH1 had an increased FK2 signal after damage as measured by increased luminescence signal, FK2 foci were visualised using IF and counter stained with γH2AX as a marker of damage. When comparing cells treated with siRNA to POH1 to a Non-targeting control (NTC) and damaged with 2 Gy IR, the cells depleted of POH1 had an increase in FK2 foci number as well as the foci being larger and brighter which co-localised with γH2AX. Unlike the small FK2 foci seen in NTC treated cells, the FK2 foci present in POH1 siRNA cells extended past the diameter of the γH2AX foci when visualised by confocal microscopy (Fig 3.3 C and D). This suggests that POH1 acts to restrict the damage-dependent Ub conjugates formed at the DSB. However as a component of the proteasome, the POH1 siRNA may be causing a defect with the proteasome which is causing the enlarged FK2 foci, rather than it being a POH1 specific defect.

Upon damage, RNF8 and RNF168 conjugate K63-linked chains at the DSB (Doil, Mailand et al. 2009, Stewart, Panier et al. 2009, Lok, Sy et al. 2011). To establish whether POH1 was acting on the K63-linked chains which form at DSBs, Joanna Morris used an antibody specific to K63-linked poly-Ub to look at foci upon POH1 knockdown by IF. U20S cells were transfected with NTC or POH1 siRNA before being exposed with 2 Gy IR, fixed and stained for K63-linked Ub. An increase in the diameter of the K63 foci can be seen in cells depleted of POH1 when compared to control cells (Fig 3.3 E and F). So, the K63-specific action of POH1 is appears to be necessary for regulation of Ub-chains formed at the sites of damage.

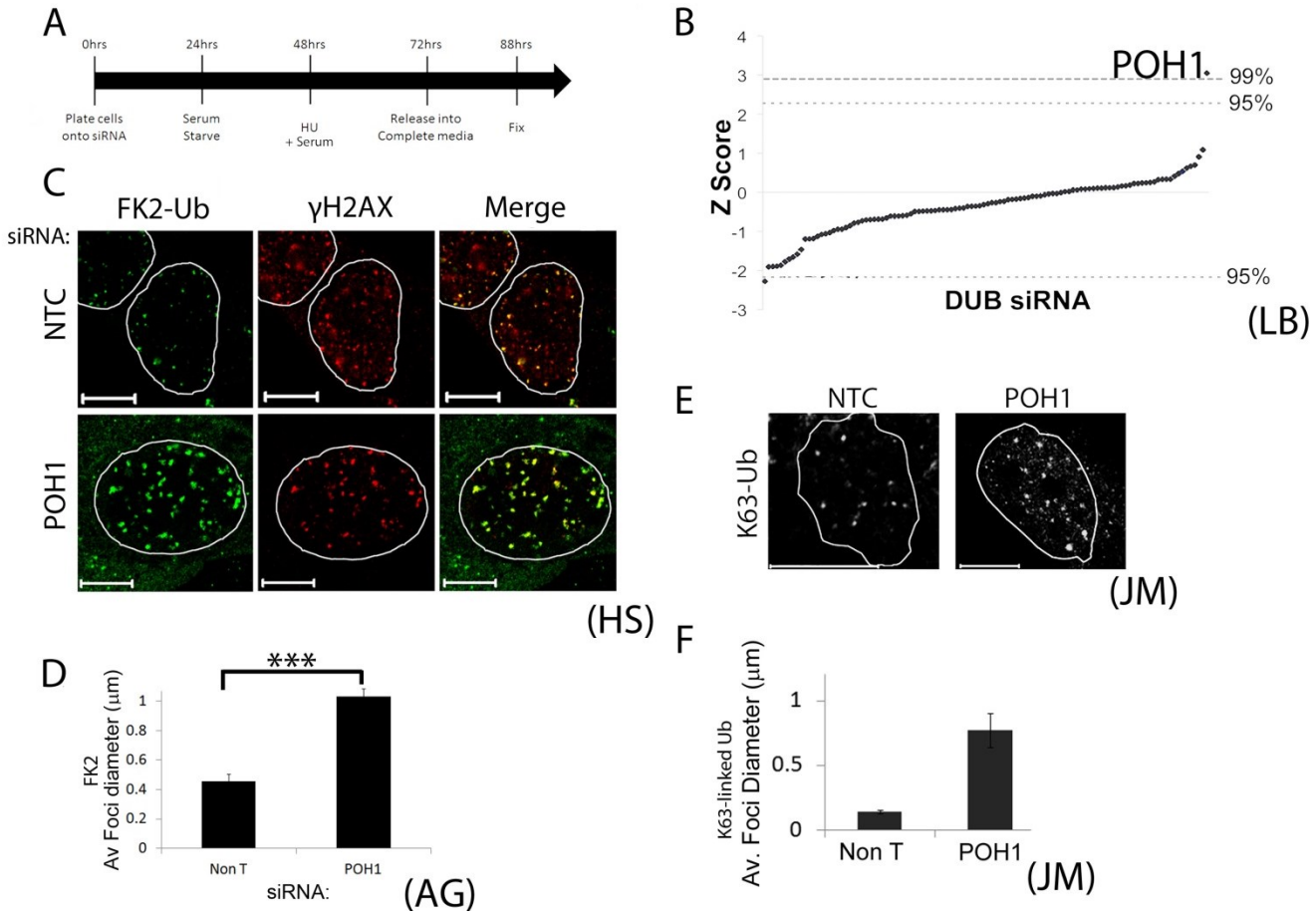


Figure 3.3 The proteasome-associated DUB POH1 increases conjugated Ub, specifically K63-linked chains at DSBs A). Schematic of the treatment course prior to measuring FK2 luminesence. **B)**. Screen 103 putative DUBs. FK2 luminesence was plotted as scatter plot of averaged Z-score from three repeats for each DUB carried out by Laura Butler (LB). **C**) Confocal images of FK2 foci after POH1 depletion after damage. U2OS cells were transfected with NTC or POH1 siRNA for 72 hrs before being subject to 2 Gy IR and fixed 1 hr later. Cells were stained for FK2 (Green) and γH2AX (Red) as a marker of damage and imaged by confocal microscopy carried out by Helen Stone (HS). **D**) Quantification of average foci diameter of FK2 foci when cells were treated with NTC or POH1 siRNA calculated with ImageJ. ***= p, 0.001 calculated by Alex Garvin. **F)** Confocal images of K63 specific foci after POH1 depletion. U2OS cells were treated with NTC or POH1 siRNA for 72 hrs, exposed to 2 Gy IR and fixed 1 hr later before staining with K63 antibody and imaged by confocal microscopy performed by Joanna Morris (JM). **E**) Quantification of K63-Ub foci size calculated using Image J.

POH1 is an active JAMM-type DUB that is a constitutive part of the 19S lid of the proteasome. In order to assess whether the constraint on damage-associated Ub-conjugates was specifically due to the catalytic DUB activity of POH1 or because of an effect of POH1 loss on the whole proteasome, Laura Butler created catalytically dead mutants. The introduction of two point mutations (H113A and H115A) within the JAMM motif rendered POH1 inactive as a DUB (termed $JAMM^{M}$) but the protein remained intact, with the mutant POH1 being incorporated into the 19S lid (Gallery, Blank et al. 2007). This incorporation of the $JAMM^{M}$ POH1 was demonstrated by expressing Flag-tagged WT-POH1 and $JAMM^{M}$ and using the Flag-tag to immunoprecipitate (IP) any proteins bound to POH1. Both WT and mutant POH1 could bind the other 19S lid component PSMD4 indicating both versions of POH1 could be incorporated into the proteasome (Fig 3.4 A).

To establish the importance of the catalytic activity of POH1 on Ub-conjugates, HeLa cells were treated for 24 hours with NTC or POH1 siRNA before transfection with either a siRNA resistant WT or the $JAMM^{M}$ version of POH1. Cells were irradiated with 2 Gy IR and fixed 1 hour later and stained with FK2 antibody. The introduction of the siRNA resistant WT POH1 plasmid caused a reduction in size of the FK2 foci, seen on POH1 depletion. Whereas cells expressing a $JAMM^{M}$ mutant version of POH1 displayed larger FK2 foci, similar to those seen on POH1 knockdown alone (Fig 3.4 B and C). This is indicative of the protease activity of POH1 acting on the Ub-conjugates.

These results demonstrate that the constituent DUB, POH1, is required to limit the amount of K63 poly-Ub chains which form when the cell senses a DSB.

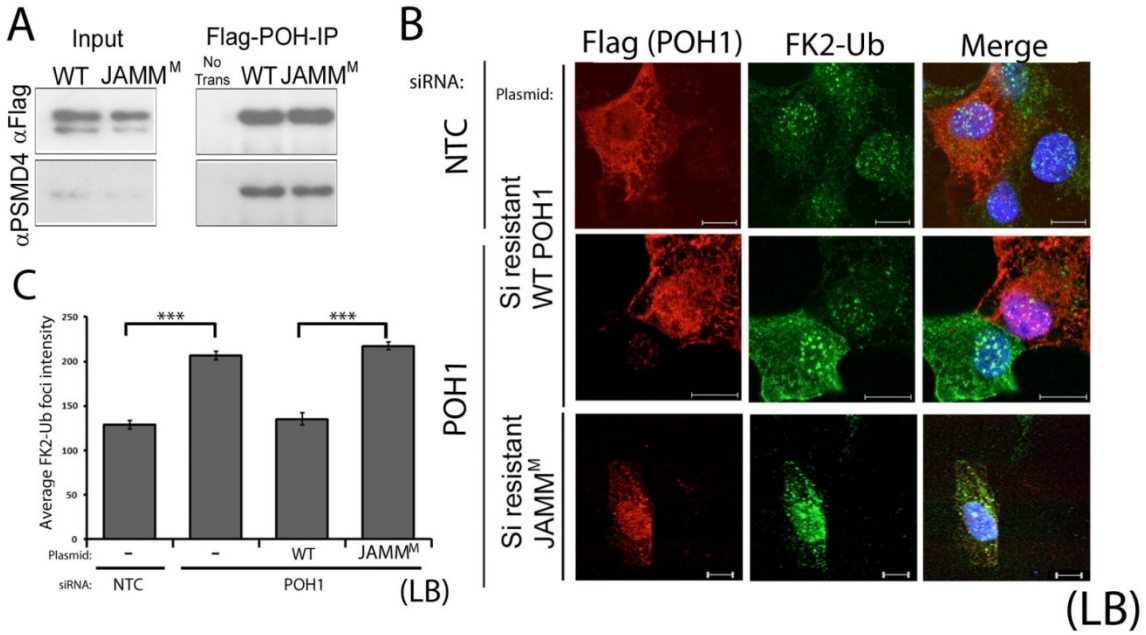


Figure 3.4 Catalytically dead POH1 cannot reduce FK2 foci size seen on POH1 knockdown. A) Flag-IP showing Flag-tagged POH1 and JAMM^M expression and associatiom with other components of the proteasome 19S lid. 293T cells were transfected with siRNA Resistant POH1 constructs and Flag-IP performed. Western blot analysis probed with anti-Flag and anti-PSMD4 antibodies **B)** FK2 confocal images upon POH1 depletion and expression of siRNA resistant version of POH1 and a catalytically dead mutant. U2OS cells were treated with NTC or POH1 siRNA then transfected with WT or JAMM^M siResistant POH1. Cells were damaged with 2 Gy IR, fixed after 1 hr and stained for Flag (Red) and FK2 (Green) and imaged by confocal microscopy carried out by Laura Butler (LB). White outlines represent DNA as seen by Hoescht staining. Scale bars = 10μM. **C)** Quantification of FK2-Ub foci intensity for cells depleted of POH1 and co-transfected with siRNA resistant WT-POH1 and JAMM^M POH1. *** = p< 0.001

3.5 POH1 restricts 53BP1 foci size

As seen in 3.3 the inhibition of the proteasome causes a loss of 53BP1 accumulation at DSBs, however the introduction of excess Myc-Ub allows the formation 53BP1 foci, with the K63 residue being necessary for foci formation (Fig 3.2 B and C). Further to this, the proteasomal DUB, POH1, has been shown to counteract the formation of K63-linked chains at sites of damage (Chapter 3 Section 3.4). Thus, the role of POH1 in controlling 53BP1 recruitment to DSBs was investigated.

Laura Butler used U20S cells transfected with NTC or POH1 siRNA for 72 hrs and stained for 53BP1 after 2 Gy IR. The cells depleted of POH1 had larger 53BP1 both in number and size much like the FK2 and K63 foci previously discussed (Fig 3.5 A-C). Two possible explanations could explain this increase in 53BP1 accumulation, either more 53BP1 is recruited to DSBs or the recruited 53BP1 is not being efficiently cleared from break sites resulting in an accumulation of the protein.

A time course looking at 53BP1 at various time points post IR was carried out by Laura Butler to determine if 53BP1 can be effectively removed in cells with reduced POH1 expression. U20S cells were transfected with NTC or POH1 siRNA before being subjected to 2 Gy IR. Cells were fixed at various time points between 0 and 24 hrs after damage and stained for 53BP1. Foci were quantified and despite the increase in number of 53BP1 foci upon POH1 depletion, the rate of foci clearance was similar between control and POH1 depleted cells (Fig 3.5 D). Efficient clearance of 53BP1 foci suggests that, rather than an accumulation of 53BP1 at DSBs, there is an increased recruitment of 53BP1 to breaks.

The enlarged 53BP1 foci suggest that, by limiting the K63-Ub at damage, POH1 restricts the amount of 53BP1 recruited to DSBs.

The increase in 53BP1 foci could be due to a number of reasons. One cause may be due to the depletion of POH1 inhibiting the proteasome, therefore there may be an increase in 53BP1 protein in the cell, resulting in the increase in size and number of 53BP1 foci after damage. This would suggest 53BP1 protein levels would be the limiting factor in recruitment to DSBs.

Another possibility is that POH1 depletion is causing a deregulation of the Ub-chains which are important in the recruitment of 53BP1, with an increase in the Ub-chains at DSBs there could be a corresponding increase in 53BP1 foci formation. This would suggest the Ub signal is the limiting factor in the recruitment of 53BP1.

To understand how POH1 was acting to limit the amount of 53BP1 binding to sites of damage, cells expressing low levels of 53BP1 were examined with and without POH1 expression. The siRNA against 53BP1 reduced but did not abolish 53BP1 protein levels. U20S cells were transfected with either NTC, POH1, 53BP1 or combined POH1 and 53BP1 siRNA. Western blot analysis showed that 53BP1 knockdown reduced the amount of 53BP1 protein in cells. Importantly it also demonstrates that POH1 depletion did not cause a gross change in 53BP1 protein expression when compared to control siRNA. Further to this, co-depletion of POH1 and 53BP1 did not restore 53BP1 protein levels as would be expected if POH1 depletion were blocking the degradation of 53BP1 and causing an increase in protein levels (Fig 3.5 E). This suggests the knockdown of POH1 is not significantly increasing the amount of 53BP1 protein in the cell and it is unlikely that a reduction in degradation of 53BP1 is responsible for the increase in foci seen upon POH1 depletion.

As 53BP1 protein levels appear unaffected by POH1 depletion, the ability of cells to form 53BP1 foci in co-depleted cells was investigated. U20S cells were transfected with either NTC, POH1, 53BP1 or combined POH1 and 53BP1 siRNA for 72 hours and then subjected

to 2 Gy IR. They were fixed after an hour and stained for 53BP1 foci. As expected POH1 depletion caused an enlargement of 53BP1 foci when compared to NTC, whereas 53BP1 siRNA significantly reduced the amount of 53BP1 foci as expected. Combined siRNA treatment of 53BP1 and POH1 caused visible 53BP1 foci despite the reduced 53BP1 protein levels, suggesting that amplified Ub-signalling may be sufficient for 53BP1 to respond to DSBs (Fig 3.5 E-G). These data support the hypothesis that the restriction of Ub-chains by POH1 is antagonising 53BP1 recruitment.

These results suggest that the POH1 DUB is tightly regulating the levels of K63 Ub-conjugates that form at damage sites. These Ub-chains are a potent signal to recruit proteins such as 53BP1, whose foci formation varies depending on the extent of these modifications.

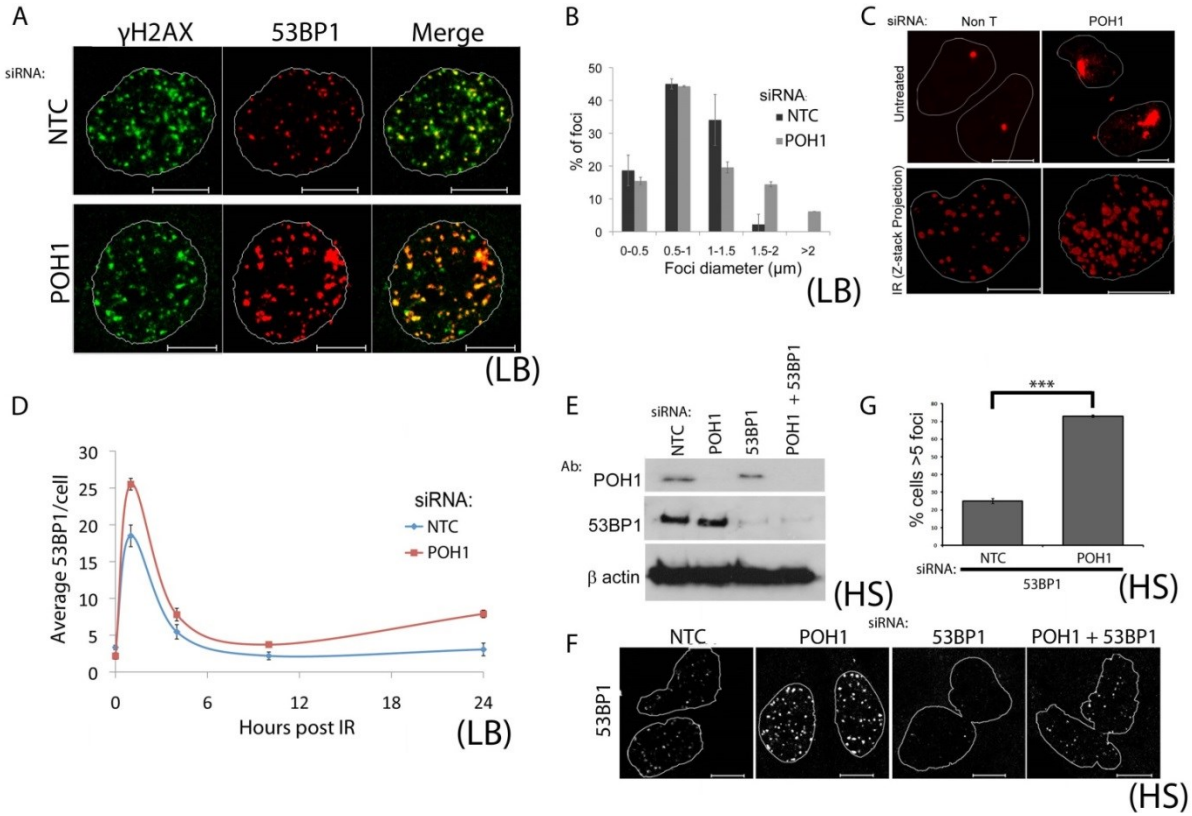


Figure 3.5 POH1 depletion increases 53BP1 recruitment to damage sites. A) Confocal images of 53BP1 (Red) and γH2AX (Green) after POH1 depletion. U2OS cells transfected with NTC or POH1 siRNA for 72 hrs before being damaged with 2Gy IR and fixed 1 hr later. **B**) Quantification ofdiameter of 53BP1 foci (n=100 foci/treatment, 2 repeats). **C)** U20S cells either untreated or treated with 2Gy IR, allowed to recover for 1 hr then fixed and stained for 53BP1. Bottom panels show Z-stack projections of 16 confocal images. **D**) Clearance of 53BP1 foci after POH1 depletion. U2OS cells transfected with NTC or POH1 siRNA, damaged with 2 Gy IR and fixed at various time points and stained for 53BP1 and average number of foci quantified at each time point (n=50 cells/time point, 3 repeats). **E**) Western blot demonstrating knockdown of POH1, 53BP1 and combined POH1 and 53BP1. **F**) Confocal images of 53BP1 foci after POH1 53BP1 and combined siRNA treatment. Cells treated as in E, damaged with 2 Gy IR, fixed 1 hr later and stained for 53BP1 **G**) Quantification of average 53BP1 foci in 53BP1 depleted and combined 53BP1 and POH1 depleted cells (>5 foci/cell n =100). White line shows DNA as stained by Hoescht. Scale bar = 10 μM.

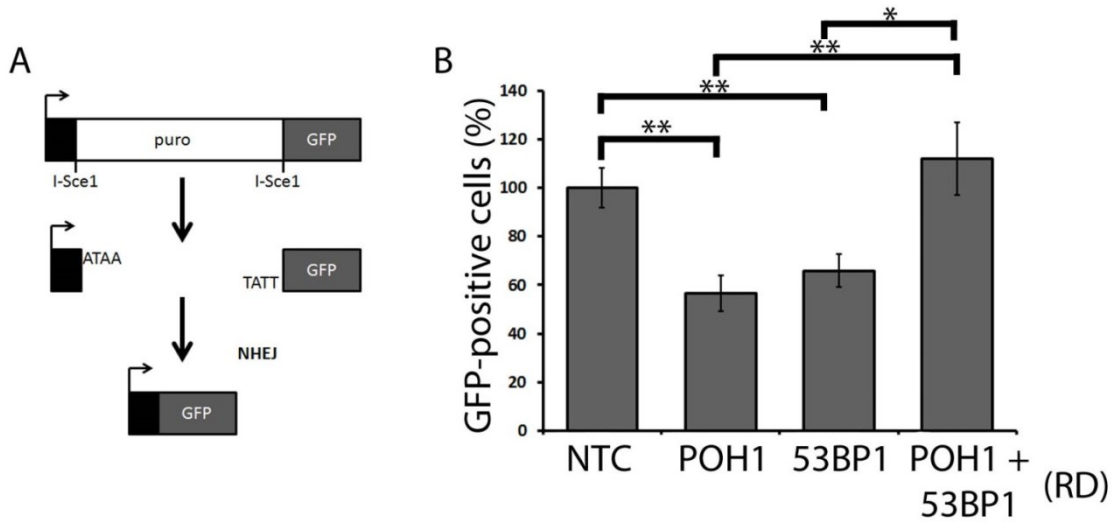
3.6 POH1 regulates NHEJ repair through 53BP1 recruitment

53BP1 promotes the repair of DNA breaks through NHEJ and opposes HR, although the exact mechanism by which 53BP1 stimulates NHEJ is not elucidated (Bothmer, Robbiani et al. 2011). As previously demonstrated, 53BP1 recruitment is increased upon POH1 depletion; therefore it is plausible that NHEJ may also be affected by POH1 knockdown. In order to determine whether NHEJ repair was affected by POH1 depletion an integrated reporter assay was utilised by Ruth Densham. HeLa cells containing an EJ5-GFP reporter, where GFP is only expressed if NHEJ is functional (Fig 3.6 A) (Bennardo, Cheng et al. 2008), were treated with NTC, POH1, 53BP1 or a combination of POH1 and 53BP1 siRNA. After cells had been incubated with siRNA for 24hrs they were transfected with the *iSce-I* to induce a DSB and an RFP- transfection control.

Unsurprisingly, reduction of 53BP1 protein caused a decrease in functional NHEJ. However, the fault in NHEJ can be recovered by the codepletion of 53BP1 with POH1. These data are consistent with the foci analysis, in which 53BP1 foci could be induced by POH1 depletion even in cells with low 53BP1 protein levels. Intriguingly, POH1 knockdown alone also caused a decrease in NHEJ, suggesting either that the excess 53BP1 in these foci is having an inhibitory effect on NHEJ or POH1 is regulating another factor that is required for NHEJ repair. Since rescue of NHEJ occurs after codepletion of POH1 and 53BP1, the reduction in NHEJ repair upon POH1 siRNA appears to be due to the increased 53BP1 at DSBs (Fig 3.6 B). Therefore the increased 53BP1 protein at DSBs may be acting to block NHEJ, perhaps by stopping access of another protein required for NHEJ to the break sites.

Ub-conjugates are important for the recruitment of 53BP1. The K63-linked chains are limited by the proteasome associated DUB POH1, which in turn restricts the amount of 53BP1 that is

recruited to breaks. By regulating the amount of 53BP1 at breaks, POH1 maintains the correct balance of repair proteins to allow efficient NHEJ to occur.



3.6 POH1 regulates NHEJ repair by limiting the recruitment of 53BP1 to DSBs. A) Schematic of the EJ5-GFP reporter assay. HeLa cells containing a GFP gene interrupted by a puromycin resistance gene inserted, stopping GFP expression. Upon transfection of *Sce1* the puromycin gene is removed and if NHEJ is proficient the GFP gene is repaired and cells express GFP. **B**) Quantification of GFP and RFP positive cells in NTC, POH1, 53BP1 or 53BP1 and POH1 depleted cells expressed as percent of NTC performed by Ruth Densham (mean of three replicates) Significance determined by t-test * = p,0.05, **= p,0.01.

3.7 Summary

Ub conjugates are a well-established signalling mechanism within DSB repair, however the DUBs that are counteracting these chains are still being revealed. The proteasome has many roles within the cell, including degrading proteins (reviewed in Adams 2003). This chapter demonstrates two distinct roles of the proteasome at DSBs.

Firstly, the proteolytic activity of the proteasome, or other proteases affected by MG132, is required for the recruitment of BRCA1 to DSBs, as proteasome inhibition causes loss of BRCA1 foci. This defect cannot be rectified by the introduction of Ub into these cells, suggesting BRCA1 recruitment involves the degradation of another substrate (Fig 3.1C).

Secondly, the Ub-processing is important for MDC1 clearance and 53BP1 recruitment. The Ub-starvation caused by proteasome inhibition, caused persistent MDC1 foci and loss of 53BP1 foci (Eytan, Armon et al. 1993, Hanna, Leggett et al. 2003). However, rescue of these foci kinetics was observed when excess Ub was introduced into the cells showing the importance of *de novo* Ub-modifications after damage (Fig 3.1 A and B, Figure 3.2). The loss of new Ub-chains does not directly implicate the proteasome for Ub processing at DSBs, but rather the Ub starvation upon proteasome inhibition is causing foci kinetic defects (Eytan, Armon et al. 1993) (Fig 3.7). In an unperturbed cell the proteasome is constantly replenishing the free Ub-pool, therefore upon damage *de novo* Ub modifications can form around the break site. This causes the clearance of MDC1 and allows the recruitment of 53BP1 and BRCA1 to allow repair to occur (Fig 3.7 A). In cells that have an inhibited proteasome these damage-dependent Ub modifications cannot be formed, subsequently causing persistent MDC1 at damage and blocking the recruitment of 53BP1. The loss of proteolytic activity also blocks BRCA1 from being recruited to DSBs (Fig 3.7 B)

However, immunofluorescent staining has shown the localisation of the proteasome at DSBs in a Ub-dependent manner, suggesting the proteasome does have a role directly at the break site (Appendix Fig. A1 and A2).

The use of Ub-mutants gives an indication of which lysines residues are likely to be involved in the foci kinetics of MDC1 and 53BP1. There was not one lysines residue within Ub which was obviously required for MDC1 clearance, with all Ub-mutants introduced causing persistent MDC1 foci. This could be due to MDC1 clearance requiring mixed poly-Ub chains or multiple distinct Ub-modifications, the introduction of a Ub lacking all lysines could be used to determine if mono-Ub is required for MDC1 clearance. However the K63 and to some extent to K6 and K27 residues appear to be necessary for 53BP1 recruitment (Fig 3.1 B, Fig 3.2 B and C). The use of the Ub-mutants can only give an idea of the linkage type as the introduction of an arginine residue may affect the interactions with Ub-binding domains which can cause unknown affects to the foci kinetics.

The results presented in this chapter suggest that K27-linked Ub may have a minor role in the recruitment of 53BP1 foci. This is in partial agreement with Gatti *et al,* however, we observe only a mild defect in 53BP1 recruitment when cells are transfected with K27R (Gatti, Pinato et al. 2012). Further to this cells, complemented with K27O Ub cannot efficiently restore 53BP1 foci numbers. Instead, we find that K63 is the major residue required for 53BP1 recruitment, but there is a minor requirement for both K6 and K27 residues.

Furthermore, other groups have suggested that 53BP1 recruitment involves K48-linked Ub in order to signal removal of chromatin bound factors by VCP (Acs, Luijsterburg et al. 2011, Meerang, Ritz et al. 2011). However, the data in this chapter shows no requirement for the K48 residue of Ub for 53BP1 foci formation. Although K48-Ub does form at sites of damage

there is no evidence presented to show a direct requirement of K48 for 53BP1 recruitment. Further to this, VCP depletion does reduce the ability of cells to form 53BP1 foci but it does not complete ablate foci formation (Acs, Luijsterburg et al. 2011, Meerang, Ritz et al. 2011). It is therefore likely that there are many mechanisms that are both direct and indirect which regulate 53BP1 localisation to DSBs; however the data presented suggest a strong reliance of K63-linked Ub as opposed to K48 poly-Ub.

Due to the defect of foci kinetics on MG132 treatment, it was assumed that proteasome inhibition was working. Western blot analysis was performed to confirm proteasome inhibition (Butler, Densham et al. 2012); this correlates with the results observed matching published results of proteasome inhibition on foci kinetics (Jacquemont and Taniguchi 2007, Shi, Lin et al. 2007).

The role of the proteasome and its associated DUB, POH1, at DSBs was also investigated during this chapter, demonstrating a role of the proteasome DUB POH1 in Ub processing at break sites. Increased Ub-conjugates are apparent both by luminescence and IF upon POH1 knockdown by RNA interference. These conjugates co-localised with the damage marker γH2AX and were additionally shown to be K63-linked chains, a chain type known to be created at DSBs by RNF8/RNF168 (Fig 3.3). POH1 was shown to limit the extent of these conjugates at DSBs, actively limiting 53BP1 foci formation.

Loss of POH1 caused an escalation in size and intensity of 53BP1 foci (comparable to the increase in FK2 foci size upon POH1 depletion) which could be rescued by knockdown of 53BP1 itself. The low level of 53BP1 expression in knockdown cells was sufficient to form foci in POH1 depleted cells but not in POH1 competent cells (Fig 3.5). Taken together these

results indicate that POH1 acts to restrict K63-chains at damage and in doing so controls the recruitment 53BP1.

The importance of this stems from the role of 53BP1 in promoting NHEJ. Cells with decreased 53BP1 protein levels have a defect in this repair pathway which can be restored by POH1 depletion. This may be due to 53BP1 being recruited to breaks. Interestingly, POH1 depletion alone also has defective NHEJ which correlates with excess 53BP1 at breaks also having a deleterious effect on repair (Fig 3.6). This might be due to excess 53BP1 binding to DNA ends and blocking other NHEJ factors, such as Artemis or XLF:XRCC4, from accessing the broken DNA ends. Immunofluorescent analysis could be performed to determine whether other NHEJ factors are excluded from break sites in POH1 depleted cells and whether this could be restored by 53BP1 knockdown. However, this chapter demonstrates that the modulation of Ub-conjugates at DSBs by POH1 creates a fine balance for recruitment of repair proteins to allow efficient repair.

As a component of the proteasome, it may be expected that POH1 depletion would cause similar repair defects as proteasome inhibition. However, proteasome inhibition had more severe phenotype than POH1 depletion, which could be explained by the amount of free-Ub in the cell. Proteasome inhibition caused a decrease in free-Ub in the pool whereas POH1 depletion did not reduce the free-Ub, therefore POH1 knockdown does not inhibit the formation of *de novo* Ub modifications (Liu, Buus et al. 2009).

This chapter therefore demonstrates a role for K63-chains in recruiting 53BP1 foci, however 53BP1 is not known to bind K63-linked chains, suggesting the recruitment of 53BP1 is not directly through the interaction of 53BP1 with the Ub-chains. The current hypothesised model is that POH1 is likely regulating another substrate which ultimately allows 53BP1 foci

formation. By limiting the K63-linked chains in the vicinity of the break, POH1 causes the ejection of chromatin factors around the DSB. By removing these chromatin factors, 53BP1 can then access these histone modifications and be recruited to the damage. However when POH1 is not present the K63-signal is amplified causing a subsequent increase in 53BP1 recruitment (Fig 3.8). This chapter shows that the proteasome has diverse functions at the DNA break with Ub regulation via POH1 being integral to 53BP1 recruitment and consequently NHEJ repair. Further insights into the mechanism of POH1 Ub-chain regulation and how this regulated DSB repair, has since been published (Butler, Densham et al. 2012)

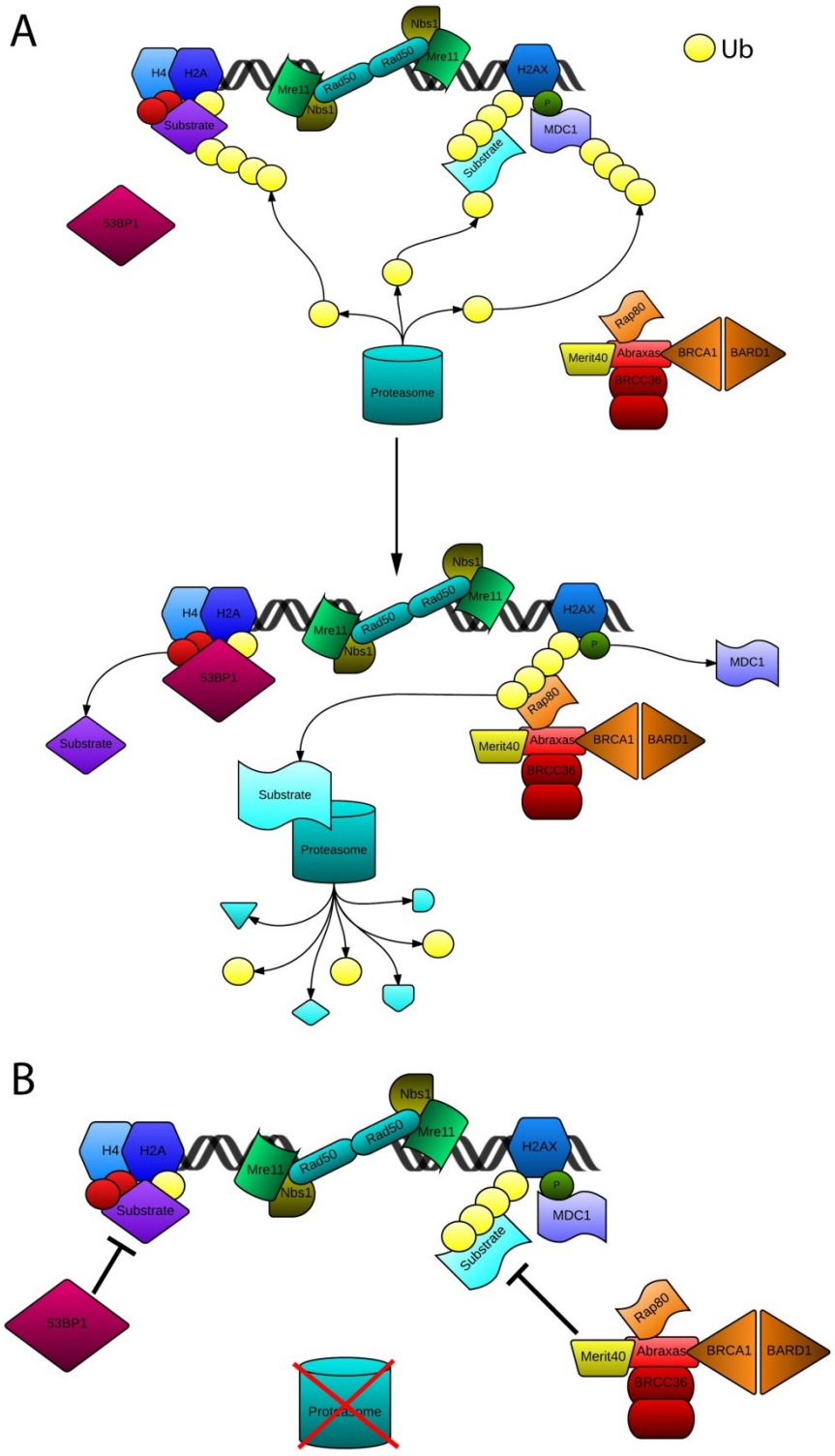


Figure 3.7 Model of how the proteasome Ub-processing regulates repair proteins. A) The proteasome is required to degrade an unknown substrate in order to allow BRCA1 recruitment. MDC1 protein clearance and 53BP1 protein recruitment require free-Ub produced by the proteasome. Suggesting damage-dependent modifications are required for correct foci kinetics. **B)** Proteasome inhibition inhibits the clearance of MDC1 and recruitment of 53BP1 due to loss of Ub-modifications. BRCA1 recruitment is blocked by the loss of degradation of a substrate.

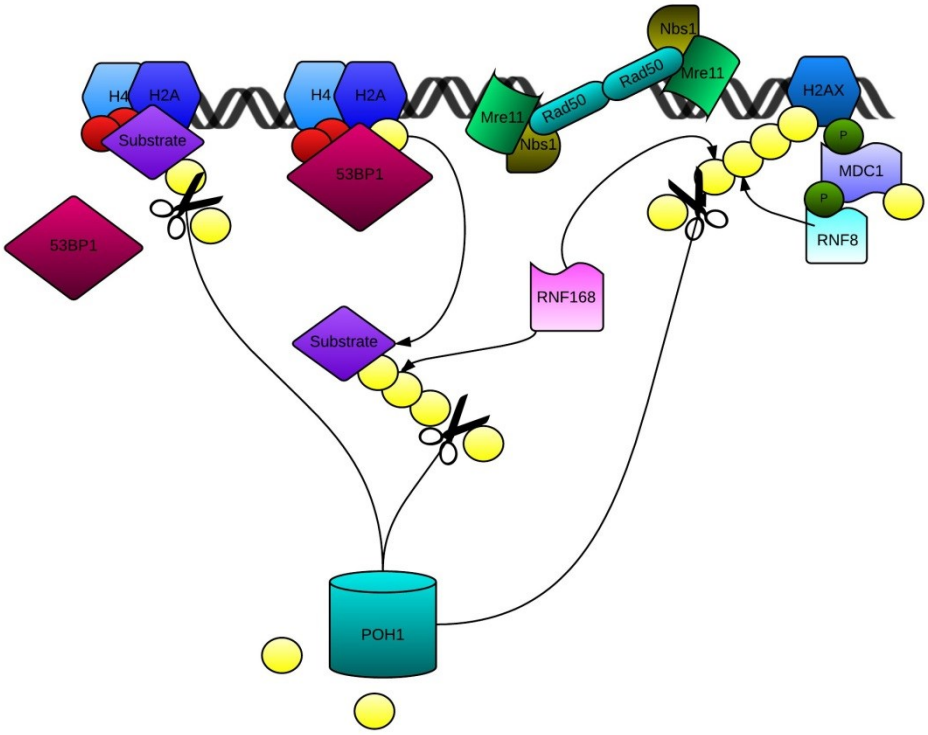


Figure 3.8 Model of POH1 actions at DSBs. POH1 is acting on the K63-linked Ub formed at DSBs, by reducing the poly-Ub modification on a substrate. It is maintaining the unknown substrate and blocking 53BP1 recruitment to chromatin marks. When the signal is increased, such as in close proximity to breaks POH1 is not sufficient to stop the substrate from being removed from chromatin and 53BP1 can bind the histone marks (red circles).

4. USP50 is an inactive DUB, whose depletion does not cause a major defect in the repair of double strand breaks

4.1 Introduction

Further to investigating POH1, the screen of 103 putative DUBs carried out by Laura Butler (Chapter 3 Fig 3.7 B) identified another DUB of interest, USP50. In contrast to POH1 siRNA, treatment with USP50 siRNA, cells showed significantly decreased FK2 luminesence, a measure of conjugated ubiquitin, after release from HU. It is unexpected that depletion of a DUB would cause a decrease in the amount of conjugated-Ub, therefore the role that USP50 plays after release from HU was investigated.

USP50 has been classified as an inactive DUB. As a UCH type DUB, USP50 requires the cysteine catalytic triad in order to cleave the isopeptide bond between Ub moieties or Ub and the substrate. However, the catalytic triad in USP50 is lacking the last aspartic acid/asparagine residue required for activity and therefore believed to be inactive, although no data was shown to prove the loss of activity (Quesada, Díaz-Perales et al. 2004).

Very little is currently known about the function of USP50 in the cell. One paper by Aressy *et al*. found that USP50 interacts with the heat shock protein, Hsp90 with this interaction causing a stabilisation of Wee1 by preventing degradation of Wee1 by the proteasome. They showed the reduction in Wee1, caused by USP50 depletion, increased G2/M checkpoint bypass. However this checkpoint bypass only occurred when the G2/M checkpoint was already compromised by overexpression of the checkpoint phosphatase Cdc25B, with USP50 depletion exacerbating this defect in the checkpoint. However, when the checkpoint was intact, with endogenous levels of Cdc25B expression, the loss of USP50 did not cause aberrant entry into mitosis (Aressy, Jullien et al. 2010). Therefore USP50 may play a role as a

back-up regulator to the G2/M checkpoint but it is unknown whether this could be the cause of decreased conjugated Ub.

The loss of enzymatic activity may account for the decreased level of Ub-conjugates after release from HU, when cells are treated with USP50 siRNA. However this requires further investigation.

As not much research has been carried out into the function of USP50 protein it was an intriguing prospect to characterise this DUB and its potential role in the DNA damage response.

4.2 USP50 probably arose due to a gene duplication of USP8

As USP50 is not very well characterised, initial investigation was performed by bioinformatic research to determine if USP50 was linked to other genes with known roles.

There have been several studies looking at the evolutionary relationship between DUBs; however the positioning of the DUBs on the evolutionary tree varies between studies. USP50 has been shown to share different common ancestors in separate studies, with USP50 being shown to be closely related to USP8 or USP39 (Ye, Scheel et al. 2009, Clague, Barsukov et al. 2013).

To determine which DUB USP50 was most related to bioinformatics research was performed. The Ensembl database showed that, in humans, USP50 lies on chromosome 15 at position q21.1. The protein comprises of a short N-terminal sequence that does not contain any currently classified domains, followed by the UCH domain, which is the only annotated domain on the Ensembl database. The active UCH DUB, USP8, which is highly conserved back to *Saccharomyces cerevisiae* with a homologue Doa4, lies next to USP50 on

chromosome 15. USP50 and USP8 have a small overlap between the C-terminal regions of both genes on the human chromosome (Fig 4.1A). The close proximity of USP50 and USP8 could be indicative of them being closely related; however as gene proximity does not necessarily equate to relatedness further research was required.

Bioinformatic studies that was performed showed that at the amino acid level there are 54% of amino acids which are either conserved, or retain the same properties as the original amino acid, between USP50 and the C-terminus of USP8 (Appendix Fig. A3). However USP8 is much larger than USP50, at 1118 amino acids (aa) to compared to 339 aa for USP50. USP8 contains an N-terminal region and a further C-terminal extension of 39 amino acids (referred to as the tail), which contains the final amino acid of the catalytic triad, not present in USP50. Along with the proximity of the two genes, the high sequence similarity at the amino acid level led to the hypothesis that USP50 arose due to a gene duplication of the C-terminal portion of USP8. The human DNA sequences of USP50 and USP8 have diverged more than the amino acid sequences. This may suggest that while the DNA sequence can still change there is a constraint on the DNA sequence that is maintaining the amino acid sequence.

To determine the structure of USP50, the known structure of the catalytic domain of USP8 (PDB 3N3K) was used to predict the structure of USP50, using the Swiss Model Workspace Suite, due to their sequence similarity. The USP50 amino acid sequence was threaded onto the structure of the catalytic domain of USP8 to produce a model of the USP50 structure (Fig 4.1 B and C). Although the structures of USP50 and USP8 were very similar there were some distinct differences, including two β-sheets which are visible in USP8 but not present in USP50. Further to the missing β-sheets, the model of USP8 has a loop between two α-helices as marked in Fig 4.1D. This loop is missing in the USP50 structure based on sequence data from Ensembl, however whilst sequencing USP50 plasmids, a stretch of six amino acids that

correspond to the loop that is observed within USP8, therefore the structure of USP50 may be more similar to that of USP8 than previously realised (Fig 4.1 D).

The Swiss PDB model shows the C-terminal tail of USP8 threads back into the active site to position the Aspartic acid residue into the proximity of the Cysteine and Histidine in the catalytic triad. This region is lacking in USP50, therefore the Aspartic acid of the catalytic triad is not complete; however the active site is more open in USP50 as it is not restricted by the tail region, as in USP8 (Fig 4.1 E).

However this structure is only a model based on the amino acid sequence, and is therefore only an approximation of the structure. To fully elucidate the structure of USP50, in depth experimental evidence would be required, including NMR or determining the crystal structure by X-ray cystallography.

The conservation of the amino acid sequence, as well as the proximity of the USP50 to the C-terminus of USP8, supports the hypothesis that USP50 arose from a gene duplication of USP8. However examination of the threaded structure suggests possible similarities and differences between USP8 and USP50 which may affect their function in the cell.

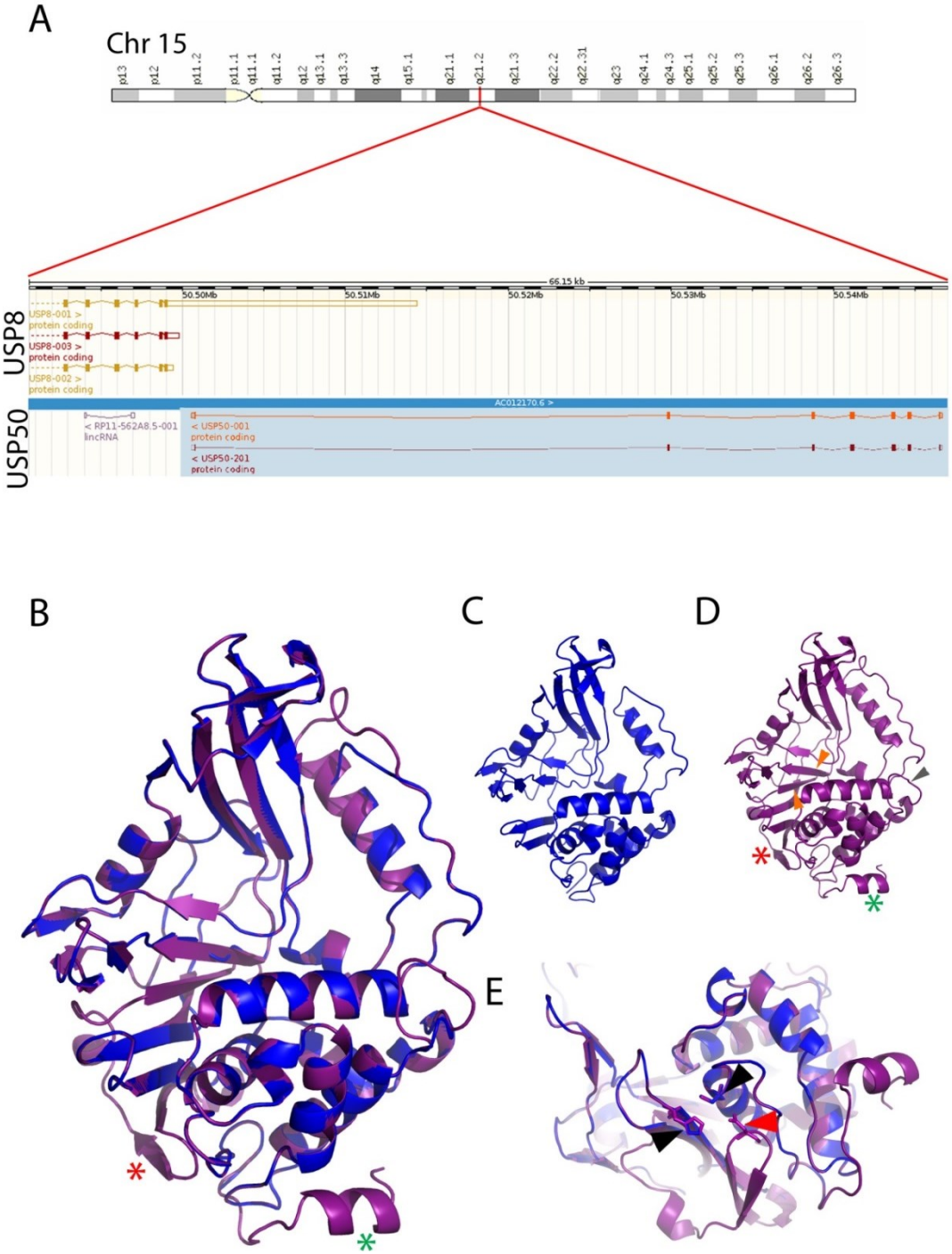


Fig 4.1 USP50 is evolutionarily related to USP8. **A)** Ensembl database map of USP50 on chromosome 15q21.1 positioned next to USP8. **B)** Ribbon model of USP50 (blue) threaded onto USP8 catalytic domain (3N3K) (purple). USP8 retains a C-terminal tail that is truncated in USP50. Green asterisk is part of the USP8 N-terminal extension. Red asterisk represents the USP8 tail. **C)** Ribbon structure of USP50. **D)** Ribbon structure of USP8 with structural differences of USP50 marked by red asterisk. Orange arrow heads represent β-sheet structural differences. Grey arrowhead represents loop that although not present in USP50 structure was represented in sequencing data. **E)** Catalytic triad of USP8. The Cysteine and Histidine residues that are present in both USP50 and USP8 are marked by black arrow heads, the Aspartic acid (red arrowhead) is a present in USP8.

4.3 USP50 is conserved across higher eukaryotic species, with a conserved Ub-binding domain

The Ensembl gene database indicates that USP50 is present in 51 species, with the earliest species known to have USP50 being fish, including Coelacanths and Platyfish. There is currently no known homologue of USP50 in *S. cerevisiae* or lower eukaryotes. Across the 40 species where the sequence is known, USP50 has a conservation of around 70% at the amino acid level, showing high conservation of most amino acids across the majority of the species (Appendix Fig. A4a). However, not all the species have retained the exact same amino acid in a particular position but may instead have an amino acid with similar properties. This high level of conservation suggests that USP50 is being maintained and protected from mutation across species. Therefore USP50 might have an important role within the cell that requires the conservation of the protein.

Although there is a high conservation of USP50 as a whole, the individual amino acids may differ in a small number of species meaning they are not 100% conserved throughout all the species included (Appendix Fig. A4b). The conservation of the individual amino acids was studied to try and identify highly conserved regions of the protein. By looking at which amino acids remain completely conserved across all 40 species it was possible to see if certain regions of the protein are more likely to be functionally important, as these residues are likely to be protected from changes throughout evolution.

A model of USP50 interacting with Ub was created in the Swiss Model Workspace Suite. To create this model, the amino acid sequence of USP50 was threaded onto the structure of USP7 bound to Ub (PDB 1NBF). This gave an estimate of the Ub-binding pocket within USP50 based on a known structure. On this model the positions of the 100% conserved residues were

mapped, these residues appeared to be in positions which are likely to interact with Ub (Fig 4.2 A and B). This suggests that the Ub-binding capacity of USP50 might be conserved. Further investigation is required to determine if these residues are required for Ub-binding.

To assess whether USP50 can bind Ub, His-USP50 protein was produced to carry out *in vitro* Ub-binding assays. Mutations were introduced using site-directed mutagenesis in regions which were hypothesised to inhibit binding to Ub. Mutations of the N240 and N241 to Aspartic acid (D) residues were believed to affect Ub-binding due to their position on the UPS50 model (Fig 4.2 C). Wild-type (WT) and mutant USP50 (ND) were cloned into the bacterial expression vector pET28a, which introduced a poly-His tag onto USP50 allowing the attachment to the Nickel (Ni^{+}) beads. The immobilised proteins were incubated on ice with K48-linked poly-Ub chains to allow binding to the USP50 proteins. Any unbound protein was washed away so that only Ub bound to the USP50 protein remained. The USP50 proteins were resuspended into 4x Lamelli buffer to denature the bonds between USP50 and any bound Ub. The amount of bound Ub was then analysed on a Western blot and probed for Ub (P4D1).

WT-USP50 was able to bind Ub, indicating that USP50 is able to bind K48-linked Ub chains *in vitro*. Interestingly, WT-USP50 appeared to only bind chains that are four Ub moieties or longer (Fig 4.2 D). The ND mutation partially restricts Ub-binding domain, with a reduced level of Ub binding to the mutated USP50 (Fig 4.2 D). As Ub-binding is not completely abolished it could suggest the N240.241 residues are involved in Ub-binding but are not essential for binding, therefore Ub can still bind but with less efficiency. Another potential cause is that the mutation from an N to a D does not cause a dramatic change in the properties of the amino acid therefore the mutant is still able to bind to Ub but with reduced efficiency. The change of residue from an N to a D does change a small polar residue into a larger

charged residue therefore they do possess different attributes which may be acting to reduce Ub-binding.

The use of *in vitro* studies to test Ub-binding gives an initial indication that USP50 is able to bind Ub; however *in vitro* assays do not fully recapitulate the role of the protein *in vivo*. Therefore to determine if USP50 could bind Ub *in vivo,* overexpression of a WT Flag-tagged USP50 in an inducible HeLa cell system, allowed the Ub-binding activity of USP50 to be tested *in vivo* (Fig 4.2 E). Treatment of these cells with 2µg/ml Dox which caused Flag-USP50 overexpression; cells were then lysed and incubated with M2 Flag agarose beads to selectively bind the Flag-tagged USP50. Any proteins interacting with USP50 would be bound indirectly through USP50. Cell lysates were probed by Western Blot for Ub (P4D1) to determine if there was enrichment for Ub in cells overexpressing USP50. In Dox treated cells, where USP50 was overexpressed, there was an increase in Ub-conjugates pulled down from cells. USP50 can therefore bind to Ub-conjugates *in vivo* (Fig 4.2 E).

Unexpectedly, it was also apparent that in whole cell extract (WCE) of Dox treated cells, which overexpressed USP50-Flag, there was an increase in high molecular weight Ub-chains (Fig 4.2 E). This could indicate that USP50 overexpression is stopping the deconjugation of these chains, potentially by blocking the chains from the proteasome and other DUBs from accessing the poly-Ub. However further work is required to confirm that the increase in Ub-conjugates observed was due to USP50 overexpression and not due to the Flag expression or Dox treatment to the cells.

The conservation of residues predicted to interact with Ub based on the USP50-Ub threaded model suggested that the role of USP50 in cells may require Ub-binding. Although USP50 is capable of binding Ub both *in vitro* and *in vivo,* whether USP50 only binds specific Ub-

linkages need further investigation. Studying endogenous USP50 Ub-binding may help identify the ubiquitinated substrate that USP50 is indirectly binding.

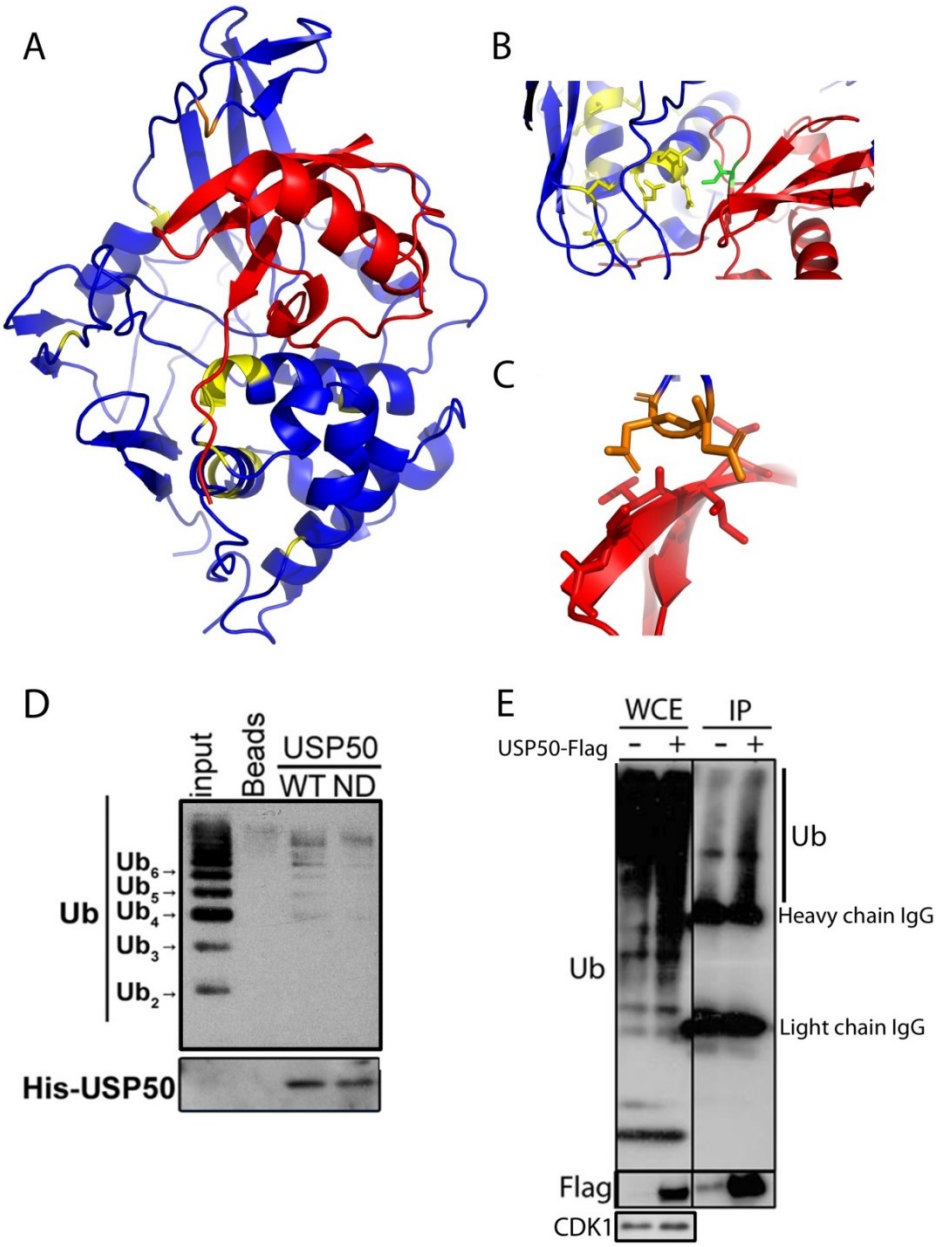


Fig 4.2 USP50 can bind Ub. A) Pymol USP50 ribbon model (blue) bound to Ub (red) as based on the USP7 bound to Ub model (PDB 1NBF), with residues conserved across 42 species marked it yellow and binding mutant residues in orange as in C). **B)** Conserved residues (yellow) in USP50 appear to interact with Ub around the Ile44 (green) interacting patch of Ub. **C)** Position of mutated residues N240-N241 (orange) which were mutated to Aspartic acid residues (D) to create a potential Ub-binding mutant (ND-USP50) **D)** *In vitro* Ub-binding assay showing WT-USP50 is able to bind K48-linked chains whereas the ND-USP50 mutant was less able to bind chains, especially of lower molecular weights, despite similar levels of both WT and ND-USP50 being present in the assays. **E)** Immunoprecipitation of Flag-tagged WT-USP50 from HeLa cells showing an enrichment of higher molecular weight Ub-chains in cells overexpressing USP50. WCE = Whole cell extract.

4.4 USP50 does not show *in vitro* DUB activity but protects Ub-chains from cleavage

As USP50 has Ub-binding capability it was investigated whether USP50 was also active as a DUB. Quesada *et al*. previously reported that USP50 displayed no protease activity; however they did not show the data to support this claim (Quesada, Díaz-Perales et al. 2004). The loss of the aspartic acid residue of the catalytic triad would imply that USP50 is no longer active as a protease. Although the aspartic acid/asparagine residue is not always necessary for protease activity, as seen with USP16 and USP30 (Nijman, Luna-Vargas et al. 2005).

The DUB activity of USP50 was assessed using purified protein, isolated from BL21 (DE3) bacteria (Avvakumov, Walker et al. 2006). Increasing concentrations of USP50 protein was incubated with linear-linked poly-Ub_{2-8} at 28°C overnight constantly shaking. USP8 protein was used as a control for positive DUB activity. Western blots were performed to determine whether the linear Ub-linkages had been cleaved into smaller moieties.

While reactions containing USP8 showed a reduction of larger Ub-conjugates and the production of mono-Ub with increasing concentrations of USP8 protein, USP50 reactions displayed no change in Ub-conjugates despite the amount of USP50 introduced (Fig 4.3 A). Therefore, although USP50 can still bind Ub-chains, as shown in Section 4.3, this data suggests it cannot cleave them. However this was only tested with linear Ub chains meaning there may be a linkage that has not been tested that USP50 is specific to, despite the ability of USP50 to bind both K63 and linear Ub.

As USP50 can bind Ub-chains but does not appear to cleave them, the ability of USP50 to protect Ub-chains from degradation by active DUBs was investigated. K63-linked Ub chains were incubated with increasing concentrations of USP8, USP50 or USP8 with the highest

concentration of USP50 at 28°C overnight and then run on a gel. Unfortunately USP50 protein could not be detected by the His-antibody, however when USP8 was incubated with USP50 protein there was less degradation of Ub-chains. There was a reduction in the generation of mono-Ub in USP8 DUB assays which contained USP50, when compared to assays containing USP8 alone. The reduction in deconjugation of chains was especially apparent in chains that were four and six Ub moieties in length (Fig 4.3 B). Therefore although USP50 was not apparent on the gel, there was a difference between the degradation of chains when USP50 is incubated with USP8. It may be that the level of USP50 is too low to be detected by the gel but is sufficient to bind to and protect chains. This supports the observation that USP50 overexpression increases larger Ub-chains in whole cell lysates (Section 4.3).

Unfortunately protein purification of USP50 was inefficient, with very low concentrations of USP50 protein purified from bacteria. *In vitro* studies using USP50 protein were hampered by the poor expression.

As USP50 is binding, but does not seem to be cleaving, Ub-chains there is a potential function to protect Ub-chains from cleavage by other DUBs.

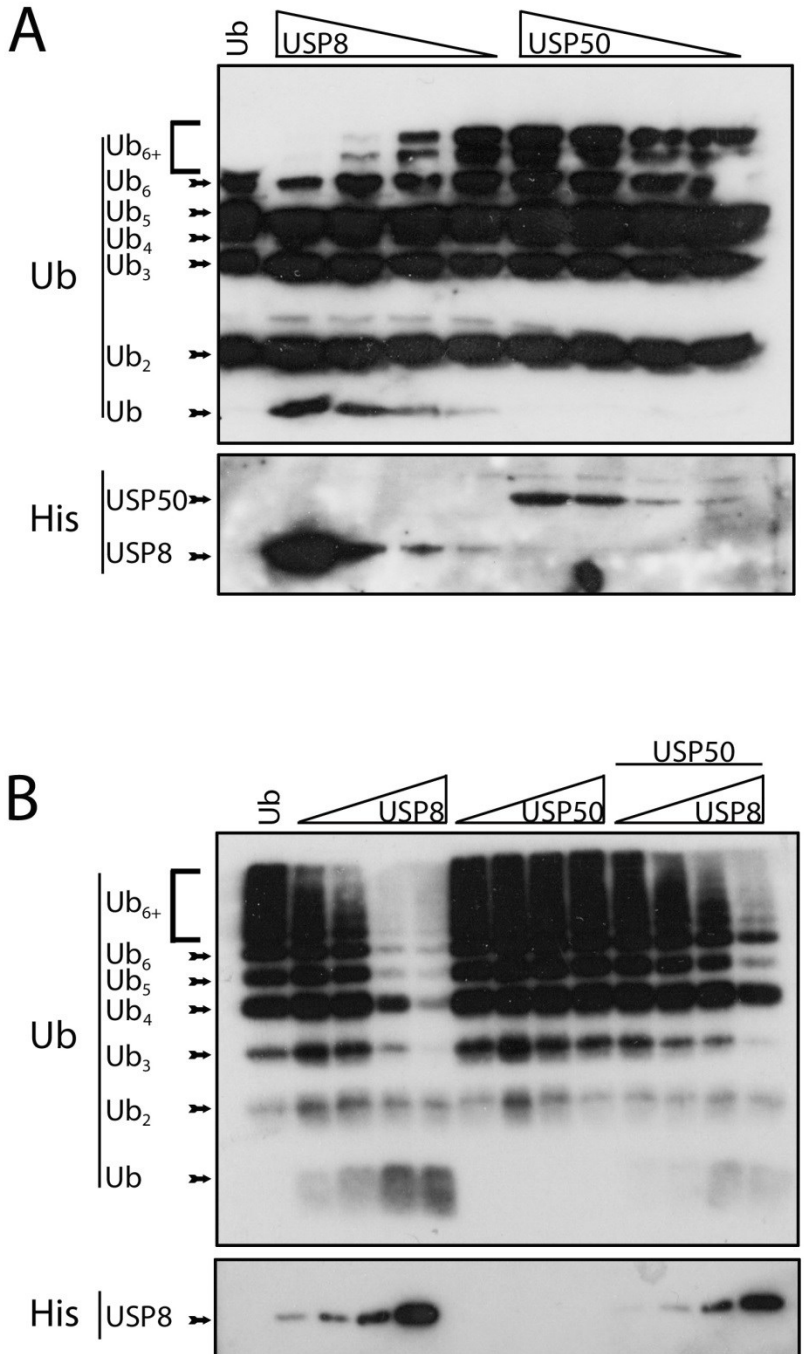


Fig 4.3 USP50 cannot deconjugate Ub chains. A) Increasing concentrations of USP8 and USP50 were incubated with linear Ub chains for 16 hours. USP8 was able to deconjugate these chains, reducing the amount of high molecular weight chains and producing mono-Ub however at no concentration of USP50 was a change in the composition of Ub-chains observed. **B)** Incubation of USP50 with USP8 reduced the amount of deconjugation of K63-linked Ub chains when compared to USP8 alone. Less mono-Ub was produced and chains of 4 or 6 Ub moieties were preferentially protected.

4.5 Generation of USP50 antibodies

As depletion of USP50 was required to determine the role of USP50 in the cell, it was necessary to determine if USP50 antibodies were able to detect USP50 proteins. To do this western blot analysis was performed using USP50 overexpression vectors to confirm the ability of the USP50 antibodies to specifically detect USP50 protein. It had already been previously published that endogenous levels of USP50 were not detectable by Western blot analysis (Aressy, Jullien et al. 2010).

Six commercially available antibodies were tested against cells expressing GFP-tagged USP50 or untransfected cells. Unfortunately, none of the commercially available antibodies was able to specifically detect USP50, producing non-specific banding patterns with no band running at the correct size for USP50-GFP expression and no band being solely in the USP50-GFP lane (Fig 4.4 A). Therefore it did not appear that these commercial antibodies were specific against USP50.

We commissioned Genscript to produce a "Western blot guaranteed" antibody. Three short peptide sequences were designed to be on the external surface of USP50 and not within the Ub-binding site based on the USP8/USP50-threading model, in order to maximise the likelihood of finding an available epitope. Six polyclonal antibodies were produced and tested against USP50 purified protein and USP50 overexpressing cell lysates. Purified protein or whole cell lysates with and without Flag-USP50 overexpression were run on Western blot and incubated with the six Genscript antibodies as well as the Flag antibody and the T7 antibody. The Flag antibody was able to distinguish the tagged overexpressed version of USP50 in the whole cell lysate, whereas the T7 antibody will identify the purified USP50 protein as it contains a T7 tag.

Whilst both the T7 and Flag antibodies where specifically able to distinguish bands of the correct size in the correct lanes, none of the Genscript antibodies were able to specifically distinguish the correct bands (Fig 4.4 B). Most of the antibodies did not produce a signal in the lane with purified protein except one antibody #15735. When running the whole cell lysates all of the antibodies gave non-specific banding patterns. None of the antibodies showed an increased signal that would correlate to the Flag-USP50 overexpression. Therefore as no commercial or commissioned antibody was able to specifically detected USP50, as purified protein or when overexpressed in cells, endogenous USP50 levels could not be analysed.

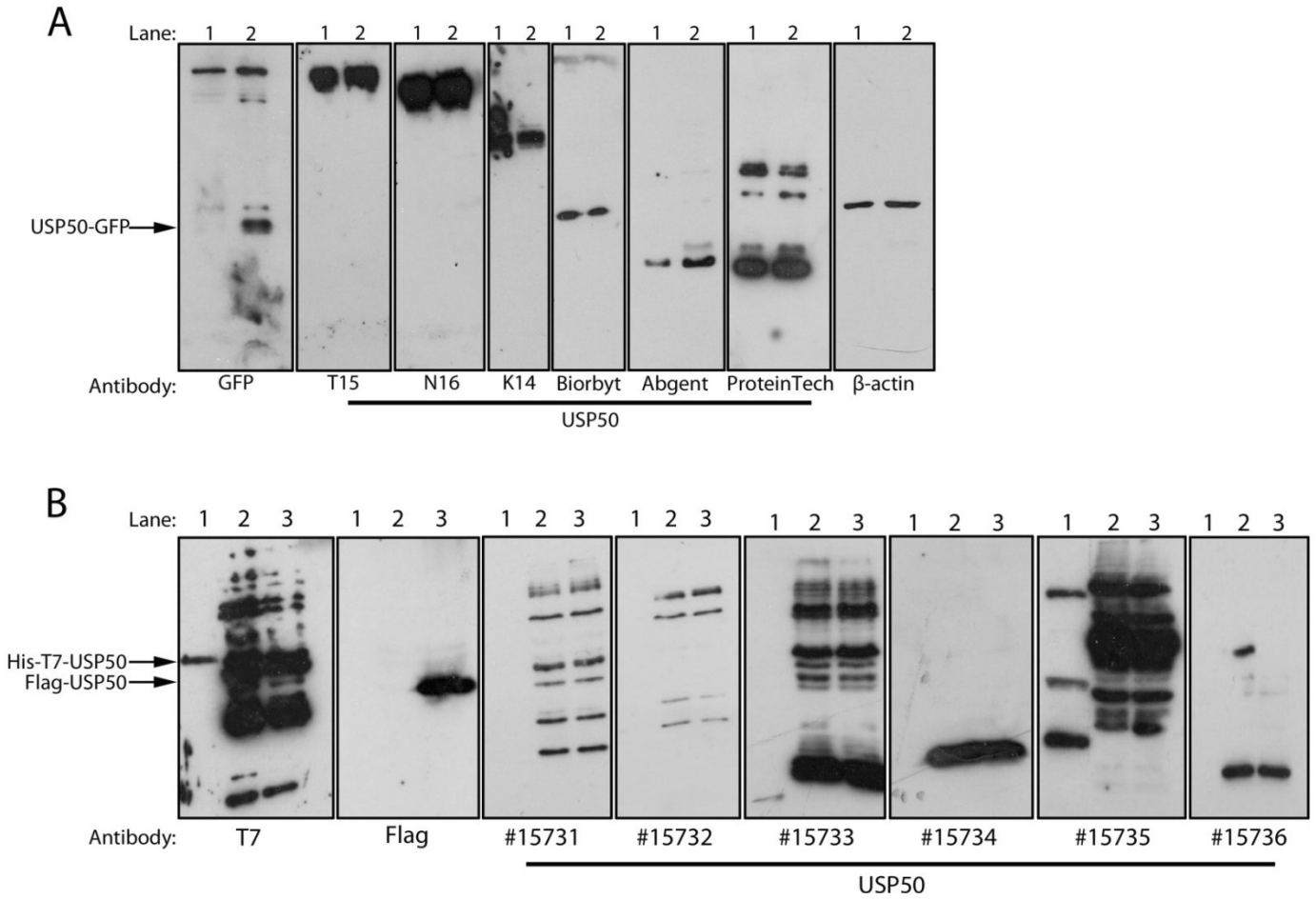


Fig 4.4 USP50 antibodies could not specifically identify USP50 protein. A) Six commercially available antibodies were tested against cells whole cell lysates of untreated cells (Lane 1) and cells overexpressing a USP50-GFP construct (Lane 2). Although the USP50-GFP protein could be detected specifically by a GFP antibody, none of the six antibodies specifically identified USP50. **B)** Six commissioned antibodies from Genscript were tested against purified USP50 protein with a T7 tag (Lane 1) as well as whole cell lysates that were untreated (Lane 2) or overexpressing a Flag-tagged USP50 protein (Lane 3). Although the purified protein could be detected using the T7 antibody and the overexpressed Flag-USP50 was visible with the Flag antibody none of the commissioned USP50 antibodies were able to specifically identify either the purified USP50 protein or the flag-tagged overexpressed USP50.

Therefore in order to confirm that USP50 siRNA could cause a reduction in the levels of a siRNA sensitive USP50, Flag-tagged protein was transiently transfected into cells. Reduction in the Flag protein levels were assessed as a measure of loss of USP50 protein. Cells were treated with either one of the four individual USP50 siRNAs and a pool of these USP50 siRNAs, and then western blot analysis performed using the Flag antibody in order to see any reduction in the overexpressed protein. The USP50 siRNAs, but not NTC siRNA, could reduce the amount of Flag-tagged protein seen in cell lysates (Fig 4.5 A and B). Therefore USP50 siRNA could reduce the levels of USP50 protein in the cell, which supports the assumption that USP50 siRNA is reducing the endogenous levels of USP50 protein in the cell.

RNA levels of USP50 were also tested after treatment with USP50 siRNAs in order to see if there was a reduction in the mRNA levels of endogenous USP50 mRNA upon siRNA treatment. mRNA was extracted from cells and reverse transcribed into cDNA, which was then amplified using USP50 specific primers and GAPDH primers as a loading control. In cells treated with USP50 siRNA there is a reduction in the amount of USP50 mRNA when compared to NTC siRNA treated cells (Fig 4.5 C).

Therefore even though there was no specific antibody available to check the levels of endogenous USP50 there is evidence that the siRNAs were working, due to the reduction in both USP50 mRNA and Flag-tagged protein.

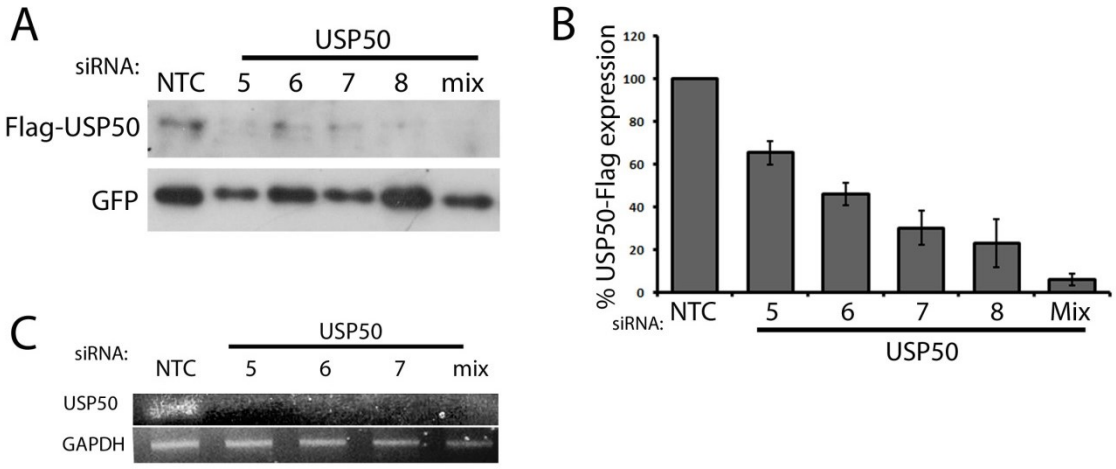


Fig 4.5 USP50 siRNA can deplete USP50 levels within the cell. A) Western blot analysis looking at levels of Flag-USP50 in cells treated with NTC siRNA, individual USP50 siRNAs or a pool of USP50 siRNAs. GFP was used as a transfection control. Percent of knockdown was determine by normalising Flag-USP50 levels to the amount of GFP in the cells and then worked out as a percent of the NTC control. **B)** Graphical representation of the Flag-expression after siRNA treatment. Averages of densitometry results from 2 individual Western blots, with Flag expression normalised against the GFP expression. Error bars = SD. **C)** mRNA was extracted from cells treated with NTC, individual USP50 or a pool of USP50 siRNAs and converted to cDNA. USP50 primers were used to specifically amplify USP50 cDNA (Full gel image in Appendix Fig. A5) and GAPDH primers were used as a control.

4.6 USP50 depletion causes increased spontaneous damage-associated foci

In vitro studies of USP50 have shown no evidence of DUB activity, likely due to the loss of the aspartic acid residue in the C-terminus (Section 4.4). USP50 can still bind Ub both *in vitro* and *in vivo*. The role that USP50 plays in cells however is still poorly understood and was therefore investigated.

As USP50 siRNA caused a decrease in FK2 luminesence after release from HU (Fig 4.6 A), immunofluorescence was carried out on USP50 depleted cells to look for a defect with the recruitment of damage proteins, BRCA1 and 53BP1. These proteins require damage dependent ubiquitination for efficient recruitment. Intriguingly, there was a significant increase in both BRCA1 and 53BP1 foci upon USP50 depletion without any exogenous damage being applied (Fig 4.6 B-E).

Repeats were carried out in different cell lines in order to determine whether this was a HeLa cell specific effect of the USP50 siRNA. A breast cancer cell line, MCF7, was treated with NTC or USP50 siRNA and stained for 53BP1. MCF7s treated with USP50 siRNA also showed a significant increase in 53BP1 foci when compared to cells treated with the NTC siRNA (Fig 4.6 F).

As previously discussed USP50 is conserved in mice (Section 4.3), therefore it was investigated whether the increase in damage-associated foci on USP50 siRNA treatment was also conserved between these species. To do this, USP50 siRNA specific for the mouse transcript was used in NIH3T3 cells. Again USP50 siRNA treated cells had significantly more 53BP1 foci without addition of exogenous damage (Fig 4.6 G).

Together these data show that the increase in 53BP1 foci occurs between cell lines and between species, suggesting deregulation of a conserved role across species and not an off target effect of siRNA treatment.

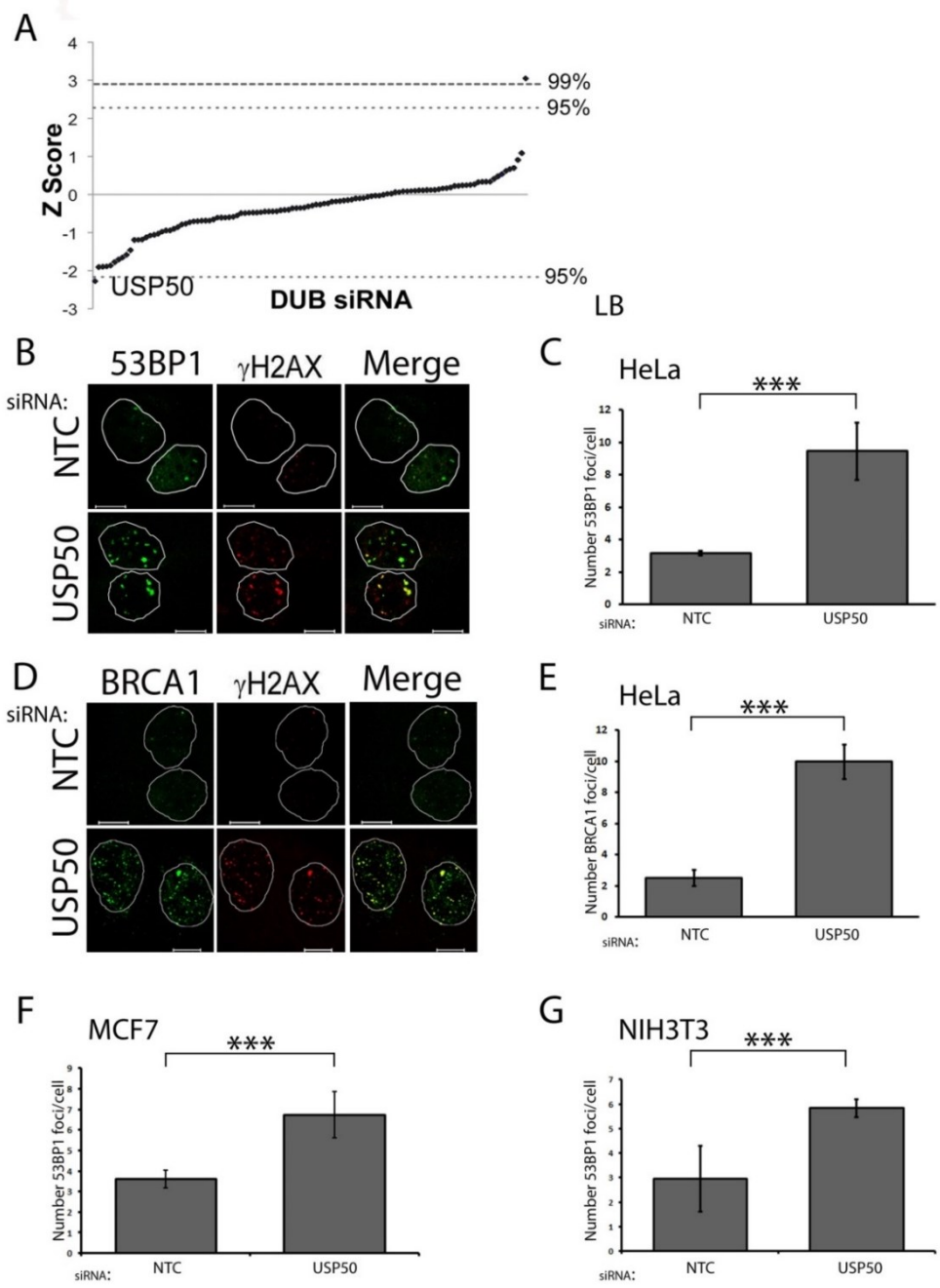


Fig 4.6 USP50 siRNA treatment causes increased DNA-damage associated foci without exogenous damage. A) Screen of 103 putative DUBs after release from HU carried out by Laura Butler. **B)** Immunofluorescent images displaying an 53BP1 foci in HeLa cells after USP50 siRNA. White line represents DNA as visualised by Hoescht staining. **C)** Quantification of 53BP1 foci in NTC and USP50 siRNA treated cells. (100 cells per treatment, n=3. **D)** Immunofluorescent images showing BRCA1 foci in HeLa cells after USP50 siRNA. White line represents DNA as visualised by Hoescht staining. **E)** Quantification of BRCA1 foci after NTC or USP50 siRNA treatment (100 cells oer treatment n=3). **F)** Quantification of 53BP1 foci in MCF7 cells after treatment with NTC or USP50 siRNA (70 cells per treatment, n =3).**G)** Quantification of 53BP1 foci in mouse NIH3T3 cells after treatment with NTC or USP50 siRNA (70 cells per treatment, n =3). *** T-test $p<0.01$.

4.7 USP50 siRNA does not grossly reduce the cells ability to repair damage

Due to the increase in damage-associated foci in all tested cell lines upon USP50 siRNA treatment, there is evidence suggesting the depletion of USP50 is causing a defect giving rise to damage foci. There are many potential reasons why these cells have increased damage-associated foci, including DSB repair defects such as, inefficient clearance of repair proteins or genomic instability due to a fault during replication or transcription. Understanding why these foci arise was consequently investigated further.

As the foci were associated with the DNA damage response, it was first investigated whether the increase in foci was due to a defect in repair of DSBs. A time course was carried out to see if 53BP1 foci could be cleared after different time points during recovery from IR. HeLa cells were treated with USP50 or NTC siRNA, damaged with 2 Gy IR and fixed at various time points after damage. Cells were stained for 53BP1 and foci quantified at each time point. Despite there being more 53BP1 foci in USP50 siRNA treated cells, the ability of cells to clear these foci happens at a similar rate as in NTC siRNA treated cells, with both siRNA treatments clearing roughly 2 foci per hour from 1 hour post IR onwards (Fig 4.7A). Therefore the increase in 53BP1 foci is unlikely to be due to an inability to clear repair proteins from damage but because more foci are arising in these cells.

Further to the time course, in order to investigate whether USP50 siRNA treated cells were less able to repair DSBs a colony survival assay was performed. HeLa cells were treated with siRNA against USP50, NTC and BRCA2 as a positive control and subjected to increasing doses of IR. Cells were plated sparsely and left to form colonies for 10-14 days before fixing with crystal violet in 50% methanol and colonies counted. The assumption for this assay is that each colony forms from a single cell, therefore a reduction in colony number is due to a decrease in the survival of cells. USP50 depletion does not cause a significant decrease in

colony forming units when compared to the NTC treated cells, therefore cells treated with USP50 siRNA are not sensitive to IR unlike cells treated with BRCA2 siRNA (Fig 4.7 B). As IR induces DSBs, USP50 siRNA treatment does not cause cells to become sensitive to DSBs, further suggesting that USP50 siRNA does not cause a defect in DSB repair.

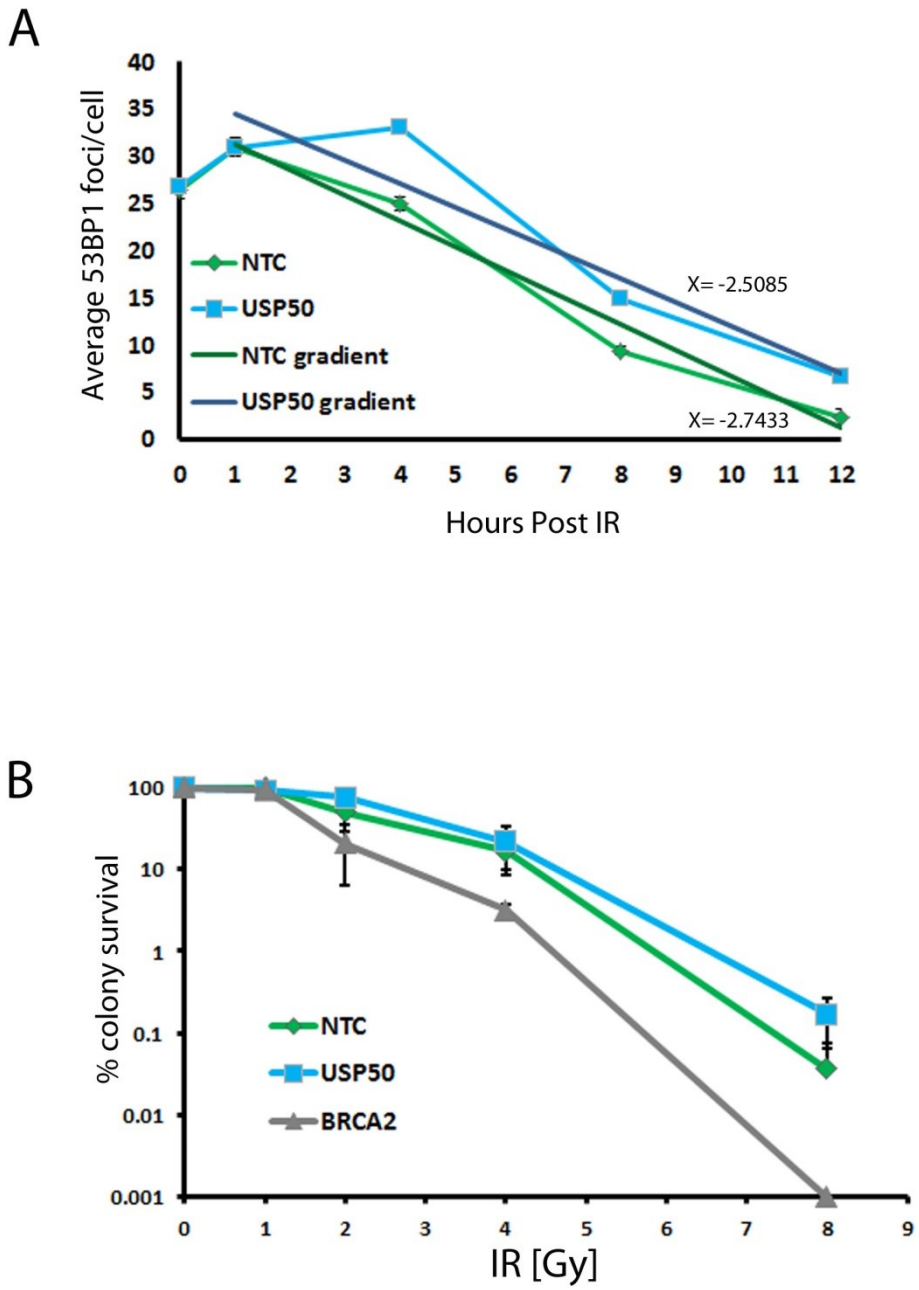


Fig 4.7 USP50 siRNA treatment does not cause sensitivity to IR. A) HeLa cells treated with NTC or USP50 siRNA were subjected to 2 Gy IR and fixed at specified timepoints during recovery. Cells were stained for 53BP1 and foci at each timepoint quantified and plotted. (70 cells per treatment n=3). **B)** Survival colony assays were performed on cells treated with NTC, USP50 or BRCA2 siRNA and subjected to increasing doses of IR. Cells were plated and left to form colonies before being fixed, stain and quantified (n=4) Error bars = SEM.

The induction of DSBs by IR occurs independently of the stage of the cell-cycle, therefore IR cannot distinguish whether there is a defect specific to either HR or NHEJ. GFP reporter assays were used to look separately at USP50 siRNA treatment on HR and NHEJ repair (Fig 4.8 A and B). U2OS cells were treated with siRNA against NTC, USP50 and BARD1 as a positive control. The restriction enzyme *iSce-I* and the transfection control, RFP, were transfected into the cells. If cells are able to repair the break induced by the *iSce-I* restriction enzyme GFP was expressed (Fig 4.8 A and B). Therefore GFP expression was measured on a FACs machine as a read out of repair. It is assumed that RFP positive cells had also been successfully transfected with the *iSce-I* enzyme and had consequently undergone DSB formation. Therefore cells that were positive for both GFP and RFP were calculated as a ratio of RFP positive cells to determine the ratio of transfected cells that had undergone successful repair. USP50 siRNA caused a slight reduction of GFP-RFP double positive cells, to roughly 80%, in both HR and NHEJ assays, but not to the extent to which BARD1 siRNA reduced double positive cells, as BARD1 siRNA is known to cause a defect in both repair pathways (Westermark, Reyngold et al. 2003). The knockdown of USP50 did not cause a significant decrease in the proportion of GFP-RFP double positive cells when compared to the NTC siRNA knockdown in either HR or NHEJ reporter assays (Fig 4.8 C and D). Therefore USP50 siRNA treatment does not cause a gross difference in the cells ability to perform HR or NHEJ repair.

Despite the increase in damage foci that appear in cells upon USP50 siRNA treatment, the repair of DSBs seems largely unaffected. As cells are still able to survive IR treatment and efficiently clear 53BP1 from damage sites, any decrease in the repair pathways does not appear to drastically affect cell viability. USP50 is therefore unlikely to have a major role in

either NHEJ or HR repair, suggesting that the increased 53BP1 foci are arising due to another pathway being deregulated in the cells.

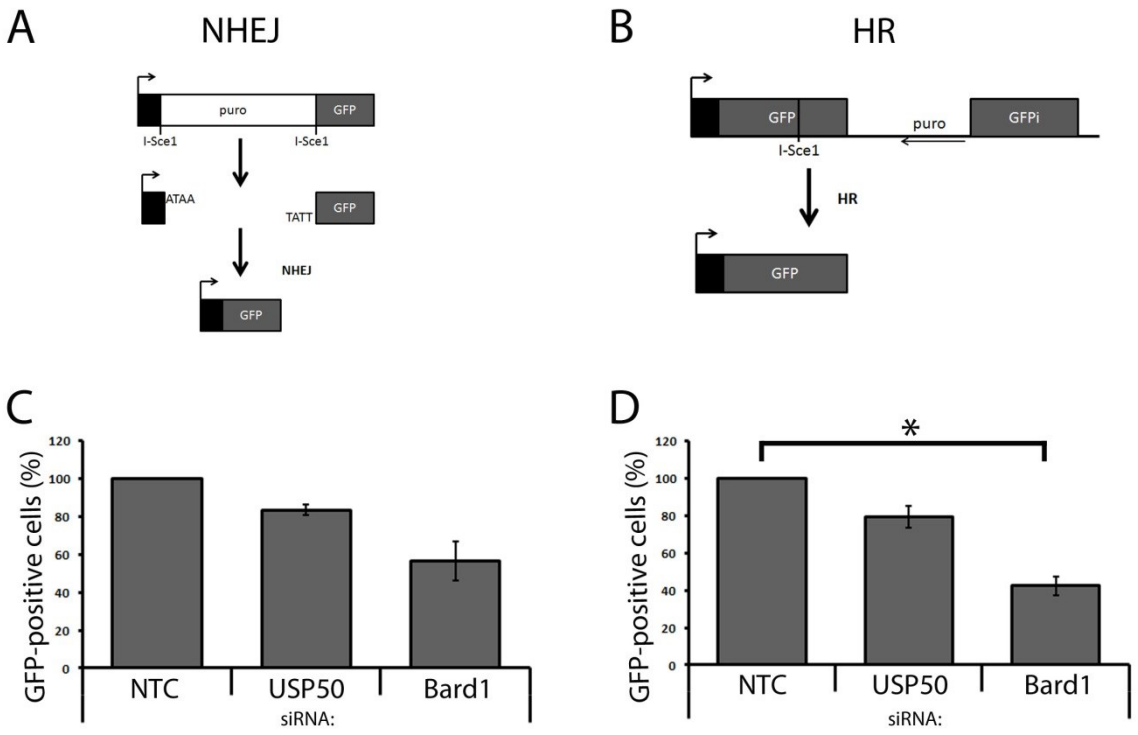


Fig 4.8 USP50 siRNA treatment does not cause a major reduction in cells ability to repair DSBs. A) Representation of NHEJ GFP assay. **B)** Representation of HR GFP assay. **C)** Percent of GFP and RFP positive cells as a proportion of NTC cells. USP50 siRNA did not cause a large decrease in the cells ability to repair by NHEJ. **D)** Percent of GFP and RFP positive cells as a proportion of the NTC control siRNA treated cells. USP50 depletion did not greatly reduce the cells ability to perform HR. Experiments were carried out in triplicate (n=3)

4.8 Generation of inducible USP50 knockdown and overexpression cell lines

In order to ensure reproducibility of results, stable cell lines were produced which had both inducible shRNA expression to induce USP50 knockdown, as well as inducible Flag-tagged siRNA resistant USP50 overexpression.

HeLa Flip-In cells were lentivirally infected with NTC or USP50 shRNA by Dr Alex Garvin and then cells selected using Puromycin to obtain stably transfected cells. Knockdown of USP50 was tested by inducing shRNA against USP50 or NTC, with 100 μM IPTG and then cells examined for 53BP1 foci. If there was the same increase in 53BP1 foci in shUSP50 cells as seen with USP50 siRNA it was assumed USP50 was being knocked down. HeLa cells containing either shNTC or shUSP50 were plated and treated with IPTG for 72 hours before fixing and staining for 53BP1 to be visualised by immunofluorescence. In shNTC expressing cells there was no significant increase in 53BP1 upon incubation with IPTG. Conversely, both clones tested, which contained shUSP50, showed a significant increase in the 53BP1 foci. However clone #22 had a higher number of foci in cells not treated with IPTG, where shRNA against USP50 had not been induced, coupled with slightly less foci per cell in cells treated with IPTG (Fig 4.9 A). This may be due to a slightly leaky expression of the shUSP50 in these cells. Therefore experiments were continued with clone #15. This result suggests that the USP50 shRNA is capable recapitulating the phenotype observed with siRNA.

To confirm that the increase in 53BP1 foci observed in shUSP50 #15 cells was due to a reduction in USP50 protein levels, cells were transfected with a transient siRNA sensitive USP50-Flag construct which was sensitive to shRNA. Western blot analysis was performed to determine if the shRNA could reduce the levels of the Flag-tagged USP50 protein. Cells expressing shNTC expressed USP50-Flag protein regardless of IPTG treatment; however shUSP50 could reduce levels of USP50-Flag protein when cells were treated with IPTG in

order to induce knockdown (Fig 4.9 B). Cells containing the shUSP50 only showed a reduction in USP50-Flag when treated with IPTG, therefore the reduction in USP50-Flag is dependent on the shRNA induction. This suggests the shUSP50 #15 contained an inducible shRNA against USP50 that could efficiently knockdown USP50 protein and give rise to increased 53BP1 foci.

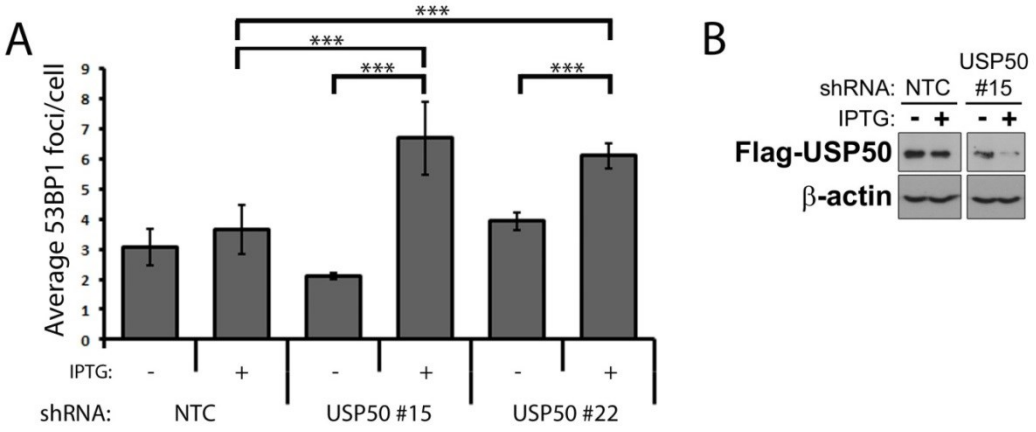


Fig 4.9 USP50 shRNA can reduce USP50 levels within the cell. A) HeLa cells stably transfected with shNTC or shUSP50 were treated with 100µM IPTG to induce shRNA expression for 72 hours. Cells were fixed and stained for 53BP1 and foci numbers quantified. Cells expressing shUSP50 displayed increased 53BP1 foci (100 cells per treatment, n=3) T-test $p<0.01$. **B)** Western blot analysis visualising Flag-USP50 protein levels after shNTC and shUSP50 exposure.

In order to determine if the damage-associated foci could be rescued by reintroducing USP50 to depleted cells, a shRNA resistant form of Flag-USP50 was expressed in an inducible manner. HeLa-Flip In cells were transfected with a shResistant Flag-USP50 in a pcDNA/FRT/TO vector and the POG44 recombinase in order to stably transform the cells. Cells were selected with hygromycin and colonies picked and expanded. Expression of Flag-USP50 was tested by treating cells with Dox in order to induce protein expression and lysates run on Western blot to determine if USP50-Flag was expressed. Dox treated cells expressed a Flag-tagged protein running the expected size of USP50, suggesting that Flag-USP50 was being expressed but was dependent on Dox treatment (Fig 4.10A).

As there was expression of the shRNA resistant Flag-USP50, cells were tested to see if the reintroduction of USP50 could prevent the formation of 53BP1 foci in cells expressing the shRNA against USP50. The cells that successfully expressed the Flag-tagged USP50 were treated with IPTG to cause expression of shNTC or shUSP50. After 24 hours cells were then treated with different concentrations of Dox to induce USP50-Flag expression and then fixed after 48 hours. Cells were stained for 53BP1 and foci were quantified. As previously demonstrated, shUSP50 cells treated only with IPTG had an increase in 53BP1 foci above shNTC expressing cells. In shNTC the addition of increasing concentrations of Dox did not significantly change the number of 53BP1 foci. However, when increasing concentrations of Dox were added to shUSP50 expressing cells, the number of 53BP1 foci decreased to levels seen in shNTC treated cells (Fig 4.10 B). This suggests that the reintroduction of shRNA resistant USP50 is reversing the effect of the USP50 knockdown. Therefore the increase in 53BP1 foci upon USP50 shRNA does seem to be dependent on the reduction of USP50 and is unlikely to be due to an off target affect as the reintroduction of USP50 can rescue the increase in 53BP1 foci.

Cells lines that could either inducibly express shNTC or shUSP50, as well as overexpress shRNA resistant USP50-Flag protein were successfully produced.

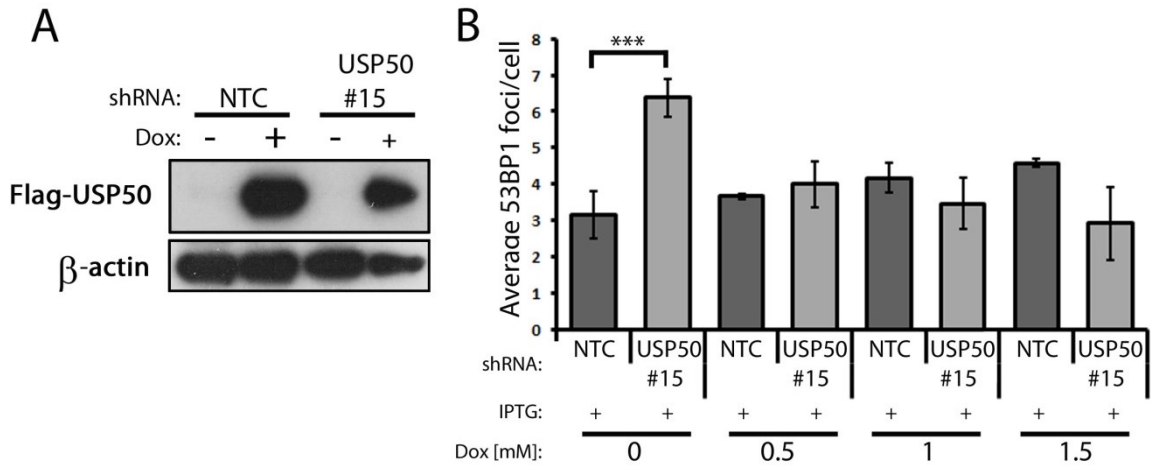


Fig 4.10 Flag-USP50 overexpression can reduce 53BP1 foci numbers in shUSP50 treated cells. A) Western blot analysis to confirm expression of Flag-USP50 in an inducible manner in HeLa-Flip In cells. **B)** HeLa-Flip In cells were treated with IPTG to induce expression of shNTC or shUSP50 for 24 hours. Cells were treated with increasing concentrations of Dox to induce expression of Flag-USP50 for 48 hours. Cells were fixed and stained for 53BP1 and foci quantified (70 cells per treatment, n = 3) T-test $p<0.01$. Error bars = SEM.

4.9 The Ub-binding activity of USP50 is required to inhibit 53BP1 foci formation

As USP50 does not appear to have DUB activity but can still bind Ub (Section 4.3 and 4.4) it was investigated whether the ability of USP50 to bind Ub was important for its role in the cell.

The ND-mutant partially inhibits Ub-binding as previously described. This mutant was introduced into the stable cell line system. Western blot analysis was performed to show that the ND-USP50 mutant was being expressed in these cell lines. Dox treatment of the cells caused expression of USP50 ND to levels similar to WT-USP50 (Fig 4.9 A).

To determine if the Ub-binding activity of USP50 was required for the prevention of 53BP1 foci, the Ub-binding mutant was expressed in cells depleted of USP50. Cells expressing USP50-ND were fixed and stained for 53BP1 foci and quantified. In cells depleted of USP50, but expressing the ND mutant there was a partial reduction in the average number of 53BP1 foci when compared to those expressing WT-USP50 (Fig 4.11 B). Conversely, the expression of WT-USP50 could significantly reduce the number of 53BP1 in cells back to roughly 2 foci per cell, which is similar to foci numbers in cells treated with NTC siRNA (Fig 4.9 A). Due to the reduction of 53BP1 upon WT expression, but only partial reduction in 53BP1 foci numbers with the ND mutation, it can be assumed that the Ub-binding activity of USP50 is required for its activity in the cell. As the ND-USP50 protein levels are comparable, although slightly reduced compared to those of WT-USP50, the incomplete reduction in 53BP1 foci is not due to there being less USP50 protein in the cells.

As the USP50-ND mutant only partially restricts Ub-binding it will be important to perform this experiment with a USP50 which has fully abolished Ub-binding to see whether this is no longer able to reduce 53BP1 foci numbers.

Complementation of USP50 into knockdown cells gives evidence against the siRNA producing an off target effect. The Ub-binding mutant only producing a partial reduction in damage-associated foci suggests that the ability to bind Ub is necessary for the function of USP50.

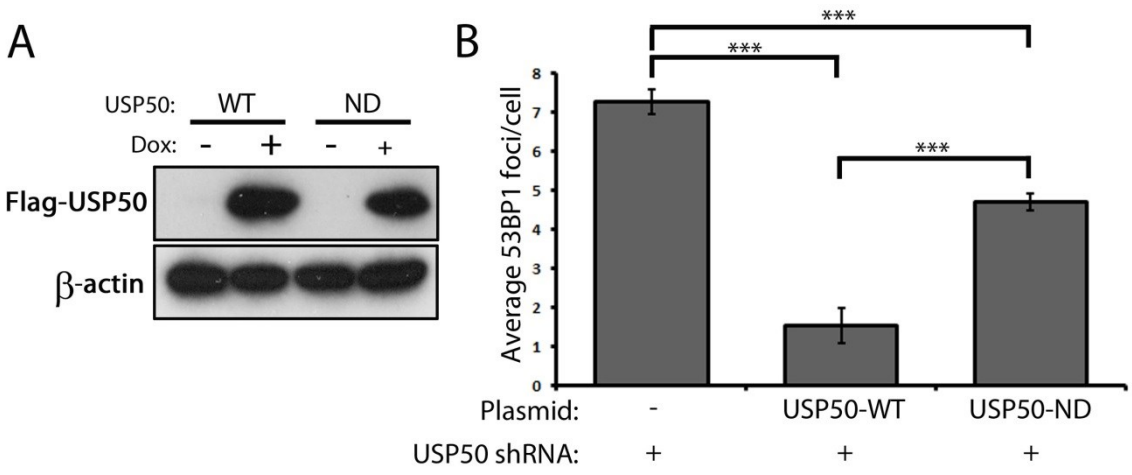


Fig 4.11 Ub-binding mutant of USP50 can only partially reduce 53BP1 foci numbers after USP50 shRNA expression. A) Western blot analysis to confirm that ND-USP50 could be expressed at levels similar to WT-USP50. **B)** HeLa cells expressing shUSP50 were then left uninduced or induced to express WT-USP50 or ND-USP50 and 53BP1 foci counted (50 cells per treatment, n=2) T-test $p<0.01$.

4.10 Summary

Within this chapter, USP50 has been characterised as an inactive DUB that has maintained the ability to bind Ub (Section 4.3 and 4.4). This small protein is likely to have arisen due to a gene duplication of the C-terminus of USP8, leading to high amino acid conservation between this region of USP8 and USP50 (Section 4.2). However, in humans, USP50 has been truncated leading to the loss of the aspartic acid which forms part of the catalytic triad, potentially explaining the lack of protease activity (Section 4.4). Interestingly, although the USP50 DNA sequence is 70% conserved across 40 species, this missing aspartic acid is actually present in some species, including mice (Section 4.3). Whether these species still retain DUB activity is unknown and is worth further investigating as it may give insights into the role of USP50. If USP50 is performing the same role in all species but acts a DUB in some species and not others, it may suggest USP50 is acting to stop access to chains either by removing the Ub-chains or by binding Ub-chains to block another Ub-binding protein.

In vivo, the loss of USP50 caused spontaneous damage-associated foci which were reproducible in two human cell lines and in a mouse cell line (Section 4.6). This phenotype can be rectified by WT-USP50 but not a mutant which can only partially bind Ub, implying the Ub-binding activity is required for USP50 function (Section 4.3 and 4.9). However, a mutant that can complete abolish Ub-binding is still required to fully elucidate the Ub-binding function of USP50 in the cell and to help determine the ubiquitinated substrate that USP50 is binding.

There were a few possible reasons for the increased damage-associated foci, a fault in DSB repair was investigated as the first possible explanation. However, no defect was detected in the clearance of damage-associated protein 53BP1 or sensitivity to the DSB inducing agent IR (Section 4.7). This coupled with only a slight reduction in the ability of cells to repair DSBs

by HR or NHEJ suggests USP50 is not playing a significant role in the repair of DSBs despite the increase in damage-associated foci. Therefore, further investigation was required into the USP50-dependent mechanism that is causing the increased 53BP1 foci as it is unlikely a DNA repair defect.

These experiments were conducted without being able to confirm knockdown of endogenous USP50 due to the lack of a specific available antibody. Moreover, endogenous USP50 is expressed at low levels making its expression difficult to detect (Aressy 2010). Therefore there is a caveat to these experiments. However knockdown of USP50 was tested using complementation assays, but also by overexpressing a tagged version of USP50, with knockdown detected by loss of the tag rather than using an antibody specific to USP50 (Section 4.5 and 4.9).

Inducible cell lines were also created to allow both inducible knockdown and overexpression of USP50. As these cells were clonally selected, all cells should display the same knockdown and overexpression efficiency allowing more reproducible results (Section 4.8).

The results of this chapter suggest that USP50 is a Ub-binding protein that functions to limit the formation of damage-associated foci through an unknown pathway. Therefore further work was performed to elucidate how loss of USP50 was causing the increase in damage-associated foci.

5. USP50 maintains genome stability during replication

5.1 Introduction

The lack of a major function in the DDR, raises the question of what is the cause of increased damage-associated foci in USP50 depleted cells. Consequently, elucidating which pathway USP50 plays a role in and how it acts to suppress the damage foci seen on its depletion was investigated.

As the damage foci do not appear to arise due to faulty DSB repair as there is no sensitivity to IR damage and 53BP1 foci can effectively be cleared (Fig 4.7), another pathway must be malfunctioning to cause the damage-associated foci. DNA damage does not require the addition of exogenous DNA damaging agents, such as IR or UV damage, but can occur due to endogenous sources including the disruption of a couple of pathways, namely replication or transcription. Replication stress is defined as the slowing or stalling of replication forks. Forks can stall in a manner of ways, including through the actions of drugs such as HU or Cpt, or through difficult-to-replicate regions of the genome. These regions consist of repetitive sequences or DNA secondary structures, including G4 quadruplexes. Further to this, DNA:RNA hybrids and protein complexes on the DNA, including transcription machinery, can cause replication stress. The cell has many mechanisms which allow stalled forks to restart, however if the fork cannot be restarted it can be cleaved into a DSB structure that can be repaired by HR-mediated repair to allow replication to continue (reviewed in Mazouzi, Velimezi et al. 2014).

Transcription mainly occurs during the G1 phase of the cell cycle but can occur in S-phase with histone genes being transcribed (Robbins and Borun 1967). There is emerging evidence that repair proteins, including Ku70 and Ku80, are required for transcription activation and

TOP2B may induce DSBs in order to initiate transcription (Mayeur, Kung et al. 2005, Ju, Lunyak et al. 2006). Therefore the increased damage-associated foci observed in S-phase may result from either replication or transcription being deregulated by USP50 loss. Determining the cell cycle phase in which the damage occurs can narrow down the pathway that USP50 is working in.

5.2 USP50 loss is not epistatic with Wee1 loss

USP50 has been implicated in preventing aberrant bypass of the G2/M checkpoint by inhibiting the degradation of Wee1 (Aressy, Jullien et al. 2010). Defective checkpoint signalling might be the cause of increased damage in the cell. If the cell enters mitosis without completing replication, the chromosomes cannot separate correctly due to under-replicated regions causing anaphase bridges, or broken chromosomes which manifests as damage in the daughter cells (Chan, Palmai-Pallag et al. 2009, Naim and Rosselli 2009)

In a paper by Aressy *et al* 2010, the loss of USP50 in cells overexpressing Cdc25B made them more prone to bypass the G2/M checkpoint (Aressy et al 2010). Aberrant phosphatase activity of Cdc25B activates the CDK1 kinase which causes entry into M phase (Lammer, Wagerer et al. 1998, Karlsson, Katich et al. 1999). Aressy *et al* attributed the checkpoint failure observed to the destabilization of Wee1 upon USP50 depletion, via Hsp90. Knockdown of USP50 was shown to decrease Wee1 protein levels, which could be rescued by MG132 treatment (Aressy et al 2010). The loss of Wee1 would cause G2/M checkpoint bypass, as normally Wee1 opposes the action of Cdc25B by phosphorylating Thr14 and Tyr15 on CDK1, causing CDK1 to become inactive and consequently stopping entry into M-phase (Lundgren, Walworth et al. 1991, Parker, Athertonfessler et al. 1992). The destabilisation of Wee1 on USP50 depletion in Aressy *et al* coupled with overexpression of Cdc25B, will be activating CDK1 thereby allowing access into M-phase in these cells.

Therefore, in cells with an impaired checkpoint, USP50 loss caused an increase checkpoint bypass, which could potentially be the cause of the damage observed (Aressy, Jullien et al. 2010). Reduction of Wee1 has already been shown to cause increased γH2AX, similar to the observed increase seen in USP50 depleted cells and therefore the increase in damage-associated foci may be caused indirectly through loss of Wee1 protein (Dominguez-Kelly, Martin et al. 2011).

One of the initial questions addressed in this project was whether USP50 knockdown was causing a loss of Wee1 protein. Western blot analysis of Wee1 protein levels was tested under different knockdown conditions. HeLa cells were treated with NTC, USP50, Wee1 or combined USP50 and Wee1 siRNA, lysed and protein levels analysed by SDS-PAGE. In cells treated with Wee1 siRNA there was a decrease in the amount of protein visible, however USP50 knockdown did not cause a dramatic drop in Wee1 protein levels (Fig 5.1 A). Thus, from this data it does not appear USP50 depletion is causing a drastic destabilisation of Wee1, unlike as previously demonstrated by Aressy *et al.*

Secondly, as the loss of Wee1 has been shown to cause increased damage (Dominguez-Kelly, Martin et al. 2011), it was hypothesised that the damage seen in USP50 depleted cells may be arising through the deregulation of Wee1. To test this, HeLa cells were treated with siRNA against USP50, Wee1 or combined USP50 and Wee1 siRNA. After 72 hours, cells were fixed and stained for 53BP1. As expected, both USP50 and Wee1 single depletions caused an increase in the number of 53BP1 foci above that of NTC control. However, the depletion of both USP50 and Wee1 increased 53BP1 foci significantly above either knock alone as shown by Students T-test (Fig 5.1 B and C). Unfortunately, there was not a suitable antibody available to confirm USP50 depletion; however the increased 53BP1 gave an indication that the siRNA was having an effect. Although the knockdown of both USP50 and Wee1 together

did cause a greater number of 53BP1 foci than either siRNA alone, the increase was not additive (Fig 5.1 C). If the spontaneous damage foci observed in USP50 depleted cells were due to a problem in the Wee1 pathway, it would be expected that there would not be a significant difference between the single siRNA treatments and the combined USP50 and Wee1 siRNA. Therefore, the spontaneous damage-associated foci in USP50 depleted cells appear to be arising from a defect in a pathway distinct from the Wee1 pathway, however as there is not an additive increase there may still be some overlap between the USP50 and Wee1 pathway that gives rise to a proportion of the damage foci seen.

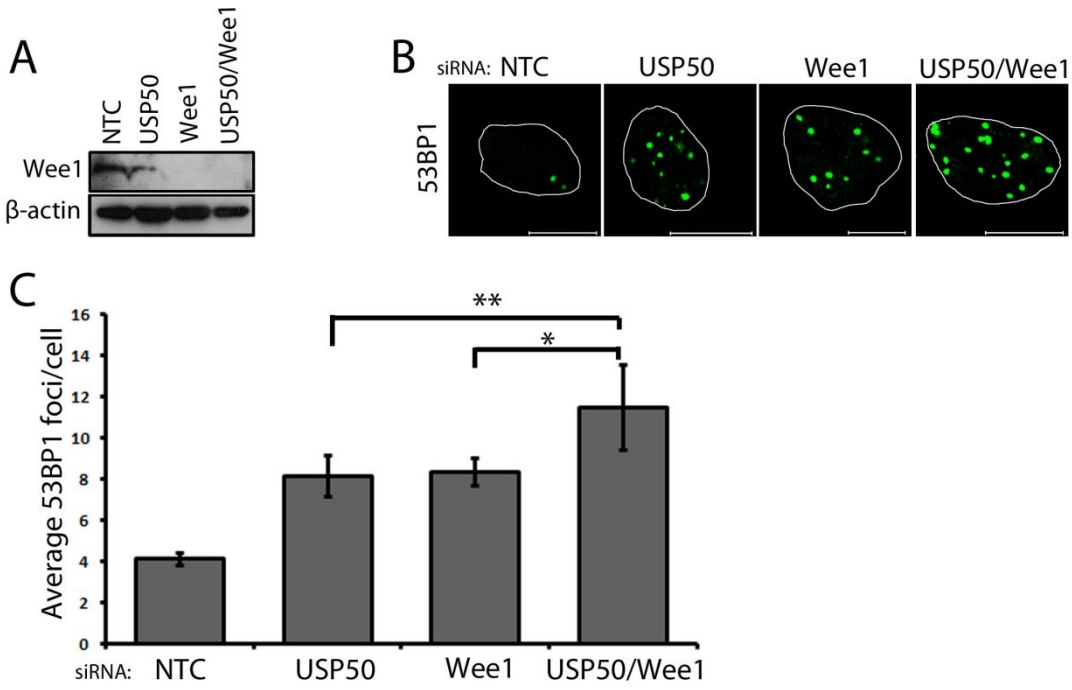


Figure 5.1 USP50 and Wee1 combined siRNA increase 53BP1 foci numbers above either siRNA alone. HeLa cells were plated and transfected with NTC, USP50, Wee1 or USP50/Wee1 siRNA and fixed after 72 hours. **A)** Western blot analysis showing Wee1 knockdown with β-actin as a loading control. **B)** Cells were stained for 53BP1 foci and imaged by confocal microscopy. White line represents DNA as marked by Hoescht staining. Scale bar = 10μM **C)** Quantification of 53BP1 foci for each siRNA treatment and analysed by students t-test (75 cells per treatment n=3) Error bars = S.E.

5.3 USP50 siRNA treatment does not cause a gross change in the cell cycle profile of cells

As USP50 depletion has already been associated with a reduced G2/M checkpoint (Aressy, Jullien et al. 2010), cell cycle analysis on USP50 depleted cells was performed.

U2OS cells were treated for 72 hours with NTC, USP50 or Wee1 siRNA before being pulsed with BrdU which was incorporated into replicating DNA. An antibody against BrdU was used to detect incorporation, and DNA was stained with Propidium Iodide (PI). The amount of BrdU incorporation can be plotted against the amount of PI staining to distinguish the cell cycle phase each cell is in. Cell cycle analysis was carried out on the Accuri FACs machine.

Asynchronous cells produce a distinctive horseshoe profile where G1 cells have low BrdU and PI staining compared to other phases of the cell cycle, S-phase cells have increased BrdU staining and increasing PI staining. Finally, cells in G2/M have low BrdU but increased PI staining. The loss of USP50 did not cause a significant change in the cell cycle profile when compared to NTC siRNA cells, although there did appear to be a slight increase in G1 cells (Fig 5.2 A). This increase in G1 cells could also potentially be early S-phase cells as it is difficult to distinguish between G1 and early S-phase cells. Despite this, there does not appear to be a gross change to the cell cycle profile when USP50 was depleted.

On the contrary, Wee1 siRNA caused a change in the cell cycle profile, with a spike of cells in G2/M phase and a greater proportion of cells in S-phase. There are also a greater number of cells that are no longer within the normal cell cycle profile, as apparent by both the horseshoe plot and the quantification. The horseshoe plot suggests many cells have increased DNA content, potentially due to re-replication of DNA. However as Wee1 siRNA causes a cell

cycle defect which is not observed in USP50 depleted cells, there is further evidence suggesting USP50 and Wee1 are not functioning in the same pathway.

If cells are perturbed during the cell cycle, cells can accumulate in one specific phase due to checkpoint activation in order to allow the cell time to overcome any defects that have arisen. If checkpoints are defective, cells can pass through the checkpoint despite any damage that has occurred, preventing damage from being repaired.

When cells were exposed to 5 Gy IR and allowed to recover for 24 hours, cells accumulate in G2/M phase of the cell cycle. Both NTC and USP50 siRNA treated cells accumulated in G2/M after 5 Gy IR (Fig 5.2 B). These results suggest that the G2/M checkpoint is intact as cells have not entered mitosis.

In addition, the use of 3 nM Aphidicolin (Aph) causes early replication specific defects due to the inhibition of DNA polymerases α and δ (Krokan, Wist et al. 1981). Therefore DNA replication cannot occur and cells cannot progress through S-phase. To examine whether USP50 depletion allowed S/G2 checkpoint bypass, cells were treated with Aph for 16 hrs before being pulsed with BrdU and fixed. The Aph treatment caused an accumulation in S-phase for both control and USP50 depleted cells. These results suggest that the S/G2 checkpoint is still active as cells cannot progress through S-phase (Fig 5.2 B).

As the cell cycle profiles of NTC and USP50 siRNA treated cells did not significantly differ from each other, there is no indication of USP50 playing a role in the regulation of the cell-cycle.

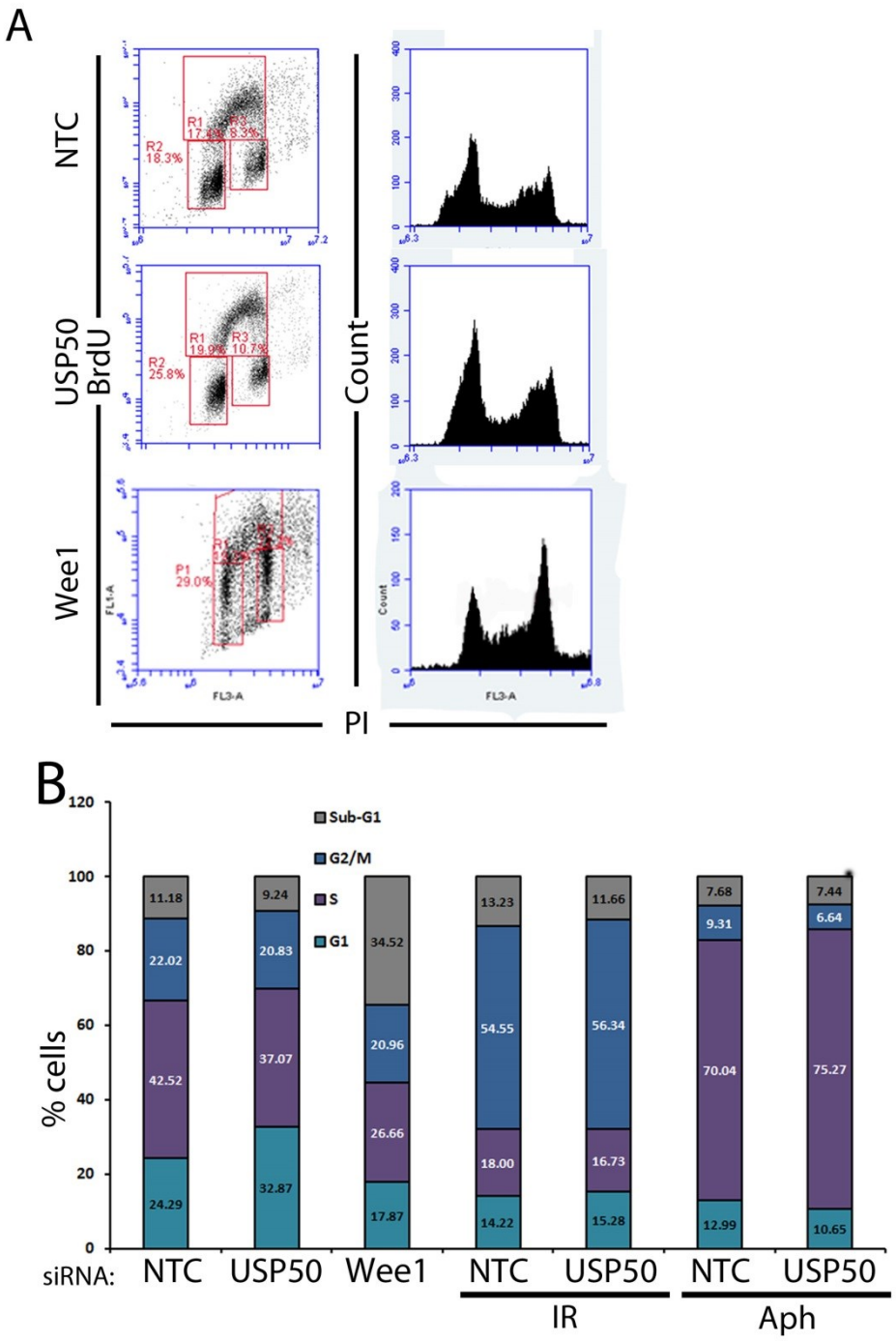


Figure 5.2 USP50 siRNA treatment does not cause a gross change in the cell cycle profile. U2OS cells were plated and treated with siRNA against NTC, USP50 and Wee1 for 72 hrs then pulsed with BrdU, fixed and stained with PI. **A)** FACs analysis of BrdU versus PI staining to give the cell cycle profile of each treatment. **B)** Prior to BrdU pulse cells were treated with 5Gy IR and left to recover for 24 hours, or 3nM Aph for 16 hours to trigger checkpoint activation. Cells were analysed by Accuri FACs and percent of cells in each phase of the cell cycle plotted (Average of 2 experiments).

5.4 The damage-associated foci observed upon USP50 siRNA treatment arise in a specific phase of the cell cycle

As a gross cell cycle defect was unlikely to be the cause of the increased damage-associated foci upon USP50 siRNA treatment, further experiments were required to understand why there was an observed increase in damage-associated foci. To determine if the foci were arising in a specific cell cycle phase, cells were counter stained with a cell cycle marker. Determining which phase of the cell cycle the damage-associated foci were arising in could indicate which pathway was faulty upon USP50 siRNA treatment. Initially to test this, HeLa cells were depleted of USP50 and stained for γH2AX, as a marker of damage and CENPF, a centromere protein, which stains cells late S and G2 phase of the cell cycle (Kao, McKenna et al. 2001).

It was apparent from this experiment that the increase in γH2AX was in CENPF positive cells. Quantification showed that there were foci in the CENPF positive cells but very few in the CENPF negative cells upon USP50 siRNA treatment (Fig 5.3 A and B). Therefore the damage foci observed were arising in a cell cycle specific manner, specifically S or G2 phase of the cell cycle.

It was hypothesised that the increased foci in G2 phase was due to a replication defect with damage being carried through into G2 phase. However as CENPF marks late S-phase cells, and G2 cells, a marker of S-phase was required to determine if the damage was arising during replication (Kao, McKenna et al. 2001).

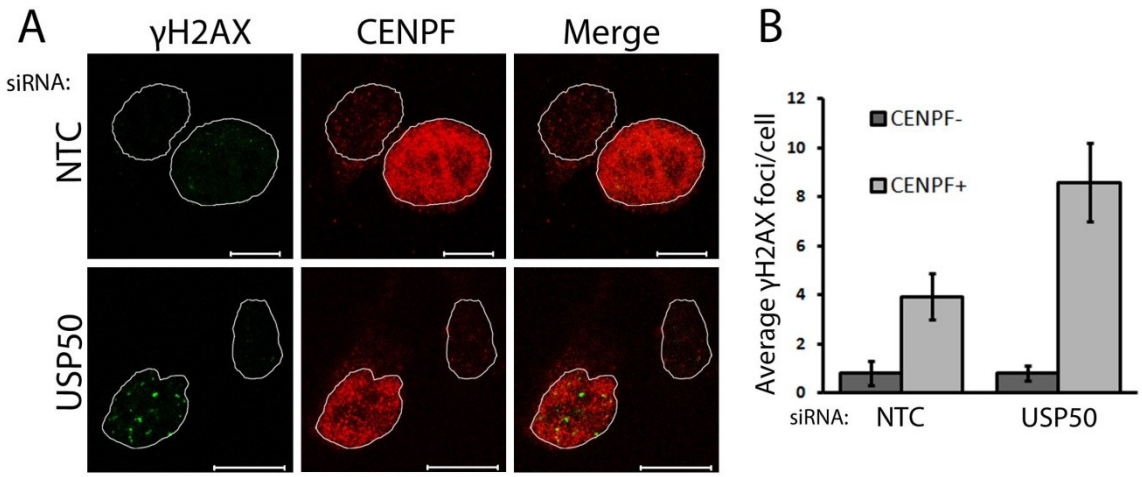


Figure 5.3. The increase in damage-associated foci in USP50 siRNA treated cells arose in a cell-cycle dependent manner. A) HeLa cells were plated and treated with NTC or USP50 siRNA for 72 hours, pre-extracted in 0.2% TritonX, fixed and then stained for CENPF as a late S/G2 marker and the damage marker γH2AX. Cells were imaged by confocal microscopy. White outline represents DNA as shown by Hoescht staining. Scale bar = 10 μM. **B)** Quantification of γH2AX foci in CENPF positive and negative cells after siRNA treatment (50 cells per treatment, n = 2)

To test whether the damage-associated foci were replication specific, HeLa cells were pulsed for 30 mins with EdU, a thymidine analogue, which is incorporated into DNA as it is replicated, thereby distinguishing S-phase cells (Chehrehasa, Meedeniya et al. 2009). Using the Click-it EdU kit, EdU was visualised by IF and counter stained for 53BP1 foci (Fig 5.4 A). Quantification of foci in EdU positive or EdU negative cells showed there were more 53BP1 foci in EdU positive cells compared to EdU negative cells upon USP50 depletion. There is also an increase of 53BP1 foci in EdU negative cells upon USP50 depletion but the majority of foci arose in the EdU positive cells indicating an S-phase dependent increase in damage-associated foci (Fig 5.4 B). Intensity of EdU staining was quantified by ImageJ software and plotted against the number of 53BP1 present in the cell. In USP50 depleted cells there were more foci in cells with more EdU incorporation, with a slight positive trend suggesting that there is a greater number of foci in cells where more EdU was incorporated (Fig 5.4 C). This result in conjunction with the CENPF staining indicates there is an increase in the number of foci in cells undergoing replication.

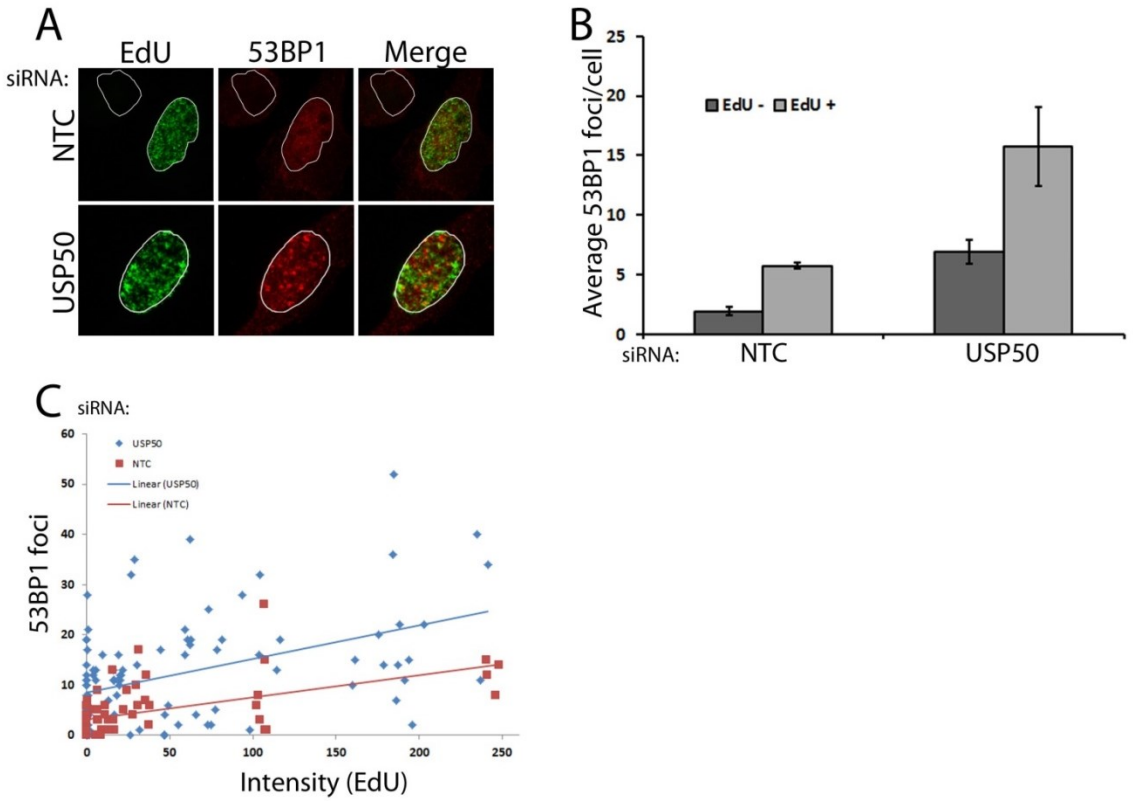


Figure 5.4. 53BP1 foci occur during S-phase. HeLa cells were plated on coverslips and treated with NTC or USP50 siRNA for 72 hours before being incubated with 10 µM EdU for 30mins. Cells were fixed and stained for EdU incorporation and 53BP1 foci. **A)** Cells were imaged by confocal microscopy. White line represents DNA as indicated by Hoescht staining. **B)** 53BP1 foci were counted in EdU positive and EdU negative cells and the average number of foci per cell plotted (40 cells per treatment, n=2) **C)** Images were analysed using ImageJ software to determine EdU intensity as a measure of S-phase progression. 53BP1 foci were counted and plotted against the EdU intensity to determine whether 53BP1 foci increased throughout S-Phase. Lines represent best fit of data fit (80 cells per treatment).

DSBs arise during replication when replication forks are stalled and cannot be restarted (reviewed in Petermann and Helleday 2010). These forks are cleaved by endonucleases; the major endonuclease is Mus81/Eme2, which displays specificity to 3'and 5' flaps as well as stalled replication fork structures. Cleavage by Mus81/Eme2 forms DSBs which are then repaired by HR repair to allow replication to continue (Pepe and West 2014). As the repair foci observed arise during S-phase, it was hypothesised that the Mus81/Eme2 endonuclease may be responsible for the increased 53BP1 foci observed on USP50 siRNA treatment. To test this, knockdowns of USP50, Mus81 and a combined knockdown of USP50 and Mus81 were carried out on HeLa cells and protein levels tested by Western blot analysis. USP50 siRNA did not appear to affect Mus81 protein levels but siRNA towards Mus81 caused a large drop in Mus81 protein in cells, confirming the knockdown (Fig 5.5 A).

Further to this, to confirm that the Mus81 endonuclease gave rise to S-phase specific 53BP1 foci, HeLa cells were treated with NTC or Mus81 siRNA before being subjected to 3mM HU for 16 hours. This exposure to HU would usually cause replication forks to collapse into DSBs (Saintigny, Delacote et al. 2001). In these experiments the loss of Mus81 protein caused a significant decrease in 53BP1 foci observed (Fig 5.5 B). Therefore the majority of 53BP1 foci arising during S-phase are Mus81-dependent.

To examine whether the damage-associated foci seen on USP50 depletion were dependent on Mus81, HeLa cells were treated with NTC, USP50, Mus81 or combined USP50 and Mus81 siRNA and stained for 53BP1. Quantification of foci showed that combined depletion of USP50 and Mus81 caused a reduction in 53BP1 foci when compared to USP50 siRNA alone (Fig 5.5 C). These results suggest that the 53BP1 foci that occur on USP50 knockdown are Mus81-dependent.

All together these results indicate that the damage foci which occur when cells are treated with USP50 siRNA arise during replication and are dependent on the endonuclease Mus81. This is consistent with a model where USP50 is required during replication potentially to stop replication forks stalling or the cleavage of stalled forks into DSBs occurs more readily.

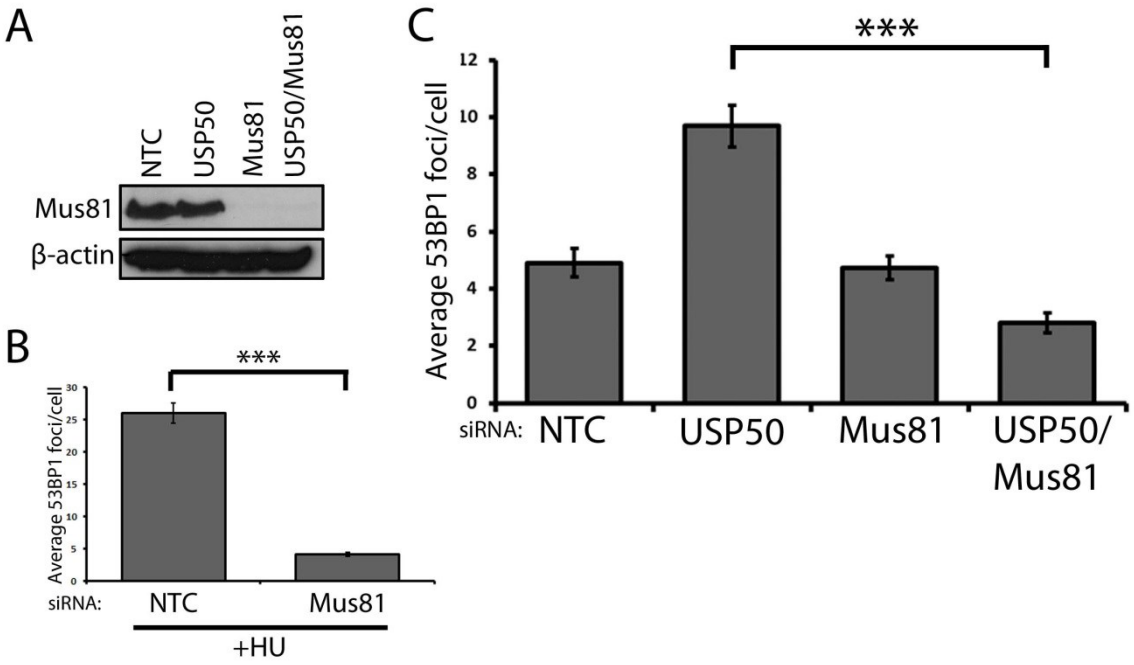


Figure 5.5 The increase of 53BP1 foci seen on USP50 depletion are dependent on Mus81. HeLa cells were plated and treated with NTC, USP50, Mus81 or a combination of Mus81 and USP50 siRNA for 72 hours. **A)** Western blot analysis showed Mus81 knockdown, β- actin was used as a loading control**. B)** HeLa cells treated with NTC or Mus81 siRNA were incubated for 56 hours. Cells were then incubated with 3mM HU for 16 hours to induce replication fork collapse. Cells were fixed and stained for 53BP1 foci. Microscopy was used to quantify the foci number and averages plotted (70 cells per treatment , n=3) **C)** Cells that were not treated with HU were fixed and stained for 53BP1 foci. Foci were quantified using confocal microscopy and numbers plotted (70 cells per treatment, n=3) Statistics were carried out using Students T-test.

5.5 USP50 siRNA treatment sensitises cells to replication-stress inducing drugs

To try and distinguish how USP50 is acting during replication, USP50 depleted cells were treated with various different drugs that disrupt replication. The process of replication can be disrupted in a variety of ways including the forks colliding with a lesion, such as crosslinked DNA, bulky lesions, depletion of nucleotides or inhibition of the polymerases (Edenberg 1976, Krokan, Wist et al. 1981, Koc, Wheeler et al. 2004). Distinguishing whether there is specific sensitivity to replication stress inducing drugs was used to give an indication into what pathway USP50 has a function in.

One cause of fork stalling is the collision with a crosslink within the DNA. This can either be an inter-strand crosslink or an intra-strand crosslink. Cisplatin is known to cause intrastrand crosslinks as well as interstrand crosslinks primarily between guanine residues. When the replication fork encounters an interstrand crosslink the two strands of DNA cannot be separated and the fork stalls (Chvalova, Brabec et al. 2007). The repair of the Cisplatin-induced damage requires HR-mediated repair, and hence BRCA2, therefore BRCA2 depletion was used to confirm Cisplatin treatment was working (Yuan, Lee et al. 1999, Bhattacharyya, Ear et al. 2000). HeLa cells treated with NTC, USP50 or BRCA2 siRNA, plated and left for two weeks before staining and colonies counted. The USP50 depleted cells showed no decrease in survival compared to NTC siRNA cells (Fig 5.6 A). BRCA2 depletion did cause increased sensitivity of cells to Cisplatin treatment, therefore showing Cisplatin was causing crosslinks within the cells. Thus, it appears USP50 depletion does not affect the cells ability to resolve the interstrand crosslinks.

UV light is known to cause replication stress by inducing thymidine dimers on one strand of the DNA. The bulky lesion caused has to be removed by NER or bypassed by TLS. A component of the NER pathway required for recognition, and subsequent repair, of bulky

lesions is the Xeroderma pigmentosum complementation group C (XPC) protein (Cordeiro-Stone and Nikolaishvili-Feinberg 2002, Melis, Luijten et al. 2011). Loss of XPC therefore causes sensitivity to UV light, so XPC depletion was used as a control to determine UV light treatment was causing the expected damage.

HeLa cells were treated with siRNA against NTC, USP50 and XPC and then exposed to increasing doses of UV light. Cells were plated at low density and left for two weeks to form colonies, stained with crystal violet and then colonies counted. Results were plotted as a percent of the colonies formed on the untreated plates for each knockdown. Although XPC depleted cells were highly sensitive to UV irradiation, USP50 depleted cells showed no significant difference between NTC treated cells (Fig 5.6 B). Therefore, despite the replication specific defect that UV light causes, USP50 does not have a role in the bypass or repair of this lesion.

The experiment was repeated using Camptothecin (Cpt), which causes replication specific damage as it binds to the topoisomerase Topo I. When the replication fork encounters the Cpt/Topo I ternary complex the single strand nick becomes a DSB (Hsiang, Lihou et al. 1989, Tsao, Russo et al. 1993). As repair of these breaks requires HR-mediated repair, loss of BRCA2 causes sensitivity to Cpt and can be used to confirm the action of the drug (Pommier, Redon et al. 2003). USP50 depletion caused increased sensitivity to Cpt at lower doses; however the sensitivity does appear to plateau at higher concentrations (Fig 5.6 C). However this result demonstrates the USP50 depletion does sensitise cells to Cpt.

Replication stress can be induced by other means, HU causes inhibition of the ribonucleotide reductase enzyme which results in a reduction of nucleotides (Koc, Wheeler et al. 2004). During replication this loss of nucleotides causes the replication fork to stall, extended

treatment with HU results in DSBs as stalled forks cannot be restarted (Petermann, Orta et al. 2010). To determine whether USP50 depletion caused sensitivity to HU treatment, HeLa cells were depleted of USP50 or BRCA2 and then exposed to increasing concentrations of HU for 16 hours. Two weeks post treatment colonies were stained using crystal violet and counted. Data was plotted as a percent of the untreated plates for each knockdown. Loss of BRCA2 causes sensitisation of cells to HU as BRCA2 is required to stabilise the Rad51 filaments that form at stalled forks and allow HR-mediated repair of collapsed forks (Davies and Pellegrini 2007, Esashi, Galkin et al. 2007). Cells depleted of USP50 formed fewer colonies than NTC control plates when treated to HU, with the percent of surviving colony forming units of USP50 depleted cells being very similar to the sensitivity caused by BRCA2 loss (Fig 5.6 D).

Together these survival assays indicate that loss of USP50 increases sensitivity to drugs that cause collapsed replication forks, rather than agents that cause cross links within the DNA.

To confirm that the replication-stress induced sensitivity was specific to USP50 loss, the inducible cell lines were utilised to knockdown endogenous USP50 and then rescue the depletion with exogenous overexpression of siRNA resistant USP50. The inducible cell lines, where USP50 depletion without overexpression of an shRNA resistant form of USP50, showed sensitivity to the HU as previously observed with siRNA depletion. However when USP50 was reintroduced to these cells the sensitivity to HU was no longer seen (Fig 5.6 E). This shows that USP50 loss is causing a replication specific sensitivity that can be rescued by a siRNA resistant form of USP50.

Therefore, the sensitivity is unlikely to be due to an off target effect of the siRNA and USP50 appears to play an important role in a replication pathway that is distinct from the repair of DNA crosslinks.

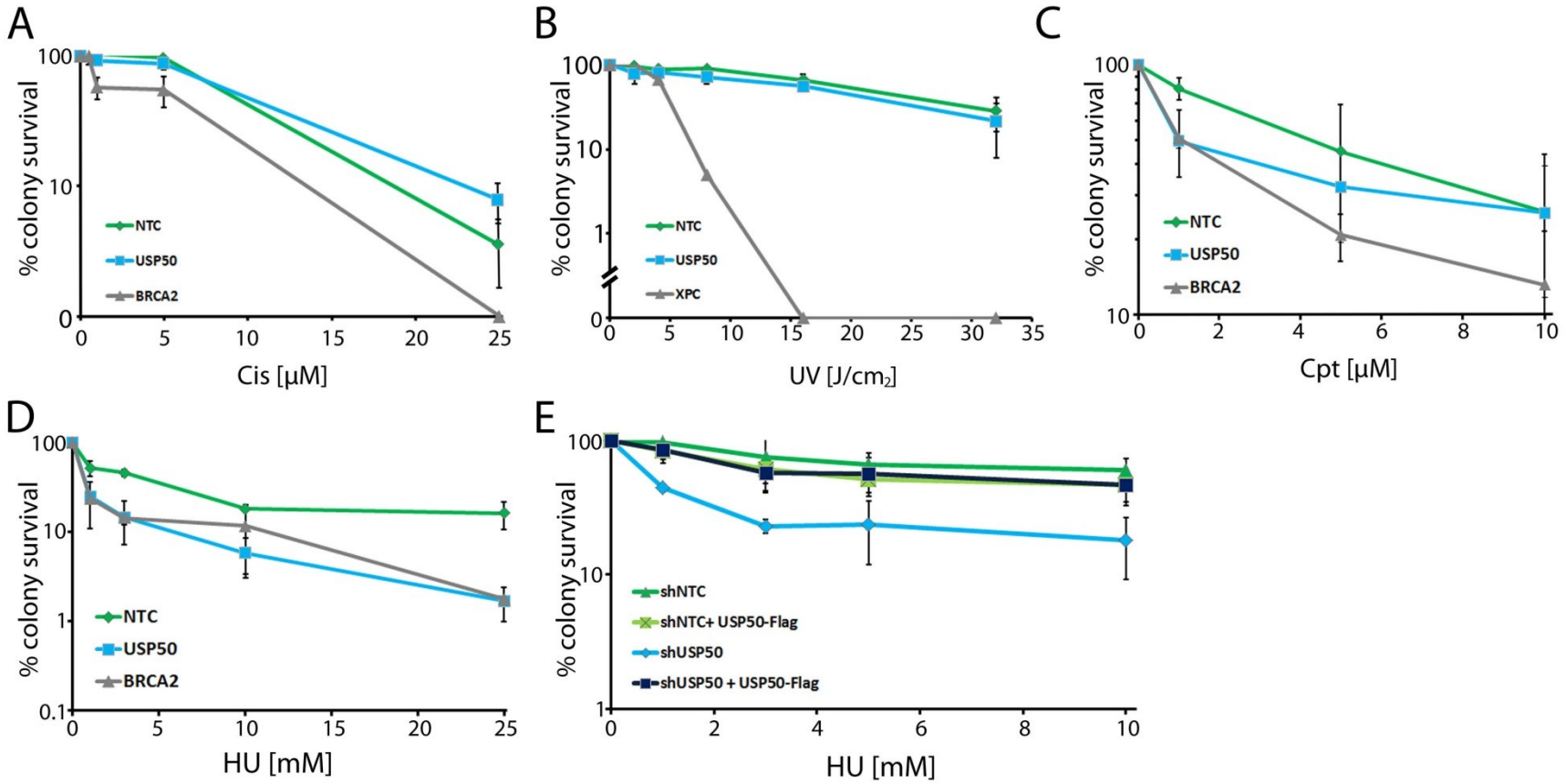


Figure 5.6. USP50 knockdown causes sensitivity to replication stress inducing drugs. HeLa cells were plated and transfected with siRNA against NTC, USP50 or BRCA2, treated as shown and incubated for two weeks. Cells were stained with 0.5% Crystal Violet in 50% Methanol and colonies counted. Percent survival was calculated from untreated colony numbers for each siRNA and plotted. **A)** Cells were incubated with increasing concentrations of Cis for 2 hours before plating at low density **B)** HeLa cells were treated with increasing doses of UV exposure before plating at low density, **C)** Cells were incubated for 2 hours in Cpt then replated at low densities. **D)** Cells were incubated with increasing concentrations of HU for 16 hours before plating at low density. **E)** HeLa stable cell lines were treated with IPTG to induce knockdown for 24 hours then treated with Dox to induce USP50 overexpression for a further 32 hours. Cells were treated with 3 mM HU for 16 hours and then plated at low (three replicates per treatment, n=4). Error bars = S.E.

5.6 USP50 depletion causes sensitivity to overexpression of constitutively active H-Ras^{V12}

Cancerous cells undergo increased proliferation and consequently replication, making cancer cells more prone to undergoing replication stress, with more damage arising during replication in cells undergoing oncogene-induced replication stress (Bartkova, Horejsi et al. 2005, Gorgoulis, Vassiliou et al. 2005). Activated oncogenes are thought to promote genomic instability by increasing the number of stalled or collapsed forks. Mutations in H-Ras that create a constitutively active oncogene has been shown to cause oncogene-induced replication stress as part of its action (reviewed in Hills and Diffley 2014).

USP50 loss sensitises cells to exogenous forms of replication stress, to test whether USP50 depletion could also sensitise cells to oncogene-induced replication stress, a human fibroblast cell line containing an inducible constitutively active h-Ras (h-Ras^{V12}) was obtained from the Petermann Group (University of Birmingham). Dr P. Kotsantis had already demonstrated the inducible Ras expression of these cells (Fig 5.7 A).

BJ-TERT- h-Ras^{V12} cells were plated and treated with Tamoxifen (4OHT) to induce h-Ras^{V12} expression or Ethanol (EtOH) as a control. Simultaneously, cells were transfected with NTC or USP50 siRNA and left for 72 hours before fixing and staining for 53BP1 foci. Quantification of the foci showed that knockdown of USP50 in EtOH treated cells increased foci. The expression of h-Ras^{V12} in NTC siRNA cells increased 53BP1 foci above that seen in EtOH cells. However, the combined knockdown of USP50 with h-Ras^{V12} expression caused a significant increase in the number of 53BP1 foci when compared to USP50 depletion in EtOH treated cells, suggesting the defect which leads to increased damage-foci on h-Ras^{v12} overexpression was exacerbated by USP50 depletion (Fig 5.7 B).

To determine whether cells were sensitive to this h-Ras^{V12} overexpression, the viability of cells was tested using the Cell titre Glo assay. Colony survival assays were attempted but due to the increased senescence upon h-Ras^{V12} overexpression there was not sufficient time for cells to form colonies that could be quantified. Therefore Cell titre Glo was used to measure the amount of ATP produced by cells as a measure of viability. BJ h-Ras^{V12} cells were plated on a 96 well plate at 1500 cells per well, transfected with NTC or USP50 siRNA and then treated with increasing concentrations of 4OHT. After 6 days, the Cell titre Glo reagent was added to the plate and readings were taken on the Victor plate reader.

Once data was plotted as a percentage of the untreated cells it was apparent that the siRNA USP50 treated cells were less viable upon h-Ras^{V12} expression than NTC. The viability appears to plateau which may be due to a threshold level of h-Ras^{V12} being expressed (Fig 5.7 C). Despite this, the USP50 depleted cells are less viable than control cells.

Together with the increase in 53BP1 foci seen in USP50 depleted cells expressing h-Ras^{V12} expression, the Cell titre Glo assay supports the theory that USP50 knockdown is sensitising cells to h-Ras^{V12} overexpression.

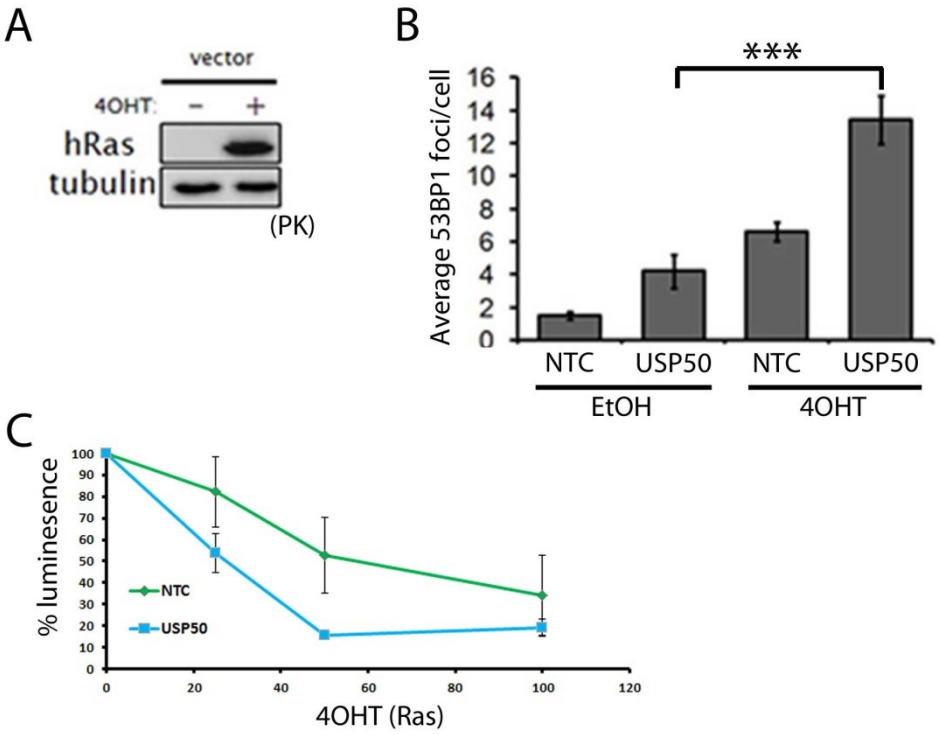


Figure 5.7. USP50 depletion increases sensitivity of cells to oncogene-induced replication stress. A) Western blot performed by Dr Kotstantis showing inducible h-Ras^{V12} expression after tamoxifen (4OHT) treatment of the Human foreskin fibroblasts BJ h-Ras^{V12} cells. **B)** BJ h-Ras^{V12} cells were plated and knockdown for USP50 or NTC and simultaneously treated with 30nM 4OHT or EtOH and incubated for 4 days. Cells were fixed and stained for 53BP1. Foci were counted and average foci number plotted (70 cells per treatment, n=3). **C)** BJ h-Ras^{V12} cells were plated in a 96 well plate and transfected with NTC or USP50 siRNA and simultaneously treated with increasing concentrations of 4OHT and incubated for 6 days. Cell titre Glo reagent was added to media and luminescence was read on the Victor plate reader. Percent luminescence was calculated from the untreated samples of each siRNA treatment and plotted. (Three replicates per treatment, n =2) Error bars = S.E.

5.7 USP50 siRNA treatment causes an increase in replication fork stalling or stopping

The spontaneous DNA damage, combined with the sensitivity to replication-stress inducing drugs, suggests that USP50 is regulating replication but the mechanism is unknown. To try and clarify how USP50 was working during replication, Fibre analysis on cells depleted of USP50 was carried out to study the kinetics of replication forks.

HeLa cells were treated with siRNA against NTC or USP50 for 72 hours before being incubated with thymidine analogue CldU for 20 mins, washed out, and then incubated for a further 20mins with IdU, another thymidine analogue (Fig 5.8 A). Cells were lysed and the DNA strands spread down a Snowcoat slide. Antibodies specific against CldU or IdU were used to distinguish replication fork progression by labelling the incorporation of the thymidine analogues. Fibres were analysed by fluorescent microscopy and structures and speeds calculated by ImageJ. The structures indicated in Fig 5.9B show different stages of replication to allow insight into how replication is proceeding after different treatments. Replication fork speed was calculated by measuring the CldU and IdU tracks of ongoing forks.

By distinguishing and quantifying the different structures, the effects of reduced USP50 expression on replication forks were analysed. There were more first label terminations in USP50 depleted cell, suggesting there were more forks stalling in USP50 depleted cells. However, there was not a significant increase in second label initiations which represent new origins firing (Fig 5.8 B). Therefore it may be that the forks are stalling but can be restarted prior to new origins being fired, or replication can be completed without the need for new origin firing.

When studying ongoing forks, the length of the CldU and IdU tracks can be used to calculate how fast replication forks are travelling. The length of the CldU and IdU tracks were measured using ImageJ and calculations performed to determine the length in μm. When plotting the fork speeds, the USP50 siRNA treated cells had the majority of forks running at between 0.6 and 0.8 kb/min, slightly slower than the control cells whose speed peaks around 1 kb/min. This can be seen by the shortening of fibre lengths, shown in Fig 5.8 C. There were also a greater proportion of forks running at the slower speeds and consequently less forks travelling at faster speeds, shifting the trend of the graph slightly left, suggesting the slowing of forks in the cells treated with USP50 siRNA. However, a peak of forks running between 1 and 1.2 kb/min goes against the trend seen in USP50 depleted cells (Fig 5.8 C). Overall however, there is a trend of forks running slower in USP50 depleted cells which indicates replication stress.

These results demonstrate that the loss of USP50 expression is causing perturbed replication, with more forks being stalled.

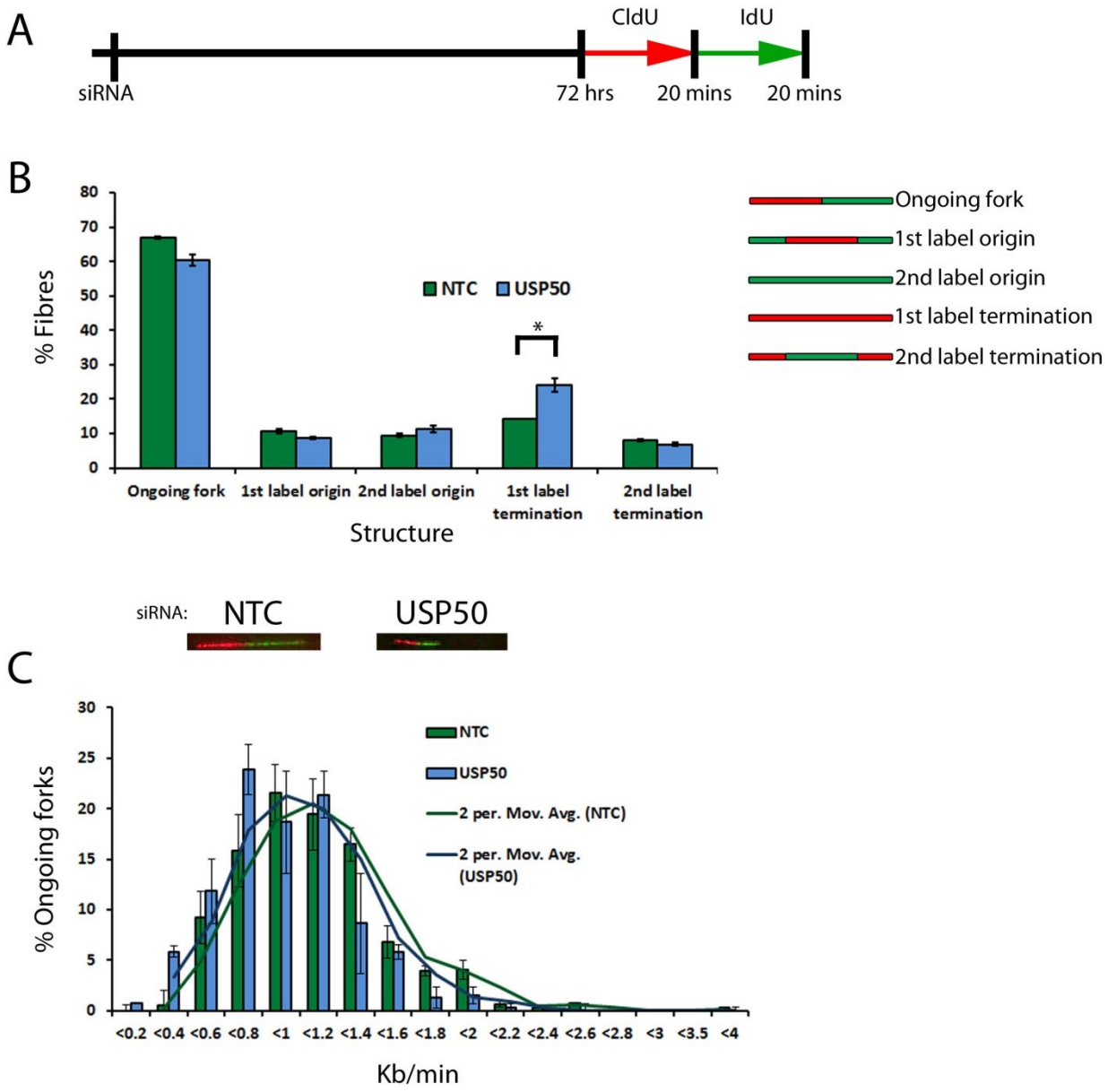


Figure 5.8 USP50 siRNA treatment causes an increase in stalled and slowed forks. A) Schematic of fibre treatment. HeLa cells were incubated for 20 mins CldU, washed and then incubated a further 20 mins with IdU. **B)** Cells were lysed and DNA spread on slides. Antibodies against CldU and IdU were used to show incorporation into DNA. Images of DNA Fibres were captured and analysed using ImageJ. Structures were counted and calculated as a percent of all structures and plotted (n=3). **C)** Fibre speeds were calculated by measuring ongoing forks in ImageJ and calculating Kb/min from fibre lengths. Representative images displayed. Results were plotted as a percent of total ongoing forks (n=3). Error bars = S.E.

5.8 USP50 siRNA treatment causes more replication forks to collapse

The fibre data shows that upon USP50 siRNA treatment there is an increase of replication forks being stalled or collapsed. The increase of 53BP1 foci in a Mus81 dependent manner suggest these are collapsed forks. When a fork stalls it becomes uncoupled from the active helicase creating long stretches of RPA coated ssDNA. This RPA signal recruits the ATR kinase, which along with other factors, stabilise the fork through phosphorylation of targets such as CHK1 and other replication fork components (Petermann, Woodcock et al. 2010, Couch, Bansbach et al. 2013) (Discussed in Chapter 1 Section.1.9.4). Therefore it was investigated whether the USP50 depletion was affecting the intra-S-phase checkpoint by looking at ATR activation. To determine if ATR was being activated western blot analysis was performed to see whether CHK1 was being phosphorylated in response to replication stress.

Cells transfected with NTC or USP50 siRNA for 70 hours were then treated with an ATR inhibitor VE-822 (ATRi), with or without 3mM HU, for 2 hours, before cells were lysed (Fokas, Prevo et al. 2014). Lysates were run on an SDS-page gel to measure CHK1 phosphorylation. Western blot analyses indicates that ATR is active upon knockdown of USP50, with CHK1 phosphorylation occurring at Ser345 in cells treated with 2 hours HU, where the HU causes stalled forks (Fig 5.9 A). This is lost upon ATRi treatment, demonstrating that the ATRi is able to effectively inhibit ATR kinase activity. When cells are not treated with HU there is no CHK1 phosphorylation regardless of siRNA or inhibitor treatment, therefore despite the increase in stalled or collapsed forks in USP50 depleted cells it is not sufficient to cause an ATR checkpoint activation that is visible by Western blot analysis. As CHK1 phosphorylation occurred following USP50 siRNA treatment when cells were treated with HU, it would seem USP50 is not causing a loss of the intra S-phase

checkpoint, as ATR is still able to phosphorylate CHK1 upon HU treatment. Therefore the increased 53BP1 foci do not arise because replication forks cannot be stabilised by ATR signalling in cells treated with USP50 siRNA.

By inhibiting ATR, stalled replication forks are no longer stabilised and are more prone to collapse into DSBs. Recent evidence suggests that loss of ATR signalling causes replication forks to actively collapse in a RNF4 and PLK1 dependent processes (Ragland, Patel et al. 2013). These collapsed forks are marked by 53BP1, hence the increase in 53BP1 foci upon ATRi treatment. To distinguish if USP50 is working downstream of the ATR kinase, USP50 knockdown was combined with ATRi. 53BP1 foci were quantified in NTC, USP50 siRNA, ATRi or combined USP50 siRNA and ATRi treated cells, after 2 hours of 3 mM HU. The use of 3 mM HU for two hours causes replication forks to stall, but not collapse into DSBs. This is due to ATR signalling being activated by the stalled forks, as previously described, which stabilises the stalled forks. The addition of ATRi should cause these stalled replication forks to collapse prematurely and cause an increase in 53BP1 foci numbers. Both USP50 siRNA and ATRi treatment caused increased 53BP1 foci above those seen in control cells, when cells were treated with HU. The ATRi treatment caused a greater number of 53BP1 foci than USP50 siRNA treatment alone; however USP50 siRNA combined with ATRi caused an additive increase of foci after 2 hours HU treatment (Fig 5.9 B and C). This would indicate that USP50 is not acting in the same pathway as ATR. It may be that loss of USP50 is leaving forks inherently unstable, which is aggravated by the loss of ATR signalling.

Despite ATR signalling being active in USP50 depleted cells it was investigated whether USP50 was causing stalled forks to collapse more readily. USP50 depleted cells were exposed to different HU exposures. HeLa cells were treated with either short (2 hours) or long (24 hours) exposure to 3mM HU (Fig 5.9 C and D). At 2 hours forks would be stalled but

stabilised by ATR, at 24 hours HU treatment stalled forks can no longer be stabilised by ATR and will collapse into DSBs (Petermann, Orta et al. 2010). If USP50 was causing accelerated collapse of stalled forks there would be an increase of 53BP1 foci in USP50 depleted cells treated with 3 mM HU for 2 hours.

Although there was an increase in 53BP1 foci in USP50 siRNA treated cells above NTC, after 2 hrs HU, the average foci per cell in USP50 depleted cells did not differ between no HU treatment and 2 hrs HU treatment, both having roughly six foci per cell (Fig 5.9 C and E). This suggests that USP50 siRNA is not destabilising stalled replication forks as if it was it would be expected that there would be more foci in the 2 hr HU treatment than no HU treatment upon USP50 siRNA treatment (Fig 5.9 C). This correlates with the fact that ATRi caused a greater increase in 53BP1 foci after 2 hrs HU than USP50 depletion. Therefore this data, along with the previous data which suggests USP50 and ATR were not in the same pathway, indicates USP50 is not required to stabilise stalled replication forks.

Conversely, USP50 depleted cells treated with 24 hours HU had a large increase in 53BP1 foci when compared against 0 and 2 hours HU treated cells (Fig 5.9 D). Therefore stalled forks do not appear to be collapsing more readily, but that there are more forks collapsing once they can no longer be stabilised by ATR.

Interestingly, when cells were transfected with NTC or USP50 siRNA and then treated with ATRi but were not subjected to HU treatment, combined ATRi and USP50 siRNA treated cells had an increase in 53BP1 foci. However, the cells treated with both USP50 siRNA and ATRi did not show a significant increase in 53BP1 foci above USP50 siRNA alone and only a slight increase above ATRi treated cells (Fig 5.9 C). This was unlike the result seen when cells were treated with 2 hours 3mM HU, where there is an additive increase in 53BP1 foci.

The lack of additive increase in USP50 siRNA and ATRi treated cells when there was no HU suggests that, without an increased number of stalled forks caused by HU treatment, the stalled forks affected by ATRi and USP50 siRNA are the same, hence there is not the increase.

The addition of 2 hrs HU to USP50 depleted cells did not increase the average number of foci per cell. However, as the experiment only measures 53BP1 foci, assumed to be collapsed forks, it is unknown if USP50 depletion is having an effect on the stalling of forks. However, it appears that the collapse of forks in USP50 depleted cells is not aggravated by 2 hr HU treatment.

As these experiments do not measure stalled forks, but markers of DSBs, there are two explanations for the increase in 53BP1 foci in USP50 depleted cells in the 24 hour HU treatment (Fig 5.9 D) as well as the combined USP50 depleted and ATRi treated cells (Fig 5.9 B and C). Firstly, more forks are stalling initially, which eventually form DSBs. Or secondly, a pathway which allows replication to continue without the formation of DSBs is no longer functional and therefore forks which could normally be restored and restarted are forming breaks. This would indicate that replication fork restart is defective upon USP50 siRNA treatment.

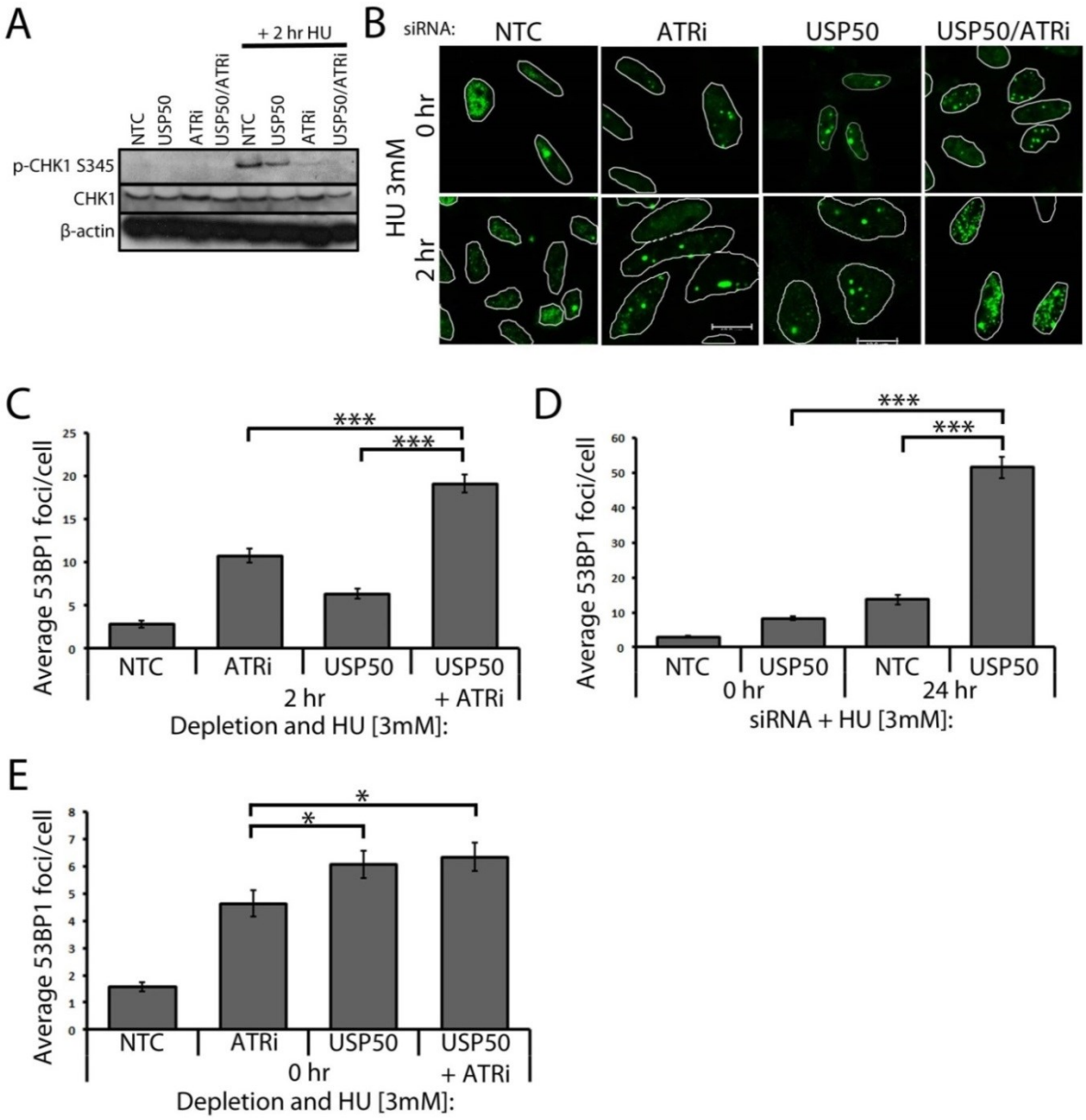


Figure 5.9 USP50 depletion does not cause a defect in the inter S-phase checkpoint. **A)** HeLa cells were plated and transfected with NTC or USP50 siRNA and left for 68 hours. Cells were treated with 5 μM ATRi (VE-821) for 2 hours. After ATRi treatment cells were treated with 2 hours 3 mM HU. Cells were lysed and western blot analysis performed, demonstrating ATR activation after HU treatment by CHK1 phosphorylation, this was inhibited by VE-821 treatment. **B)** Cells were fixed and stained for 53BP1 Average foci number per treatment were plotted (50 cells per treatment, n=2) **C)** Quantification of 53BP1 foci in ATRi treated cells. **D)** HeLa cells were plated and transfected with NTC or USP50 siRNA for a total of 72 hours, prior to fixing cells were either left untreated or incubated with 3 mM HU for either 2 or 24 hours. Cells were fixed and stained for 53BP1and average foci number plotted. **E)** Cells treated as in B) and C) but not treated with HU. Average 53BP1 foci plotted. (50 cells per treatment, n=2) Error bars = S.E.

5.9 USP50 and WRN depletion are epistatic for 53BP1 foci

Stalled replication forks can be restarted once the block on replication has been resolved or bypassed (Discussed in Chapter 1 Section1.9.4). However, if the fork cannot be restarted the replication fork can collapse into a DSB forms which can be repaired by HR-mediated repair to allow replication to continue (Saleh-Gohari, Bryant et al. 2005). One mechanism that allows replication restart prior to break formation is through fork remodelling by helicases, including the WRN and SMARCAL1 helicases (reviewed in Petermann and Helleday 2010).

The WRN helicase and its interacting partner WRNIP are known to be ubiquitinated, which could potentially mean it is a substrate for USP50 binding (Bish, Fregoso et al. 2008, Li, Wang et al. 2010). It has been demonstrated that WRN depletion causes an increase in DNA damage during replication without causing a cell-cycle-defect (Sidorova, Li et al. 2008, Patro, Frohlich et al. 2011).

Further to this, depletion of SMARCAL1 increases spontaneous DNA damage foci (Bansbach, Betous et al. 2009). As reduction in these helicases cause phenotypes similar to those observed on USP50 depletion, it was hypothesised that USP50 might work in the same pathways as one of these helicases.

HeLa cells were plated and transfected with NTC, USP50, WRN, SMARCAL1 single siRNAs or combined USP50 and WRN or combined USP50 and SMARCAL1 siRNA for 72 hours. Cells were fixed and then stained for 53BP1. Foci were counted and the average number plotted on a graph. The depletion of USP50 caused an increase in 53BP1 foci, as did WRN and SMARCAL1 knockdown, with all the siRNAs alone giving a similar average number of 53BP1 foci per cell. When USP50 and WRN were knocked down together there was not a significant difference in the number of 53BP1 foci between the co-depleted cells

and either siRNA alone (Fig 5.10 A and B). Conversely, the co-depletion of USP50 and SMARCAL1 showed a significant increase of 53BP1 foci when compared to the individual siRNA treatments. This increase was not additive, but indicates that USP50 and SMARCAL1 are giving rise to 53BP1 through different pathways, though there may be some functional overlap of these pathways. Due to the increase in 53BP1 foci on co-depletion of USP50 with SMARCAL1 this pathway was not studied any further. As there was no increase in average 53BP1 foci when USP50 and WRN were co-depleted in cells, it suggests that the 53BP1 foci are arising through the same mechanism which required further investigation.

To confirm the knockdown of WRN, a Western blot was performed to investigate protein levels. It was apparent that WRN protein levels were reduced in cells treated with siRNA against WRN. However, WRN protein was visible in both NTC and USP50 siRNA transfected cells (Fig 5.10 C). As well as confirming the knockdown of the protein, it also demonstrated that USP50 depletion was not causing a loss of WRN protein.

The epistatic nature of the 53BP1 in USP50 and WRN co-depleted cells when compared against either siRNA alone is indicative of these proteins acting in the same pathway. This suggests USP50 may have a role in restoring stalled forks through the WRN helicase to prevent collapse into a DSB.

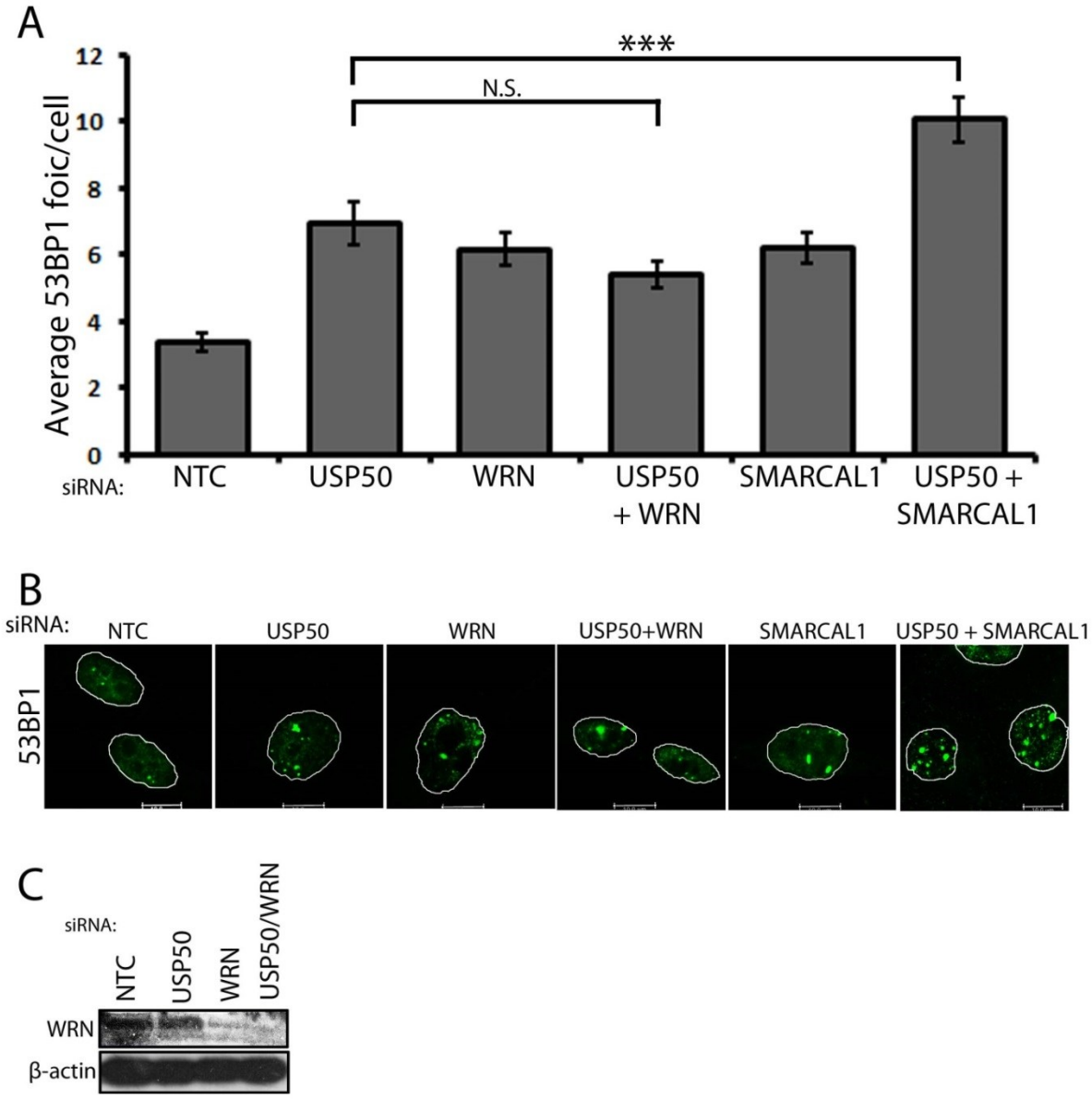


Figure 5.10 USP50 appears to work in the same pathway as WRN. HeLa cells were treated with NTC, USP50, WRN, SMARCAL1 or USP50 and WRN or USP50 and SMARCAL1 siRNA for 72 hours. **A)** Cells were fixed and stained for 53BP1. Foci were quantified and averages plotted (70 cells per treatment, n=3) Significance was determined by Students T-test, Scale bars = S.E. **B)** Images showing representative 53BP1 foci per treatment. White line represents DNA as visualised by Hoescht staining. Scale bar = 10μM **C)** Western blot analysis confirming reduction in WRN protein upon siRNA treatment.

5.10 Proteasome inhibition can reduce 53BP1 foci in USP50 depleted cells

Ubiquitination is a major signal for the proteasome and as USP50 Ub-binding appears to be important for its role within the cell (Chapter 4 Section 4.3), it was tested whether proteolytic degradation also played a role in regulating the pathway which USP50 is functioning in.

MG132 treatment has been shown to inhibit 53BP1 foci formation (Jacquemont and Taniguchi 2007); however introduction of Ub can restore foci as discussed in Chapter 3 Section 3.3 (Fig 5.11 A). The loss of the proteolytic activity is coupled with a loss of free Ub (Hanna, Leggett et al. 2003). The formation of poly-Ub chains at sites of damage is required for 53BP1 to be recruited, by removing the chromatin bound protein JMJD2A, allowing 53BP1 to access the H4K20me2 mark (Mallette, Mattiroli et al. 2012). 53BP1 also interacts directly with the mono-Ub mark on H2AK20 via its UDR (Fradet-Turcotte, Canny et al. 2013). Therefore in proteasome inhibited cells 53BP1 foci can form in the absence of the proteolytic activity, as long as there is an excess of Ub present (Chapter 3 Section 3.3).

In order to determine if the increase in damage-associated foci in USP50 siRNA treated cells was linked with degradation by the proteasome, cells depleted of USP50 for 24 hours were transfected with Myc-Ub for a further 48 hours to create an excess of Ub within the cells, allowing Ub-signalling to occur despite the inhibited proteasome. Before fixing, cells were treated with MG132 for a further hour. Quantification of the foci showed that exposure to MG132 reduced the number of 53BP1 regardless of USP50 status. However, when cells were treated with MG132 but supplemented with Myc-Ub, 53BP1 foci cannot be restored upon USP50 depletion despite the ability of the cell to form 53BP1 foci (Fig 5.11 B). Therefore the 53BP1 foci that arise upon USP50 depletion appear to be dependent on the proteolytic activity of the proteasome.

As proteasome inhibition reduces 53BP1 foci upon USP50 depletion, there may be a role for USP50 in blocking the proteasome from degrading a substrate.

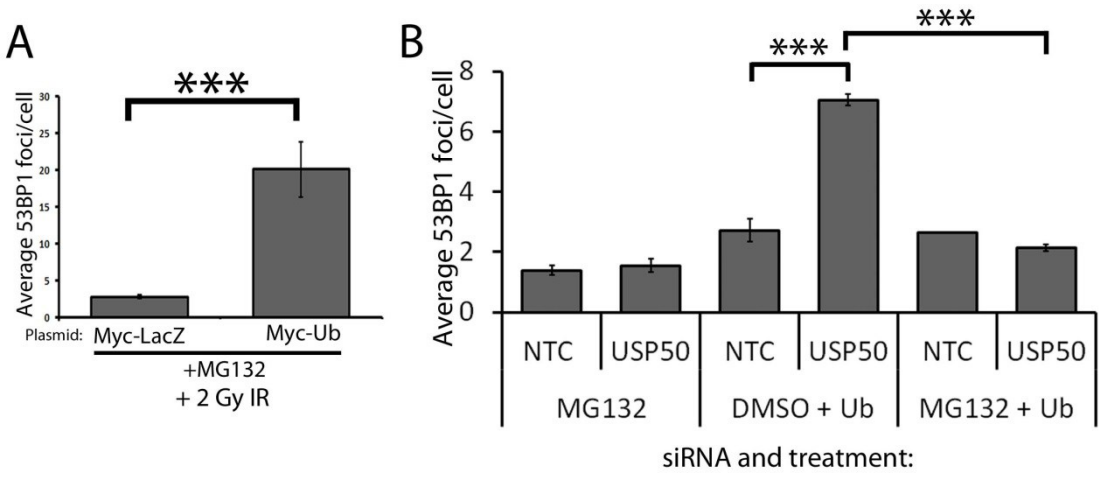


Figure 5.11 Increased 53BP1 foci formation seen on USP50 siRNA treatment is no longer observed upon MG132 treatment. HeLa cells were treated with NTC or USP50 siRNA and 24 hours later transfected with Myc-LacZ or Myc-Ub and incubated for 43 hours. Cells were treated with 5 μM MG132 before treatment. **A)** Cells were subjected to 2 Gy IR then allowed to recover for 1 hour before fixing. Cells were stained for 53BP1 and foci counted. Average foci numbers were plotted (70 cells per treatment, n=3). **B)** Cells were fixed, cells and stained for 53BP1 and foci numbers quantified. Average foci number was plotted, significance was determined by Students T-test (50 cells per treatment, n = 2) Error bars = S.E.

5.11 Summary

The results of this chapter demonstrate that USP50 depletion causes an increase in damage-associated foci arising during S-phase of the cell cycle in a Mus81-dependent manner (Fig 5.5). The increase in 53BP1 foci seen in EdU negative cells upon USP50 depletion may be 53BP1 bodies which represent damage carried through the cell cycle into G1, but the damage may still have arisen during replication (Fig 5.4) (Lukas, Savic et al. 2011). Thus there appears to be a defect in the replication of the cells treated with USP50 siRNA as the majority of 53BP1 foci do occur in an S-phase specific manner (Fig 5.3 and 5.4). The fault in replication causes an increased sensitivity to not only drugs that cause replication stress, but to overexpression of h-Ras^{V12}, which provides a model of oncogene-induced replication stress (Fig 5.7). However, the mechanism by which USP50 is facilitating faithful replication is still under investigation despite results in this chapter have given an indication to the pathway involved.

In USP50 depleted cells the intra-S-phase checkpoint still remains intact as ATR is still active, suggesting stalled replication forks can still be stabilised through ATR signalling (Fig 5.9). Although when ATR is no longer sufficient to stabilise the stalled replication forks, the collapse of the forks is exacerbated by USP50 loss. Stalled forks can be restarted by mechanisms including helicase dependent fork remodelling (reviewed in Petermann and Helleday 2010). As 53BP1 foci formation appears to be epistatic with WRN depletion in USP50 knockdown cells, it is likely that USP50 is functioning as part of the same pathway that WRN acts in (Fig 5.10). The increase in DSBs could consequently be stalled forks that could not be restored by the WRN helicase and thus are converted into DSBs before repair.

Although USP50 appears to be part of the WRN pathway, it is unknown what role USP50 plays in restoring stalled replication forks. As the WRN protein does not appear to be lost

upon USP50 depletion it is unknown how USP50 could be regulating this pathway. Therefore more research is required to elucidate the mechanism of USP50 control within the WRN pathway.

By studying 53BP1 foci, there is an assumption of DNA damage, corroborated by the loss of 53BP1 upon Mus81 depletion; however no evidence to show increased DSBs has been demonstrated. Further to this, by studying 53BP1 foci, it cannot be said whether there is an increase in stalled forks in USP50 depleted cells that eventually form DSBs or whether there is the same number of stalled forks but USP50 depletion prevents restart by other mechanisms. Although fibre data can go some way to show that there is increased first label termination, again it is unknown if these are stalled or collapsed replication forks (Fig 5.8). If USP50 is working in the WRN pathway it would suggest that normally USP50 is enabling fork restart after stalling and therefore depletion causes increased fork collapse, however this needs to be further tested.

The WRN protein is known to be recruited to the replication fork (Su, Mukherjee et al. 2014); however the localisation of USP50 has not been shown. Exogenous GFP-tagged USP50 is usually cytoplasmic but has been shown to translocate to the nucleus upon damage (Aressy, Jullien et al. 2010). In our hands toxicity of overexpressing the protein has led to problems studying the localisation of USP50, however further work, including iPOND may be utilised to determine if USP50 is at the fork, or is regulating another factor away from the replication fork.

Similarly to Chapter 3, MG132 has been used as a proteasome inhibitor, the loss of 53BP1 foci indicate that the inhibitor is working but there is no evidence into the reduction of proteolytic activities (Fig 5.11). The role of the proteasome in the USP50 pathway will also

require further investigation, potentially using fibre analysis to determine if the decrease in foci also correlates with a rescue of other defects seen on USP50 depletion including replication fork structures and fork speed. This can be used to determine whether USP50 is acting to protect a substrate, which is required for fork stability, from degradation.

The use of cells which overexpress h-Ras^{v12} are used as a model of replication stress, although the overexpression of this oncogene can cause other defects within the cell (Fig 5.7). Without evidence that the decrease in viability of cells upon USP50 depletion is due to the increased replication stress rather than other faults caused by h-Ras^{V12} overexpression it can only be suggested that USP50 is sensitising cells to oncogene-induced replication stress. This suggestion is supported by the decreased survival of USP50 knockdown cells to exogenous replication stress inducing drugs, such as HU and CPT (Fig 5.6). Therefore further work could be performed using other models of oncogene-induced replication stress to confirm USP50 causes sensitivity to other causes of replication stress.

In addition, it would be interesting to see the role of USP50 in non-cancerous cells as most of the experiments to date have been carried out in cancer cell lines. This would be interesting as it may prove a useful target for cancer therapies if USP50 could specifically sensitise cancer cells to replication stress, especially to oncogene-induced replication stress.

Although the mechanism by which USP50 allows faithful replication is still not elucidated, the role of USP50 in replication has not been shown before, presenting a novel role for this Ub-binding protein. Current experiments suggest a model where stalled forks that can no longer be stabilised by ATR are restructured in a USP50/WRN dependent manner to allow restart and when USP50 is lost the fork cannot be restarted by this mechanism and is

converted into a DSB (Fig 5.12). USP50 also could provide a potential therapeutic target that could specifically sensitise cancer cells to replication stress, either exogenous or endogenous.

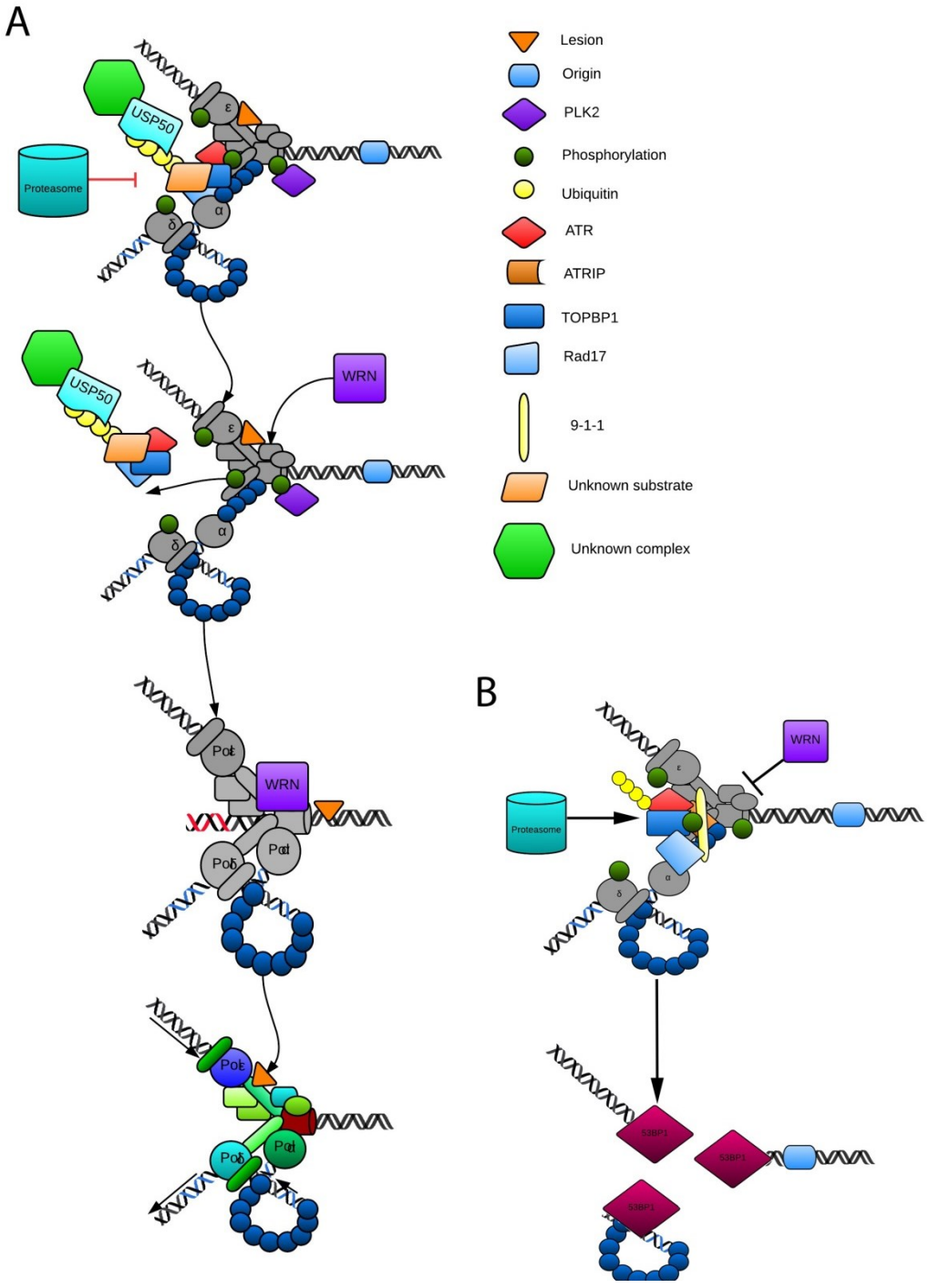


Fig 5.12 Hypothesised model of USP50 function during replication. A) USP50 binds to a ubiquitinated substrates and as part of a larger complex, possibly VCP. USP50 works after ATR signalling is no longer stabilising forks and promotes WRN fork remodelling to allow replication to continue without forks collapsing into DSBs. B) When USP50 is not present WRN cannot remodel the fork and therefore the fork collapses into a DSB.

6.1 Introduction

Ub is an important signalling molecule in eukaryotic cells and the role it plays in the repair of DSBs has been extensively studied, with K63-linked chains being required for protein recruitment to breaks (Sobhian, Shao et al. 2007, Doil, Mailand et al. 2009). Conversely, the function of Ub in replication is an emerging field, with evidence for ubiquitination being necessary for the bypass of lesions by the replication fork (Hoege, Pfander et al. 2002, Stelter and Ulrich 2003, Kannouche, Wing et al. 2004, Watanabe, Tateishi et al. 2004). More recent work has also demonstrated the need for ubiquitination of Mcm7 at replication termination to remove fork components from the DNA (Maric, Maculins et al. 2014, Moreno, Bailey et al. 2014).

DUBs have been shown to be important for the regulation of these Ub signals, with loss of DUBs having negative effects on the pathways involved. There are already several DUBs that have been implicated in DSB repair discussed in Chapter 1 Section 1.8.4 (Panier and Durocher 2013). The JAMM-type DUB, BRCC36, localises to damage through the Rap80 complex, acting on the K63-linked chains produced by RNF8 (Dong, Hakimi et al. 2003, Shao, Lilli et al. 2009). USP3 is required for the removal of Ub from histones H2A and H2B, and consequent regulation of repair protein recruitment (Nicassio, Corrado et al. 2007, Doil, Mailand et al. 2009). Another USP-type DUB—USP44—has been shown to displace RNF168 from damage sites and its depletion causes a slight increase in conjugated Ub at DSBs (Mosbech, Lukas et al. 2013). This shows that more than one DUB is likely to be involved in controlling the Ub at damage site. Further to this, both OTUB1 and OTUB2 have been shown to inhibit the formation of the Ub chains at DSBs, both acting to reduce the amount of Ub conjugates at DNA damage. OTUB2 reduces chains via its catalytic activity, whereas OTUB1 does not require its enzymatic activity to inhibit chains (Kato, Nakajima et

al. 2014). Instead, OTUB1 interacts with the E2 enzyme, Ubc13, stopping the formation of RNF8/RNF168-dependent K63-linked chains (Nakada, Tai et al. 2010). As many components of the repair pathway are ubiquitinated, there is a requirement for many DUBs at DSBs to ensure tight regulation of repair. The high level of regulation of proteins at DSBs means that aberrant repair signalling is unlikely to occur.

As the role of Ub in replication is less characterised than it is in DSB repair, there are fewer DUBs known to be involved. USP1 is known to deubiquitinate PCNA once the clamp protein has promoted the bypass of lesions blocking the fork (Huang, Nijman et al. 2006). Recent work has also shown that USP20 is phosphorylated by ATR in response to replication stress. Once phosphorylated, USP20 is stabilised and in turn deubiquitinates and stabilises Claspin to maintain the fork in response to stalling (Yuan, Luo et al. 2014). Similarly to DSB repair, it is likely that there are many DUBs involved in controlling replication in order to guarantee correct DNA duplication. However, as less is known about Ub in replication, it follows that the DUBs have also not been characterised.

Results of an siRNA screen against 103 putative DUBs in the human genome indicated a role for two DUBs in response to HU treatment. The experiments discussed within this thesis show a role for POH1/PSMD14/rpn11, a component of the proteasome (Glickman, Rubin et al. 1998), at DSBs. Further to this, a poorly characterised DUB, USP50 is involved in faithful replication—potentially in promoting fork stability.

6.2 The proteasome and its associated DUB, POH1, in DSB repair

6.2.1 Functions of the proteasome at DSBs

The proteasome is an integral component of the DSB repair pathway (Krogan, Lam et al. 2004, Jacquemont and Taniguchi 2007). The 19S lid of the proteasome contains a JAMM

DUB called POH1, which removes the Ub-chains from substrates and, therefore, provides the Ub for the free-Ub pool (Yao and Cohen 2002).

Proteasome inhibitors are commonly used to study the proteolytic activity of the proteasome, however, the loss of degradation is coupled with a depletion of free-Ub (Hanna, Leggett et al. 2003). The reduction in free-Ub means new Ub modifications cannot be formed, causing defects that might be attributed to the proteolytic activities of the proteasome. Previous studies have demonstrated that proteasome inhibition disrupts the formation of 53BP1 and BRCA1 foci at DSBs (Jacquemont and Taniguchi 2007, Shi, Ma et al. 2008).

Both BRCA1 and 53BP1 proteins are recruited to DSBs via Ub-chains, however the mechanisms of recruitment is different for these repair proteins. BRCA1 is recruited by K63-linked Ub ligated by RNF8 and RNF168 through the interaction of BRCA1 with Rap80. The tandem UIM motifs in Rap80 bind to the K63-chains bringing in BRCA1 as part of the BRCA1-A complex (Kim et al. 2007, Sobhian et al. 2007, Wang et al. 2007)

Despite the recognition of mono-Ub by the UDR in 53BP1, there is only *in vitro* evidence of 53BP1 interacting with K63-linked di-Ub chains (Gatti M et al 2015). 53BP1 also binds to modified histones via its Tudor domain, binding to H4K20me2. In undamaged chromatin there are two currently-researched proteins that bind to the H4K20me2 mark that could be blocking 53BP1 recruitment—JMJD2A and L3MBTL1 (Acs, Luijsterburg et al. 2011, Mallette, Mattiroli et al. 2012).Current research suggests that the modification of both JMJD2A and L3MBTL1 by poly-Ub facilitate the removal of these proteins thereby allowing access to H4K20me2 by 53BP1 (Acs, Luijsterburg et al. 2011, Mallette, Mattiroli et al. 2012)

A study by Shi *et al.* showed that MDC1 clearance from breaks was dependent on the proteasome, claiming the proteolytic activity was the necessary activity. Without the

degradation of MDC1, BRCA1 could not be recruited (Shi, Ma et al. 2008). However, as demonstrated in Chapter 3 Section 3.2 the removal of MDC1 was not solely dependent on the proteolytic activity of the proteasome, as Myc-Ub transfection could significantly reduce the number of MDC1 foci in cells treated with MG132 prior to irradiation. This suggests that the clearance of MDC1 was possible despite the impaired proteolytic action of the proteasome. Therefore MDC1 clearance requires Ub-modifications in order to be cleared, which is not possible in cells treated with MG132 due to the loss of the free-Ub pool (Hanna, Leggett et al. 2003).

Shi *et al* demonstrated that BRCA1 foci formation was disrupted by proteasome inhibition, which they attributed to MDC1 persistence at break sites. I also showed that BRCA1 foci could not form in MG132 treated cells and this could not be restored by the introduction of excess Ub into the cell. BRCA1 recruitment therefore does require the proteolytic activity of the proteasome. Yet, as previously discussed, MDC1 could be cleared to levels seen in control cells by the introduction of Myc-Ub into MG132 treated cells; suggesting MDC1 is not the block that is stopping BRCA1 recruitment (Chapter 3 Section 3.2). It is therefore likely that the recruitment of BRCA1 requires the degradation of an upstream protein, however further research is required into what this substrate might be as these results suggest MDC1 is not the block on BRCA1 recruitment.

On the other hand, 53BP1 foci recruitment was restored by the transfection of Myc-Ub (Chapter 3 Section 3.3). This observation demonstrates that while the proteolytic action of the proteasome is still impaired, 53BP1 foci could form. The recycling of Ub back into the free-Ub pool is an essential role of the proteasome for 53BP1 recruitment.

The use of MG132 although previously shown to block degradation by the proteasome, western blot analysis to confirm that degradation could not be carried out by the cell was not performed as part of this project. However, the defects seen in repair protein kinetics upon MG132 treatment, such as MDC1 persistence and loss of BRCA1 and 53BP1 foci formation matches previously published faults in repair foci kinetics upon proteasome inhibition. Further to this there was no analysis of free-Ub levels within cells treated with MG132 to confirm that the affect observed could be due to a loss of the free-Ub pool. However, the rescuing of certain phenotypes, such as MDC1 clearance and 53BP1 recruitment, upon addition of Myc-Ub indicates the defects seen upon MG132 treatment was due to Ub starvation rather than loss of proteolysis.

These results demonstrate that distinct pathways are required for the recruitment of 53BP1 and BRCA1 to DSBs despite both relying on the poly-Ub that forms.

6.2.2 Ub linkages in the DSB response

The use of Ub mutations converting K to R were used in Chapter 3 Section 3.2 and 3.3 to try and determine which linkages of poly-Ub were important, firstly in MDC1 clearance and, secondly in 53BP1 recruitment. While I was not able to confirm if a single specific Ub lysine residue was needed for the MDC1 clearance, the K63 residue was important for 53BP1 foci formation. K63-linked chains are formed at DSBs by two E3 ligases—RNF8 and RNF168—signalling the recruitment of repair proteins such as BRCA1-A complex (Kolas, Chapman et al. 2007, Mailand, Bekker-Jensen et al. 2007, Wang and Elledge 2007, Doil, Mailand et al. 2009). Thus, as K63-linked chains are thought to be the predominant chain type at DSBs it follows that 53BP1 recruitment relies on the K63 residue within Ub. Despite the importance of the K63 residue, K6 and K27 also appear to play a minor role in the recruitment of 53BP1.

53BP1 foci recruitment was partially reduced by the introduction of K6R-Ub, suggesting this residue was also important for 53BP1 foci formation. However, foci numbers are not reduced to levels seen in MG132 treated cells, nor K63R transfected cells, suggesting there is only a partial reliance on the K6 residue. The reciprocal experiments with only the K63 residue showed that 53BP1 foci could not be fully restored by Ub with this residue alone, whereas the reintroduction of K6-K63 only Ub showed a greater increase in foci, though still not completely back to WT levels. These results demonstrate a need for both K63 and K6 residues in Ub. Therefore both K63 and K6 chains and chains of mixed K6 and K63 linkages are required for 53BP1 recruitment to DSBs.

Recent work has suggested that the K27 poly-Ub chains are created by RNF168 and are a major signalling molecule for 53BP1 recruitment (Gatti M et al 2015). The experiments performed in this thesis show that K27O-Ub is not sufficient to restore 53BP1, however the introduction of K27R-Ub did show a decrease in 53BP1 formation to a similar extent as K6R. These results show that while the K27 residue does not appear to be the major linkage as suggested in Gatti *et al* it may play a role in the recruitment of 53BP1. As 53BP1 foci cannot be fully restored by K6-K63O-Ub further experiments could be performed to determine if K6-K27-K63O-Ub could fully restore 53BP1 foci. However the work performed indicates that unlike in Gatti *et al*, K63 is the major linkage required for 53BP1 recruitment which is consistent with Doil *et al* 2009 and Stewart *et al* 2009.

Although the use of Ub mutants cannot provide conclusive information on the linkage-type of poly-Ub chains, important residues can be distinguished. K to R Ub mutants have previously been shown to be stable, with linkages being formed through the remaining lysines but the introduction of an incorrect residue may have unknown effects (Baboshina and Haas 1996). The introduction of a mutation may effect interactions with other Ub moieties or how Ub is

bound by Ub-binding domains. Therefore the K to R Ub mutants, though useful for indicating which linkages are likely to be important, cannot provide a definitive answer.

6.2.3 POH1 is a K63-specific DUB that regulates DNA damage dependent Ub

The recruitment of the proteasome to DSBs means it is perfectly located to regulate Ub-conjugates at sites of damage (reviewed in Gudmundsdottir, Lord et al. 2007). Other DUBs have already been shown to act on the Ub-chains at repair; however, the importance and complexity of the repair pathway means there are multiple mechanisms working to control this process (discussed in Chapter 1 Section 1.8.4). To identify potential DUBs involved in DSB repair, a screen of siRNAs against 103 putative DUBs showed that siRNA against POH1 caused an increase in conjugated-Ub, as measured by luminescence from the FK2-HRP antibody, after release from HU.

POH1, as a constitutive component of the 19S lid of the proteasome, is known to cleave Ub chains *en bloc* from substrates destined for degradation (Yao and Cohen 2002). The loss of this action is unlikely to be the cause of the increased FK2 luminesence signal as the *en bloc* activity is not damage specific. Further to this, the FK2 antibody used to detect the Ub-conjugates also detects unanchored poly-Ub chains which would be produced by POH1 cleaving at the proximal Ub (Fujimuro, Sawada et al. 1994). Therefore it is unlikely that the increase in conjugated-Ub after release from HU was due to the loss of the *en bloc* mode of action (Chapter 3 Section 3.4).

POH1 has also been shown to specifically cleave K63-linked Ub-chains in addition to its *en bloc* activities. A reduction in K63-specific activities could be responsible for the observed increased FK2 luminescence, as there could be an accumulation of K63 poly-Ub chains when POH1 is depleted (Cooper, Cutcliffe et al. 2009). The results of Chapter 3 Section 3.4

demonstrate that the loss of POH1 increases Ub conjugates, specifically at sites of damage, with larger K63-Ub foci forming at damage. In response to DSBs, two E3-ligases, RNF8 and RNF168, are known to form K63-linked chains, which are responsible for the recruitment of repair factors (Mailand, Bekker-Jensen et al. 2007, Doil, Mailand et al. 2009). One such repair factor is 53BP1, in Chapter 3 Section 3.5 it was shown the K63-residue is required for 53BP1 recruitment, suggesting K63-linked poly-Ub is important in 53BP1 foci formation. As POH1 is limiting the K63-linked chains at DSBs it would suggest that POH1 is restraining 53BP1 recruitment through limiting the extent of these poly-Ub chains. Further work outside this thesis has demonstrated that when POH1 is knocked down, JMJD2A was removed from chromatin regardless of the damage status of the cells. Consequently, there is more H4K20me2 available for 53BP1 binding, causing an escalation in 53BP1 recruitment.

Therefore POH1 seems to be one regulatory mechanism in a highly controlled cellular pathway. The regulation of RNF168-dependent chains has also been seen by two HECT E3-ligases, TRIP12 and UBR5, which also act to limit the spread of 53BP1 on chromatin after damage. However TRIP12 and UBR5, rather than acting to reduce the poly-Ub chains at DSBs, cause decreased stability of RNF168. Therefore the reduction of RNF168 limits the amount the Ub conjugates at the sites of DSBs. The increase in poly-Ub chains at DSBs when TRIP12 and UBR5 are depleted causes enlarged 53BP1 foci (Gudjonsson, Altmeyer et al. 2012), as seen with POH1 depletion. Combined POH1 and TRIP12 depletions have been shown to further increase the size of 53BP1 foci beyond either depletion alone (J.Morris unpublished results). Therefore there are multiple layers of regulating the Ub at sites of damage and consequently the recruitment of repair proteins, with the POH1 DUB being one of the factors involved.

53BP1 is thought to promote NHEJ repair through blocking resection, although the exact mechanism of how 53BP1 causes this block is still under investigation. 53BP1 interacts with a number of proteins that are also shown to block resection and therefore stop commitment to HR repair. One such protein is Rif1 which requires 53BP1 to be recruited to DSBs by interacting with the N-terminal region of 53BP1 and then antagonises resection at these breaks (Chapman, Barral et al. 2013, Di Virgilio, Callen et al. 2013, Zimmermann, Lottersberger et al. 2013). Downstream of Rif1, another protein Rev7 has been shown to block resection (Boersma, Moatti et al. 2015).

Separately to Rif1 and Rev7, another interactor of 53BP1, which can promote NHEJ, is PTIP. By interacting with phosphorylated Ser25 on 53BP1, PTIP can block resection independently of Rif1 and Rev7 (Munoz, Jowsey et al. 2007, Callen, Di Virgilio et al. 2013). PTIP interacts with the endonuclease Artemis in order to block end resection and therefore stop the commitment to HR repair (Wang, Aroumougame et al. 2014). However Artemis can process the ends of the DNA in order to create compatible ends that can be ligated back together (Ma, Pannicke et al. 2002). Therefore 53BP1 acts to promote NHEJ through two independent pathways which block resection and stop HR repair occurring. Intriguingly in Chapter 3 Section 3.6, the loss of POH1 actually caused a defect in NHEJ despite the increase in 53BP1 at sites of damage. Therefore instead of solely blocking HR, the excess 53BP1 recruitment could potentially be inhibiting the access of NHEJ repair proteins to the DSB as well as blocking resection. Therefore, it is possible that POH1 is maintaining a fine equilibrium of 53BP1 recruitment to breaks in order to allow efficient NHEJ. From the work demonstrated in this thesis, as well as further work performed in our lab, the current working model for the function of POH1 at DSBs is that the DUB activity of POH1 restricts the RNF8/RNF168-dependent K63-linked chains at breaks. The length of the chains close to the break is still

sufficient to remove chromatin bound proteins in order to allow 53BP1 to be recruited to damage. However, the Ub-chains further from the break are shortened by POH1 and therefore the histone bound proteins are not removed, meaning 53BP1 is not recruited to these more distant histones. The increase in 53BP1 protein recruitment in POH1-depleted cells blocks the recruitment of other repair proteins, such as Artemis, to the break therefore inhibiting NHEJ (Chapter 3 Fig 3.8).

This work has subsequently been published (Butler, Densham et al. 2012).

6.3 USP50 is a poorly characterised DUB that is involved in replication

Very little is currently known about the role of USP50 within the cell. Due to the low levels of endogenous expression and the believed lack of enzymatic activity, this protein has been largely disregarded (Quesada, Díaz-Perales et al. 2004, Aressy, Jullien et al. 2010). However, as Ub regulation is a major signalling molecule in the cell, USP50 could have an important role independent of its DUB activity.

6.3.1 USP50 is not enzymatically active but still retains Ub-binding activity

It has been previously reported that USP50 does not have enzymatic activity as a DUB, although the evidence for this was not shown within the paper (Quesada, Díaz-Perales et al. 2004). The loss of the Asp/Asn residue of the catalytic triad is the likely cause of the loss of enzymatic activity. Although there are other DUBs that have been shown to retain enzymatic activity, specifically USP16 and USP30, despite not having all three residues of the catalytic triad (Nijman, Luna-Vargas et al. 2005). However, in Chapter 4 Section 4.3 and 4.4, it is shown that, USP50 lacks enzymatic activity against linear and K48-linked Ub *in vitro*, but nevertheless can still bind Ub both *in vitro* and *in vivo*. The results shown in Chapter 4 Section 4.3 show USP50 is probably regulating a ubiquitinated substrate, as an impaired Ub-

binding mutant only partially rescues the DNA damage-associated foci seen upon USP50 knockdown. Determining what the ubiquitinated substrate that USP50 is regulating will give greater insight into replication stability as another aspect of the pathway will be elucidated.

Previously published DUB interactome studies have suggested that USP50 interacts with VCP; however, the use of USP50 overexpression could be providing an artefactual results (Sowa, Bennett et al. 2009), as the overexpression of USP50, as previously shown, causes aberrant binding to Ub-chains, potentially binding ubiquitinated substrates (Chapter 4 Section 4.4). The AAA+ ATPase, VCP, is known to interact with Ub-binding proteins as adaptor modules (Mullally, Chernova et al. 2006, Schuberth and Buchberger 2008), however, recent work has shown that a Ub-binding protein, Spartan, interacts with VCP in order to inhibit replication stress (Davis, Lachaud et al. 2012, Ghosal, Leung et al. 2012, Mosbech, Gibbs-Seymour et al. 2012). Therefore there is an adaptor of VCP already known to carry out one hypothesised role of USP50.

Despite this, bioinformatic research as part of this project has shown that, apart from the Ub-binding pocket, there is a hydrophobic patch visible on USP50—indicating a potential protein binding domain (Appendix Fig. A6). This patch may bind to VCP meaning USP50 could be an interactor for a specific process regulated by VCP, or this patch may bind another protein making USP50 a Ub-binding adaptor for another complex required in replication fork stability. Further research is required to identify whether USP50 is part of a larger complex and, if so, what this complex is.

Hence the role of USP50 may be as part of a larger complex where enzymatic activity depends on another subunit but the targeting of the complex to a specific substrate relies on USP50 binding Ub-chains of four or more moieties. However, until further research can be

performed to find out if USP50 is acting as a complex there is still the possibility USP50 may be working solely as a Ub-binding protein.

6.3.2 USP50 siRNA causes the formation of spontaneous damage-associated foci during S-phase

Results in this thesis demonstrate that USP50 siRNA caused an increase in damage-associated foci, which was not consistent with a defect in the DNA damage repair pathway (Chapter 4 Section 4.7). A previous report showed that USP50 regulates the degradation of Wee1, with loss of USP50 destabilising Wee1 protein, which can be rescued by proteasome inhibition (Aressy, Jullien et al. 2010). As Wee1 knockdown causes genome instability and cell cycle defects, the loss of USP50 could have been causing the damage-associated foci indirectly through a loss of Wee1 (Dominguez-Kelly, Martin et al. 2011). However, in Chapter 5 Section 5.2, there was no large loss of Wee1 protein on USP50 knockdown, as demonstrated by Western blot analysis. This coupled with an increase in 53BP1 foci seen upon USP50 and Wee1 combined knockdown, suggests that Wee1 and USP50 are working in different pathways to suppress the damage. Therefore the increase in 53BP1 foci observed was due to a fault in another pathway.

However, the increase in 53BP1 foci in the double knockdown of USP50 and Wee1 was not additive when compared against either siRNA alone. This suggests that there may be some overlap in the pathways and some of the damage foci seen will be arising through the Wee1 pathway. This correlates with the slight decrease in Wee1 protein seen upon USP50 depletion, suggesting USP50 siRNA is destabilising a small portion of Wee1 and causing some of the 53BP1 foci observed. Although these results indicate the Wee1 pathway is not the main source of the damage-associated foci seen upon USP50 siRNA treatment.

Unfortunately knockdown of endogenous USP50 could not be assayed as no antibody tested, including three commercially available and six Genscript commissioned antibodies, were able to specifically detect USP50. Western blot analysis was used to detect purified USP50 protein via the T7 tag on the protein. Further to this cells over expressing a flag-tagged version of USP50 were also lysed and tested for USP50 expression using the Flag antibody to confirm that USP50 protein was present, but no USP50 antibody was able to specifically detect either the purified protein or the overexpressed protein. Therefore to confirm USP50 knockdown it was shown that USP50 siRNA could reduce levels of a flag-tagged version of USP50. Consequently it was assumed that siRNA would also be reducing endogenous levels of USP50 protein.

Aressy *et al* implicated USP50 in the G2/M checkpoint, with loss of USP50 causing bypass of the checkpoint in cells where the checkpoint was already impaired by the overexpression of Cdc25B. Hence USP50 may be acting as a secondary regulation to the checkpoint (Aressy, Jullien et al. 2010). However, Chapter 5 Section 5.3 shows that when only USP50 is lost there is no gross change in the cell cycle profile. Therefore although USP50 may act as a back-up checkpoint regulator when the G2/M checkpoint is compromised; it is unlikely that the damage-associated foci observed were due to a cell cycle defect.

Further investigation into the cause of the damage showed a S-phase specific rise in damage foci numbers (Chapter 5 Section 5.4). This increase, coupled with the specific sensitivity to replication stress-inducing drugs, shows a role for USP50 in replication (Chapter 5 Section 5.5). The sensitivity to HU may also go some way to explaining the initial reduced FK2 result upon USP50 knockdown. As FK2 luminescence was not normalised to DAPI staining to ensure the same number of cells were being analysed and USP50 depleted cells are sensitive

to HU, there may have been increased cell death in these cells, hence giving a reduced signal for conjugated Ub (Chapter 3 Section 3.4).

The cell-cycle-specific nature of the role of USP50 may mean that USP50 is regulated in a cell cycle specific manner, to control its activity. The potential interaction of USP50 with the Wee1 kinase (Aressy, Julian et al 2010), could mean USP50 is phosphorylated, or if USP50 is involved in fork stability it could be a target of ATR phosphorylation. Bioinformatic searches show that there are three putative phosphorylation sites which could be targets of kinases (Appendix Fig. A7). Additionally, there may be other posttranslational modifications, including ubiquitination or sumoylation, which would limit the action of USP50 to the S-phase of the cell cycle.

The S-phase specific increase in damage suggested a fault in replication. Replication forks can collapse into DSBs, marked by 53BP1, when they stall and can no longer be stabilised by ATR, as part of the intra-S-phase checkpoint (Tercero and Diffley 2001, reviewed in Cimprich and Cortez 2008). In Chapter 5 Section 5.8 it was demonstrated that the ATR-dependent intra-S phase checkpoint is still functional in USP50 siRNA treated cells. When cells were treated with both USP50 siRNA and an ATR inhibitor, and then drugged with HU, there was a significant increase in 53BP1 foci when compared to cells treated with either USP50 siRNA or ATR inhibition alone in conjunction with HU. These results indicate ATR signalling is active upon USP50 depletion and can stabilise stalled replication forks. Once ATR is no longer sufficient to maintain the replication forks, there are more DSBs that form in USP50 siRNA treated cells.

The additive increase seen in HU perturbed cells when USP50 is depleted and ATR is inhibited could be explained by two potential causes.

1) There are more stalled forks initially, which collapse into DSBs once ATR can no longer stabilise the forks. This theory would indicate that USP50 is acting to remove lesions before the fork interacts with them or USP50 is acting to stop the forks from stalling.
2) The same numbers of forks are stalling but are less stable or cannot be resolved by a means that does not result is a DSB. This theory would suggest that USP50 is acting to stabilise stalled replication forks.

In cells that were not exposed to HU but treated with combined USP50 siRNA and ATRi there was no increase in 53BP1 foci when compared to either USP50 siRNA or ATRi treatment alone (Chapter 5 section 5.8). Therefore without HU treatment the formation of 53BP1 appears to be epistatic between USP50 and ATR inhibition, indicating USP50 and ATR are in the same pathway. However this is contrary to the results shown in cells treated with HU, when there is an increase in 53BP1 foci upon dual inhibition of USP50 and ATR. This suggests that there are only a limited number of stalled forks in these unperturbed cells, which had not been treated with HU. Therefore the combined inhibition of ATR and USP50 cannot cause an additive increase in 53BP1 foci as all the stalled forks have already formed DSBs in the single treatments of USP50 siRNA or ATRi.

Fibre analysis in Chapter 5 Section 5.7 suggests that there are more stalled or collapsed forks upon USP50 depletion; however, the increase is small and is not coupled with an increase in new origin firing. This small increase although significant suggests that there is a limited number of stalled or collapsed forks in USP50 siRNA treated cells. If ATRi cells had the same number of first label terminations it would support the hypothesis that in cells untreated with HU there is only a small number of stalled forks that can collapse into DSBs in USP50 siRNA, ATRi or combined USP50 and ATRi treated cells. The results in Chapter 5

Section5.7 suggests there is a significant number of forks stalling or collapsing in USP50 depleted cells, but more research is required to fully understand how USP50 is working to stabilise the replication fork.

Replication forks collapse into DSBs once they can no longer be stabilised by ATR signalling. RNF4 has been shown to increase the amount of collapsed forks in ATR deficient cells, further indicating a role for Ub in replication fork stability (Ragland, Patel et al. 2013). The regulation of replication by Ub is still an emerging subject and the characterisation of a Ub-binding protein involved in replication fork stability demonstrates part of a pathway, which is not fully elucidated. RNF4 could be producing Ub chains which are bound by USP50, however there may be other ligases, both already implicated in replication, or not currently identified as having a role in replication, which produce the poly-Ub that is bound by USP50. To fully understand the pathway that USP50 is acting in, the E3 Ub-ligase also needs to be identified.

In cells that still have ATR expression there are many mechanisms that allow the restart of stalled replication forks (discussed in Chapter 1 Section 1.9.4), one mechanism is through fork restructuring by helicase enzymes (reviewed in Petermann 2010) The WRN helicase is one such enzyme, which has shown similar defects upon depletion as USP50 knockdown—with increased damage but no change in cell cycle (Sidorova, Li et al. 2008, Patro, Frohlich et al. 2011). The WRN helicase is known to be ubiquitinated, along with its interacting partner WRNIP, providing a potential docking site for USP50 (Bish, Fregoso et al. 2008, Li, Wang et al. 2010). Combined depletion shown in Chapter 5 Section 5.9 showed no increase in damage foci, which suggests the knockdowns are epistatic and therefore USP50 and WRN are working in the same pathway. However, these are preliminary results which still require

further investigation into how USP50 is regulating this mode of replication fork restart, including whether USP50 is directly binding WRN.

Further preliminary work indicated that the reduction in USP50 causes increased 53BP1 foci in a proteasome dependent manner. Inhibition of the proteasome by MG132 treatment reduced the number of 53BP1 foci in USP50 siRNA treated cells, even when cells were transfected with Myc-Ub. This leads to the hypothesis that when USP50 is depleted, the Ub chains which USP50 should be binding to, are prone to attack by the proteasome. More work is required to fully understand the requirement of the proteasome in the pathway, but the potential targeting of the proteasome to the ubiquitinated substrate, that is no longer bound by USP50, could give an indication into the Ub-linkage that is being bound by USP50.

Although USP50 appears to be protecting an unknown substrate from degradation, the cellular localisation of USP50 and hence the substrate, is not known. USP50 is a cytoplasmic protein but it has been shown to enter the nucleus upon DNA damage (Aressy, Jullien et al. 2010). The results presented in Chapter 5 show a role for USP50 in replication fork stability, preventing collapse into DSBs but this does not mean that USP50 itself is at the replication fork. USP50 could be acting to anchor a substrate in the cytoplasm until it is required in the nucleus, or could be acting directly at a substrate at the fork. Currently, iPOND studies have not demonstrated USP50 being present at the fork (Sirbu, Couch et al. 2011, Dungrawala, Rose et al. 2015). This could be due to a number of reasons.

1) USP50 is not at the fork and therefore would not be seen by iPOND

2) USP50 is transiently at the fork and therefore cannot be captured at high enough frequencies at the fork by the iPOND method

3) USP50 is known to be expressed at low levels in the cell and therefore, USP50 is at the fork but at such low levels that it is not detected at a high enough frequency by iPOND

Therefore to understand how USP50 is working to stabilise replication forks requires investigation into the cellular localisation of USP50.

Stabilising replication forks is important especially in cancer cells as these cells generally undergo more proliferation and subsequently more replication than non-cancerous cells, they are also more prone to oncogene-induced replication stress (Bartkova, Horejsi et al. 2005, Gorgoulis, Vassiliou et al. 2005). The role USP50 plays in potentially stabilising the replication fork should, therefore, have a more important role in cancerous cells than non-cancerous cells. Almost all experiments carried out in this thesis have been performed in cell lines generated from cancers, such as the HeLa cervical cancer cells or the osteocarcinoma U2OS cells (Scherer, Syverton et al. 1953, Kanzaki, Hilliker et al. 1994). USP50 depletion has been shown to sensitise cells to a model of oncogene-induced replication stress in human fibroblasts cells containing an inducible constitutively active h-Ras shown in Chapter 5 Section 5.6. However, activated h-Ras may be causing other defects in the cell that are not due to oncogene-induced replication stress. Thus, USP50 could become a potential target for cancer therapies.

6.3.3 USP50 is conserved across higher eukaryotic species

As USP50 plays an important role in preventing replication fork collapse into DSBs it would be expected that this function is conserved across the species which contain USP50. Bioinformatic research shows USP50 is conserved across 42 species including mice; however, the mouse homologue of USP50 has retained the catalytic triad unlike the human

version (Chapter 4 Section 4.3). The loss of enzymatic activity has been attributed to the loss of the Aspartic acid residue, so potentially; the mouse USP50 could still be active. It was shown in Chapter 4 Section 4.5 that USP50 depletion in NIH3T3 cells still causes the increase in 53BP1 foci as seen in human cells suggesting a conserved function. It would be intriguing to know whether the mouse USP50 is still active and, rather than merely blocking the proteasome or another complex, is actually removing the chains to stop the signal.

Recent work from the International Mouse Phenotyping Consortium has shown that mice which have a heterozygous mutation in USP50 display distinct differences from their wild-type counterparts. The USP50 heterozygotes are larger with abnormal bone density as well as a decreased startle reflex. Whether these phenotypes are due to the loss of the same pathway that has been alluded to in this thesis remains to be examined. Further to this the propensity of these mice to cancer would be of interest.

As preliminary data suggest USP50 is working in the same pathway as WRN, the phenotype of the WRN mouse was investigated. Although the WRN mouse phenotype was not available on the IMPC database, a study by Lebel and Leder discussed some phenotypes of homozygous mice. Homozygous Wrn -/- mice were born below the expected mendelian ratio, but those which survived did not display severe defects when compared to littermates. One wrn-/- mouse developed myocardial fibrosis and another developed a T cell lymphoma but apart from these defects no other phenotypes were characterised (Lebel and Leder 1998). However, until more extensive phenotyping data is available for wrn-/- mice it is not possible to do a full comparison between Usp50-/- and wrn-/- mice.

Although more work is required to fully elucidate the role of USP50, the hypothesised model of USP50 action is to allow fork restart once replication forks have stalled. We propose that

USP50 is acting as part of a larger complex, with USP50 binding to Ub-modifications potentially at replication forks. The complex blocks aberrant degradation of the ubiquitinated substrates by the proteasome. USP50 then acts to allow the remodelling of the replication fork by the WRN helicase to allow fork restart. When USP50 is no longer present in the cell, the stalled replication fork cannot be remodelled and instead, once ATR can no longer stabilise it, collapses into a DSB. The break can then be repaired to allow replication to continue and cell survival (Chapter 5 Fig 5.12).

The work presented has shown that USP50 has a role in replication which was not previously known, although further investigation is required to fully elucidate its function.

6.4 Future Questions

The POH1 story has been published, explaining how the proteasome associated DUB regulates the K63-linked chains at DSBs in order to allow faithful repair, however there are still some open questions surrounding this project (Butler, Densham et al. 2012). The role USP50 plays during replication still has many unanswered questions that require further investigation.

6.4.1 Proteasome future questions

Despite being published, there are still questions about how the proteasome and POH1 regulate the Ub-chains and consequently DSB repair. When studying the function of the proteasome at sites of damage we did not explore how BRCA1 recruitment requires proteolytic activity. As proteasome inhibition blocked the recruitment of BRCA1 to DSBs it is hypothesised that a substrate must be degraded by the proteasome in order to allow BRCA1 recruitment to DNA breaks, but what this substrate may be requires work. In order to determine what the degraded substrate possible experiments include:

A. Determining likely candidates that are present upstream of BRCA1 at DSBs and test the clearance of these proteins in MG132 treated cells. The candidates should not be cleared at later time points after DNA damage if the proteasome is required to remove them.
B. Carry out ChIP analysis to determine if any proteins are more highly associated with DSBs after MG132 at later time points
C. If any potential candidates arise from the previous two experiments, carry out depletion of this protein in MG132 treated cells and visualise whether BRCA1 foci can form when this protein is reduced.

Work by Shi et al suggested MDC1 was degraded by the proteasome in order to promote BRCA1 recruitment. However work in this thesis suggests MDC1 clearance does not require the proteolytic activity of the proteasome, instead Ub-modifications cause MDC1 to be removed from the DSB. The work using K to R Ub mutants did not give an indication of a specific linkage was required for MDC1 clearance, therefore further investigation is required to determine whether a specific poly-Ub linkage is required for MDC1 clearance. This could be investigated by:

A. Visualising MDC1 clearance as described in Chapter 3 Section 3.4 but using a Ub where all lysines are mutated to arginines meaning poly-Ub chains cannot form. If MDC1 can still be cleared it is likely to be a mono-Ub modification that is required for clearance
B. Carry out a pull down of MDC1 and then carry about western blot analysis to see if there is mono-Ub or poly-Ub forming on MDC1
C. If poly-Ub is forming on MDC1 carry out western blot analysis with K48 or K63 antibodies to determine if it is either of these linkages that are forming on MDC1

D. If it is neither K48 or K63 poly-Ub that is forming on MDC1, carry out UbiCREST digestion on the MDC1 specific chains to work out which linkage specific DUB can cleave the chains that form on MDC1

Unlike MDC1 there use of Ub K to R mutants did give an indication of which poly-Ub chain type is required for 53BP1 recruitment to DSBs. However, as 53BP1 recruitment to DSBs cannot be fully restored by K63O-Ub or K63-K6O there is potential that another linkage, potentially K27, is required for 53BP1 foci formation. The requirement of K27 poly-Ub is suggested by recent work by Gatti et al. however this research indicates K27 is the main linkage required which is not supported by data in this thesis (Chapter 3 Section 3.3). Therefore it would be interesting to further investigate which poly-Ub chains are necessary for 53BP1 recruitment by carrying out immunofluorescent staining using K6-K27-K63O in MG132 treated cells to see if 53BP1 can be restored to wild-type levels

6.4.2. POH1 future question

As well as studying the proteolytic role of proteasome at DSBs the requirement of proteasome-associated DUB, POH1, in the repair of breaks was also studied. This work showed that loss of POH1 increased K63-linked poly-Ub at DSBs and consequently 53BP1 recruitment. Surprisingly though the loss of POH1 reduced NHEJ repair, which can be restored by 53BP1 reduction. As 53BP1 usually promotes NHEJ more work is required to determine how the increase in 53BP1 is inhibiting NHEJ rather than promoting it. The data presented in this thesis suggests that excess 53BP1 is somehow blocking NHEJ, but in order to determine the mechanism behind this further experiments are needed, such as:

Possible experiments:

A. Carry out high resolution microscopy on 53BP1 foci to determine whether the kinetic of the foci change after POH1 depletion
B. Study the recruitment of downstream NHEJ factors after POH1 depletion to determine whether their recruitment is affected by POH1 siRNA.
C. If any downstream NHEJ factors are affected by POH1 depletion, carry out the same experiment but with codepletion of POH1 and 53BP1 to determine if recruitment can be restored.

6.4.3 USP50 future questions

The role of USP50 in replication still requires further investigation in order to determine the mechanism by which USP50 prevents replication forks collapsing into double strand breaks. As USP50 lacks enzymatic activity but still retains Ub-binding activity, it may be acting as a Ub-binding module for a larger complex. It has already been reported that USP50 interacts with VCP and therefore may be another Ub-binding component for VCP during a role in replication.As previously mentioned, there are external hydrophobic residues on the opposite face of the protein to the Ub-binding pocket (Appendix Fig. A6), which suggest USP50 is binding another protein or a complex of proteins. In order to determine whether USP50 is interacting with another protein that is required during replication there are several possible experiments that can be carried out:

A. Create mutants of USP50 in which individual hydrophobic residues in the putative binding domain are changed. Mutated USP50 can be reintroduced into USP50 depleted cells and cells stained for 53BP1 foci to determine if the mutated USP50 can rescue the increase 53BP1 foci seen on knockdown or if overexpression of the mutant USP50 are no longer able to rescue the damage-associated foci.

B. If any mutations in USP50 cannot rescue the increase in 53BP1 foci using the binding pocket mutants, carry out mass spectrometry with both wild-type USP50 and mutant USP50 to compare what interactions are lost in order to determine the binding partner of USP50.

As well as USP50 functioning as part of a larger complex, it is likely that USP50 is binding to a ubiquitinated substrate which is involved in the stability of the replication fork. As Ub-binding appears to be required for the function of USP50, understanding what the ubiquitinated substrate that USP50 is binding to will help elucidate the pathway that USP50 is working in and give further insight into how USP50 promotes replication fork stability. The experiments that can be performed to determine what the ubiquitinated substrate are:

A. Create Ub-binding mutants which completely abolish the Ub-binding activity seen by *in vitro* binding assays. Once Ub-binding mutants have been established, perform IF on cells depleted of USP50 and transfected with the mutant USP50, to confirm that Ub-binding mutants cannot rescue the 53BP1 foci formed.
B. Once mutations that abolish Ub-binding are confirmed, carry out mass spectrometry using USP50 wild-type and USP50 Ub-binding mutants to determine which interactions are different. This will need to be carried out in stable cell lines, as overexpression of USP50 appears to cause aberrant binding to Ub conjugates, which would therefore cause incorrect results (Chapter 4 Section 4.3) and, therefore, expression levels of USP50 will have to be controlled

The substrate that USP50 is binding to must be being ubiquitinated by an E3 Ub-ligase. To fully understand the pathway that USP50 is working in to prevent DSB breaks forming during replication, the E3 Ub-ligase must be identified by carrying out experiments such as:

A. Carry out RNF4 codepletions with USP50 and study fork kinetics by fibre analysis to determine if there is an epistatic effect in codepleted cells, including looking at first label terminations. Further to this investigation, fork speeds can be analysed to see if fork progression is effected more by the codepletion of USP50 and RNF4.
B. If RNF4 is not epistatic with USP50 depletion then orther ligases can be investigated. To determine if a Cullin ligase is involved, an inhibitor against neddylation, such as MLN4924 (Soucy TA 2009, Brownell JE 2010), can be used to determine if there is an epistatic effect on 53BP1 foci with USP50 depletion. If the neddylation inhibitor proves to be epistatic, then investigation into Cullin ligases can be carried out beginning with Cullins known to be involved in replication such as Cul4A.
C. If the Cullin inhibitor does not yield any potential ligases, determining the ubiquitinated substrate could provide insight into the ligase, by investigating if any ligases are known to target the substrate of USP50 binding. To determine if USP50 is binding chains created by the identified ligase, immunofluorescence staining for 53BP1 can be performed on cells codepleted of USP50 and the identified ligase. If foci formation is epistatic, a probable ligase will be identified. Fibre analysis can also be performed to see whether fork structures and speeds are affected by ligase inhibition and if they reproduce the phenotype seen on USP50 siRNA treatment.
D. If no known ligase is found for the substrate, investigation into any known ligases are involved in replication fork stability can be undertaken. Whether the ligase is involved in the same pathway as USP50 can be determined by carrying out codepletions with USP50 and seeing if there is an epistatic formation of 53BP1 foci. Then carry out fibre analysis to determine if the fork structures and speeds are similar between USP50 depleted cells, ligase depleted and codepleted cells.

Further to elucidating the ligase which is modifying the substrate, understanding the linkage specificity of USP50 can give insights into the signalling network which USP50 is acting in or whether it is a signal for degradation. The type of linkage is important to signal a specific pathway and therefore potential experiments to work out the modification type include:

A. Perform *in vitro* binding assays with purified USP50 protein attached to Ni^{+} beads and incubate with different Ub linkages such as K63, K48 and linear Ub chains and determine if USP50 has an affinity for a specific linkage or chain length
B. Use the inducible Flag-tagged USP50 to perform an *in vivo* flag pull down and carry out a Western blot of the bound Ub. By using antibodies specific to K48 and K63 it can be seen if there is an enrichment of either of these chain-types.
C. If there is no enrichment for K48 or K63-linked Ub, use UbiCrest to determine which linkages USP50 is binding to.

As USP50 appears to be involved in the stabilisation of replication forks, it could be hypothesised that USP50 is required at the fork. However USP50 has not be shown to be at the replication forks in previously studies. Another theory is that USP50 is acting on an upstream factor away from the replication fork that has a detrimental effect on the replication fork. To determine how USP50 is acting to stabilise replication forks, specifically if USP50 is at the replication fork, requires investigation, including:

A. Use tagged USP50 to carry out IF staining to determine cellular localisation of USP50, cells can be counter stained with EdU to see if localisation changes during S-phase
B. Pull down tagged USP50 and test whether it is binding to any replication fork components, either by western blot analysis of known ubiquitinated components or mass spectrometry of the bound proteins.

The specificity of USP50 to specific pathways during replication-stress suggests that there must be a signalling mechanism that controls this specificity. Therefore it could be hypothesised that USP50 is itself modified in response to the replication fork stalling. The major signalling mechanism to stalled replication forks is activation of the kinase ATR. This leads to the hypothesis that USP50 itself could be phosphorylated by ATR in response to fork stalling. Two potential phosphorylation sites have been identified that may be targets for ATR. It is intriguing to know whether USP50 is itself regulated to limit its role purely to S-phase, by carrying out the following experiments:

A. Using stable expressing USP50-Flag cell lines, carry out Western blot analysis using phosphatase inhibitors and determine if there is a band shift on the gel indicative of phosphorylation
B. Carry out site directed mutagenesis of the putative phosphorylation domains and reintroduce mutated USP50 to depleted cells and stain for 53BP1 foci to see whether the phosphorylation sites are important for replication fork stability
C. If there is no indication of phosphorylation, Western blot analysis can be carried out using N-Ethylmaleimide, to inhibit cysteine DUBs and SENPs (Sommer S 2013), to determine if a 8kDa band shift is apparent.

The translational implications of understanding the role of USP50 in response to replication stress is not understood. As cancer cells are more prone to undergo oncogene-induced replication stress, USP50 could be a potential target for cancer therapies. However, the sensitivity of non-cancerous cells to the loss of USP50 must be investigated before therapies can be created. To determine if a USP50 inhibitor would be a good anti-cancer therapy, distinguishing if there is a cancer specific sensitivity to USP50 inhibition must be investigated by carrying out potential experiments, including:

A. Use cell lines which were not originally derived from cancer and deplete USP50 and stain for 53BP1 foci. Quantify the foci numbers and determine whether there is an increase in foci number and if so, if it is as large as in cell lines derived from cancers
B. Fibre analysis can be used in non-cancerous cell lines to see if the same amount of stalled forks are occurring in these cells upon USP50 knockdown as occurs in cancerous cell lines
C. Test sensitivity of USP50 depleted non-cancerous cell lines to replication stress-inducing drugs, such as hydroxyurea and Aphidicolin, by colony assay. Compare the sensitivity of USP50 depleted non-cancerous cells with cancerous cells and see if there is a change in sensitivity

6.5 Conclusions

In this thesis two DUBs—POH1 and USP50—have been identified to have novel roles in DSB repair and replication, respectively. Ub is emerging as a major signalling molecule for many processes in the cell, including DSB repair and replication. However the role of Ub in the DDR is much more established than the role of Ub in replication (Ulrich and Walden 2010). Results in this thesis demonstrate the importance of the poly-Ub chains in the kinetics of repair proteins, specifically MDC1 and 53BP1. While the proteolytic activity of the proteasome is important for BRCA1 recruitment, the Ub processing function of the proteasome also plays a key role in allowing the progression of DSB repair (Chapter 3). The processing of Ub conjugates at DSBs, by the proteasome associated DUB, POH1, is shown to limit the spread of the K63 damage-dependent chains. By limiting the amount of K63-linked chains at DSBs there is a subsequently restriction of 53BP1. By restraining the amount of 53BP1 that accumulates at DSBs, POH1 maintains a fine balance of repair proteins at the sites

of damage that allows NHEJ to progress. The poorly characterised DUB—USP50—has been shown to have a novel role in replication fork stability in this thesis. A role for USP50 in replication has not been demonstrated before; instead a role in maintaining the G2/M checkpoint is the only published action (Aressy, Jullien et al. 2010). Within this thesis, there is evidence indicating this inactive DUB is able to bind Ub and through this mechanism maintain genomic stability. While USP50 does not have an obvious effect on the intra-S checkpoint, the loss of this Ub-binding module causes replication forks to collapse, an effect that is exacerbated by loss of ATR signalling. The stabilisation of replication forks may be through binding an unknown substrate and protecting it from early degradation by the proteasome. Preliminary results suggest that USP50 may be working to restructure replication forks through the action of the WRN helicase, in order to allow fork restart without collapsing into a DSB. As USP50 is a small Ub-binding module it is likely that it is part of a larger complex. USP50 may therefore act as an adaptor for a larger complex that regulates a ubiquitinated substrate from replication forks but only once ATR is no longer stabilising the fork, allowing WRN to remodel the fork (Chapter 5 Figure 5.13). Sensitivity assays provide evidence that there are distinct pathways for restarting stalled forks depending on the type of block encountered by the fork, as USP50 is not involved in the repair of ICLs despite their impact on replication fork progression (Chapter 5). Although the role of Ub in replication is still emerging, this work provides more evidence for the importance of ubiquitination in protecting replication forks.

Increased replication and oncogene-induced replication stress mean that most cancer therapies escalate the damage caused during replication to selectively kill cancer cells (Helleday, Petermann et al. 2008). Current research is finding new ways to sensitise cells to replication stress, including RPA inhibition (Glanzer, Liu et al. 2014). The increased sensitivity to

replication stress, both endogenous and exogenous, when USP50 is depleted makes it an intriguing therapeutic target.

Specific inhibitors to DUBs are already being investigated as cancer therapies due to the specificity of the inhibitors and the reduction in toxicity to standard treatments (Lim and Baek 2013). Small molecule inhibitors of USP8 have already been developed. With the homology between USP50 and the USP8 C-terminus, there is potential for the development of USP50 inhibitors. Inhibition of USP8 has been shown to induce apoptosis in cells, with small molecule inhibitors causing decreased cell growth (Byun, Lee et al. 2013). USP8 inhibitors have been shown to be specific to USP8, despite its homology with USP50, with the treatment of non-small cell lung cancer being effectively treated by USP8 silencing (Guedat and Colland 2007, Byun, Lee et al. 2013). The lack of the tail of USP50 means that although there is strong conservation between USP50 and USP8, they are distinct and could, therefore, be targeted specifically by inhibitors (Chapter 4 Section 2).

Members of the Ub pathway are attractive therapeutic targets, so the discovery of a potential Ub-binding protein required for replication fork stability after stress makes USP50 a potential new therapeutic target.

Appendix

A1 Localisation of the proteasome at DSBs

HeLa cells were plated and transfected with either Ub-Myc or lysine-less Ub (K0-Ub-Myc) for 24 hours. Cells were damaged with 2 Gy IR allowed to recover for 1 hour before being pre-extracted with YG buffer (20 mM HEPES pH 7.5, 20 mM NaCl, 1 mM DTT, 5 mM $MgCl_2$, 0.5% NP40) for 3 minutes before fixing in 4% PFA for 30 minutes. Cells were stained for γH2AX as a marker of damage and the proteasome using the Sug1 antibody.

In cells transfected with Myc-Ub there is localisation of the γH2AX and the proteasome, however in cells transfected with K0-Ub-Myc show reduced colocalisation of γH2AX and the proteasome. This suggests that there is a requirement for poly-Ub chains for the recruitment of the proteasome to DSBs.

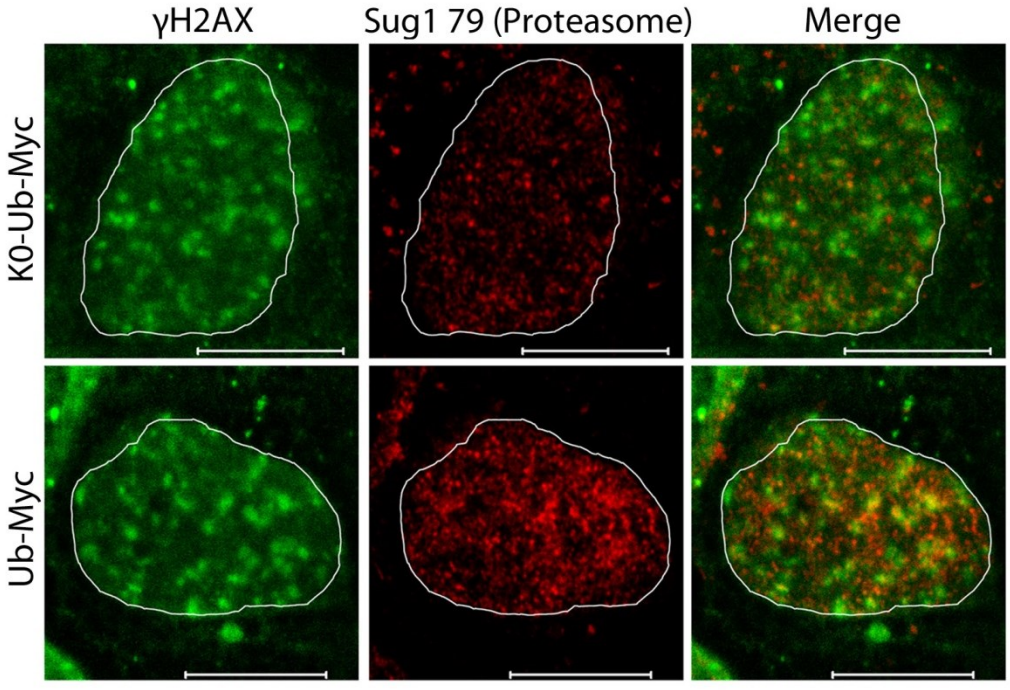


Fig A1. Proteasome recruitment to DSBs is reduced in cells expressing K0-Ub-Myc. Confocal images of HeLa cells transfected with Ub-Myc and K0-Ub-Myc and stained for γH2AX (green) and the proteasome with Sug1 (red). White line represents DNA based on Hoescht staining. Scale bar = 10 μM

A2 Localisation of the proteasome to DSBs in MG132 treated cells

HeLa cells were transfected with either Ub-Myc or Myc-LacZ for 24 hours prior to treatment with MG132 for 1 hour before being damaged with 2 Gy IR. Cells were left to recover for 1 hour before being pre-extracted with YG buffer (20 mM HEPES pH 7.5, 20 mM NaCl, 1 mM DTT, 5 mM $MgCl_2$, 0.5% NP40) for 3 minutes and then fixed in 4% PFA for 30 minutes. Cells were stained for γH2AX as a marker of damage and the proteasome using the Sug1 antibody.

In cells transfected with Myc-LacZ no foci formation of the proteasome is apparent. Conversely, cells expressing Ub-Myc were able to form proteasome foci that can partially localise to sites of damage. This supports the notion that proteasome recruitment to DSBs requires Ub conjugates.

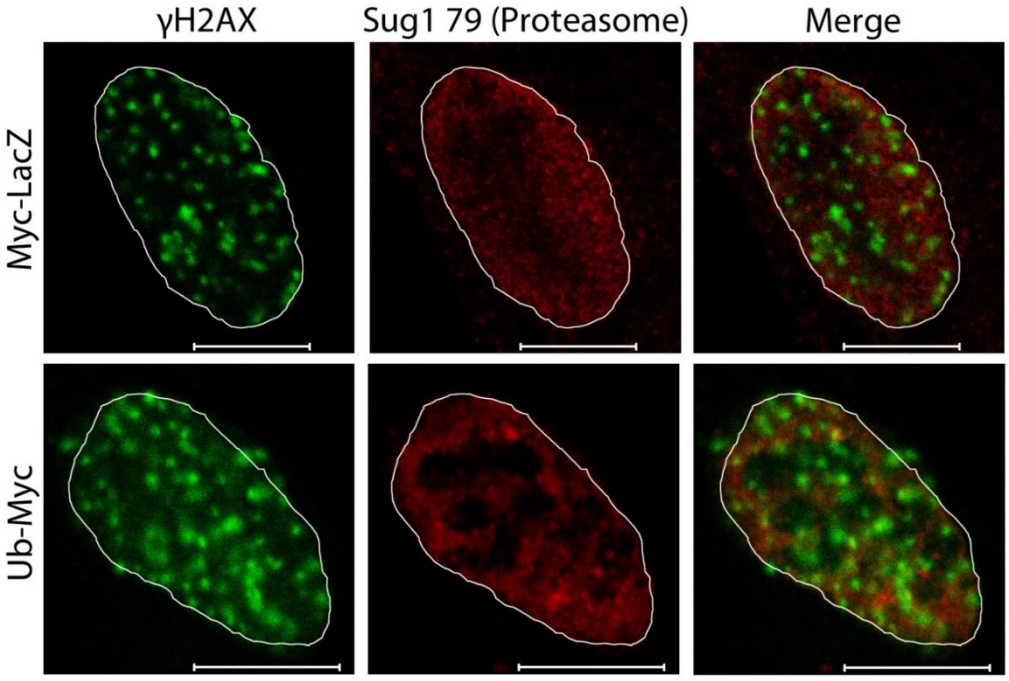


Fig A2. The Proteasome can form foci in MG132 treated cells when overexpressing Myc-Ub. Confocal images of HeLa cells transfected with Myc-LacZ and Ub-Myc and stained for γH2AX (green) and the proteasome with Sug1 (red). White line represents DNA based on Hoescht staining. Scale bar = 10 μM

A3 Amino acid alignment of USP50 and USP8

USP50 and USP8 amino acid sequences were input into t-coffee software and aligned. Within USP50, 54% of amino acids were either conserved or had the same properties as those in the same position as USP8

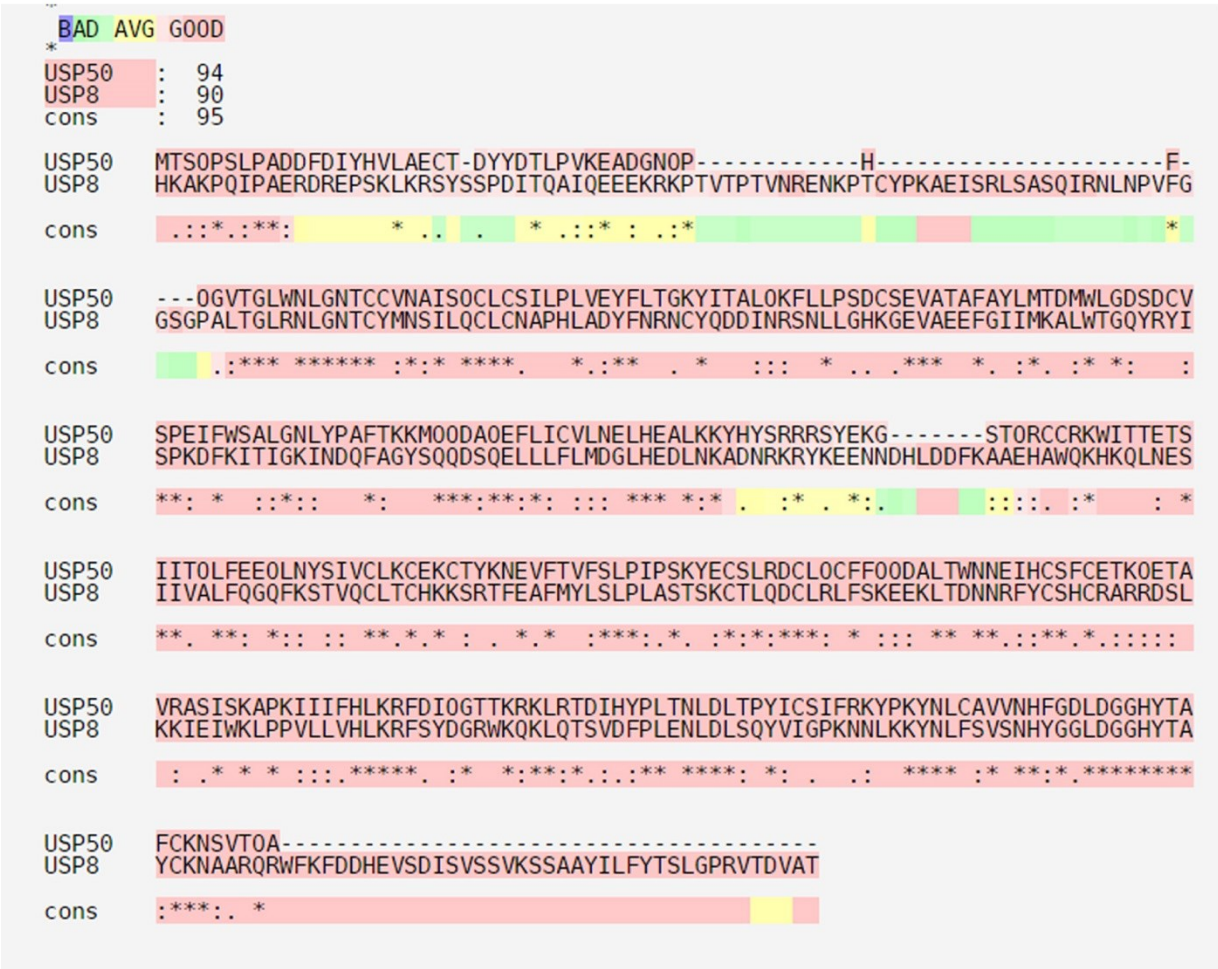
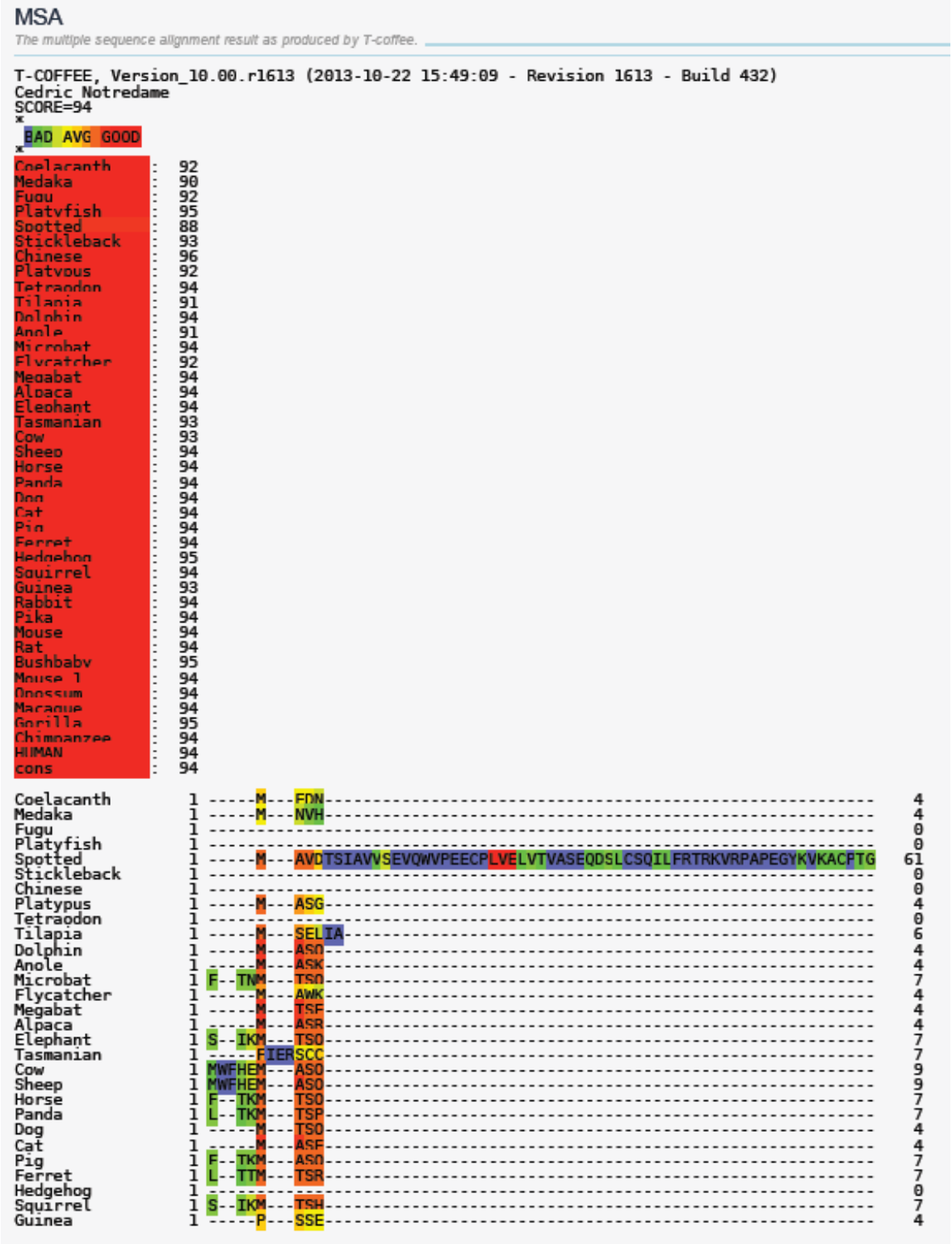
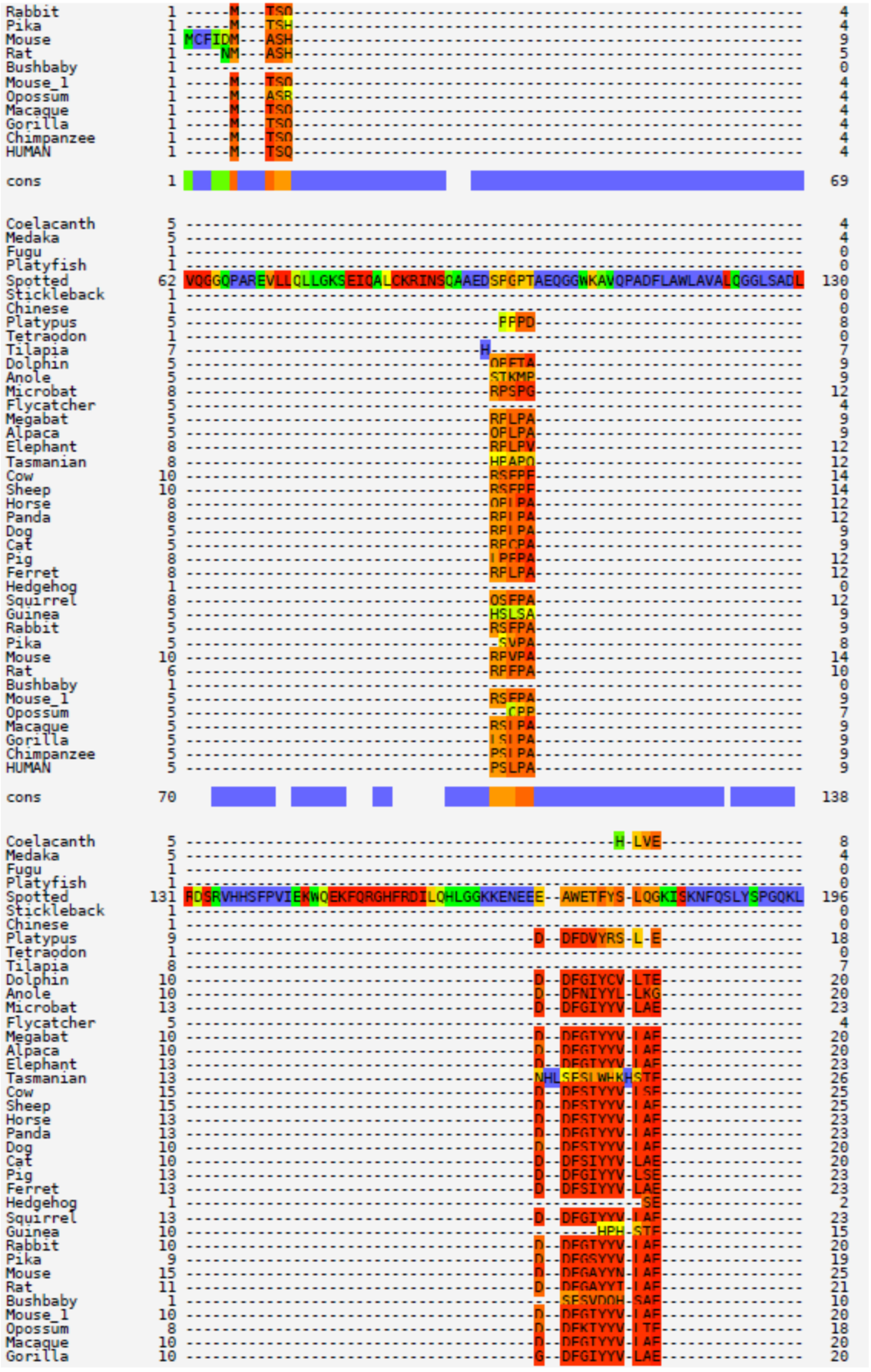


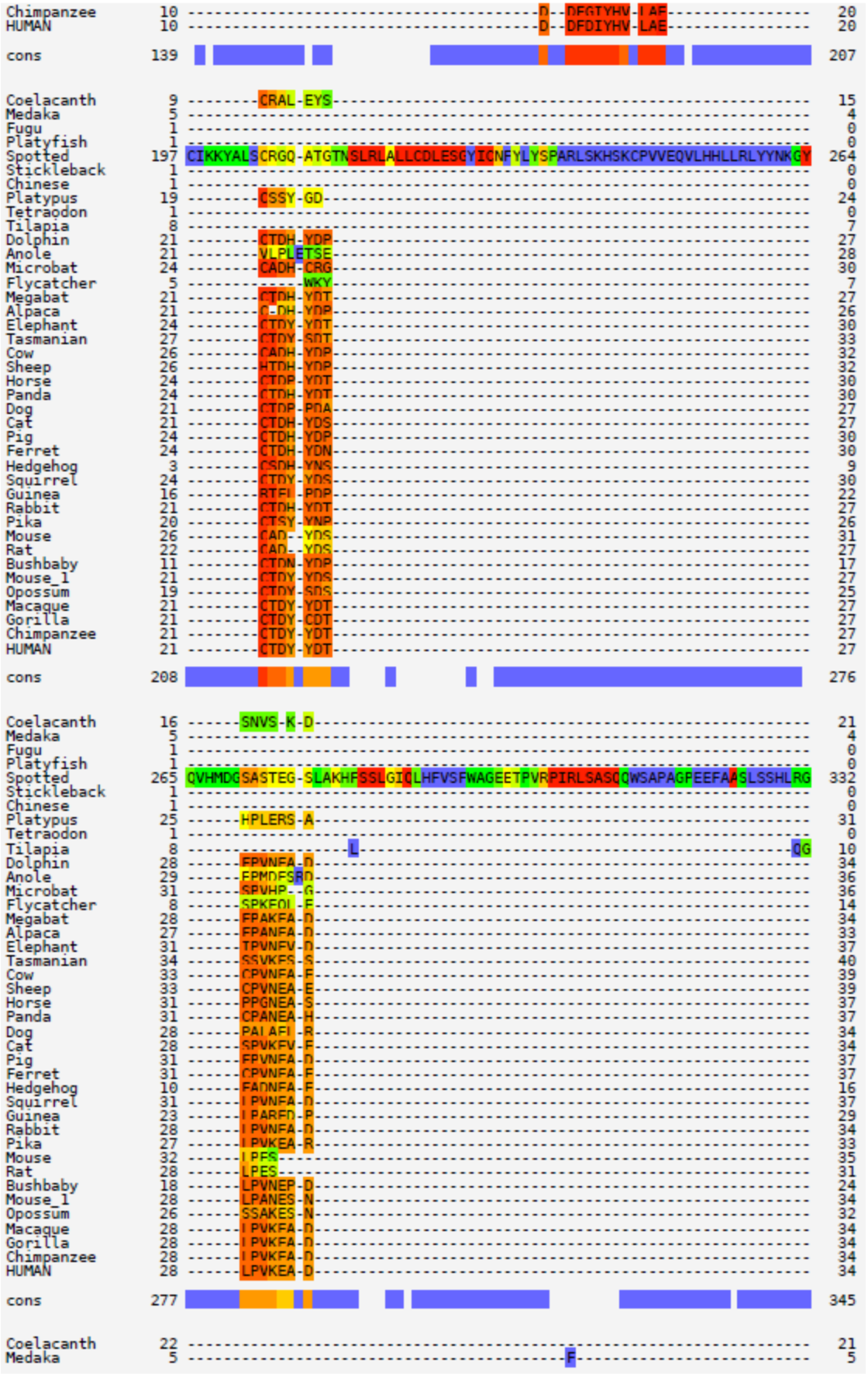
Fig A3 T-coffee alignment of USP50 and USP8. Amino acid sequence of USP50 and USP8 input into t-coffee software to calculate alignment and conservation.

A4 USP50 alignment between 40 species

Alignment of USP50 amino acid sequences from 40 different species to work out alignment and conservation of amino acids. Sequences were input into the T-coffee software.



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Coelacanth 9 15
Medaka 5 4
Fugu 1 0
Platyfish 1 0
Spotted 197 264
Stickleback 1 0
Chinese 1 0
Platypus 19 24
Tetraodon 1 0
Tilapia 8 7
Dolphin 21 27
Anole 21 28
Microbat 24 30
Flycatcher 5 7
Megabat 21 27
Alpaca 21 26
Elephant 24 30
Tasmanian 27 33
Cow 26 32
Sheep 26 32
Horse 24 30
Panda 24 30
Dog 21 27
Cat 21 27
Pig 24 30
Ferret 24 30
Hedgehog 3 9
Squirrel 24 30
Guinea 16 22
Rabbit 21 27
Pika 20 26
Mouse 26 31
Rat 22 27
Bushbaby 11 17
Mouse_l 21 27
Opossum 19 25
Macaque 21 27
Gorilla 21 27
Chimpanzee 21 27
HUMAN 21 27
cons 208 276
Coelacanth 16 21
Medaka 5 4
Fugu 1 0
Platyfish 1 0
Spotted 265 332
Stickleback 1 0
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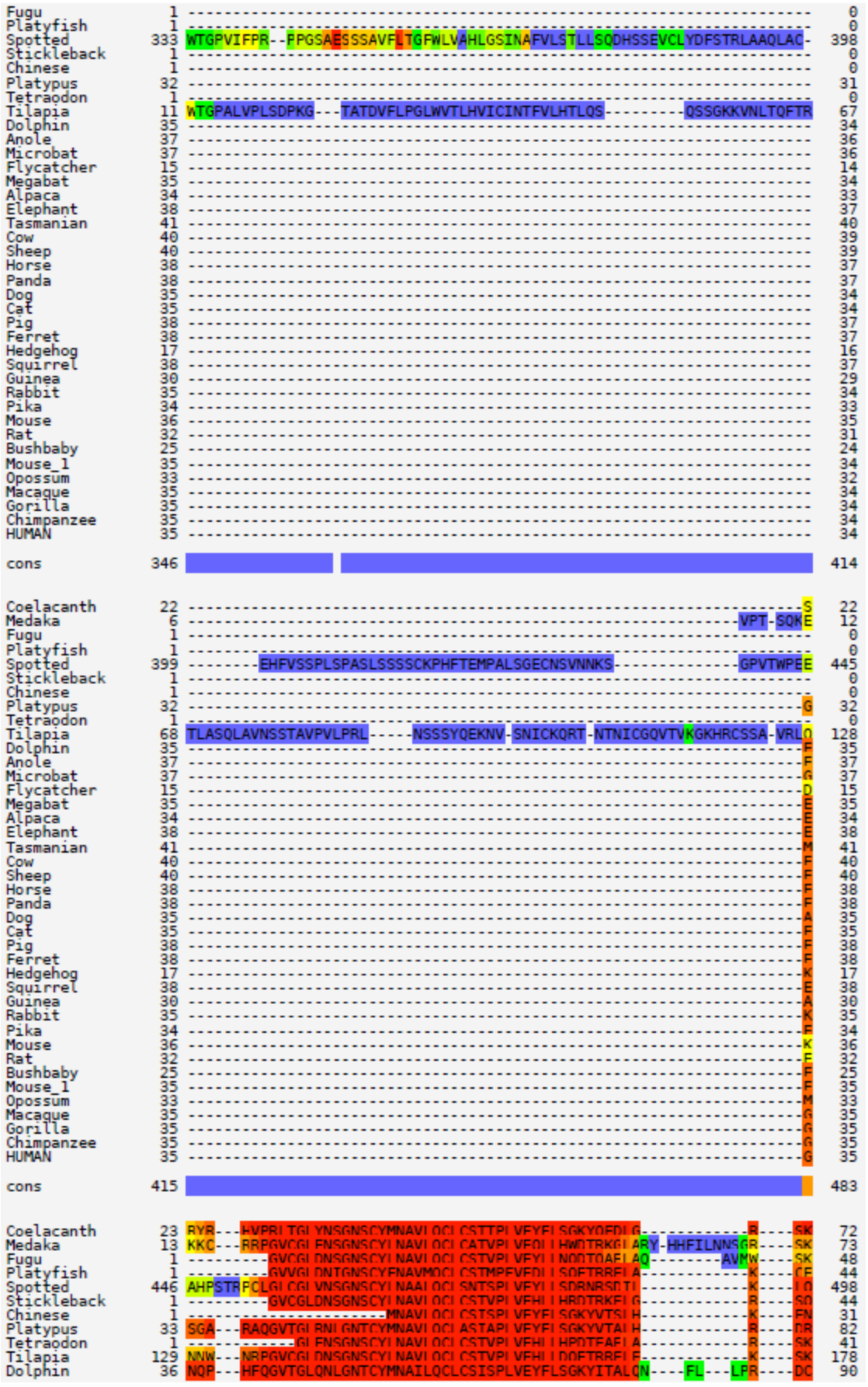
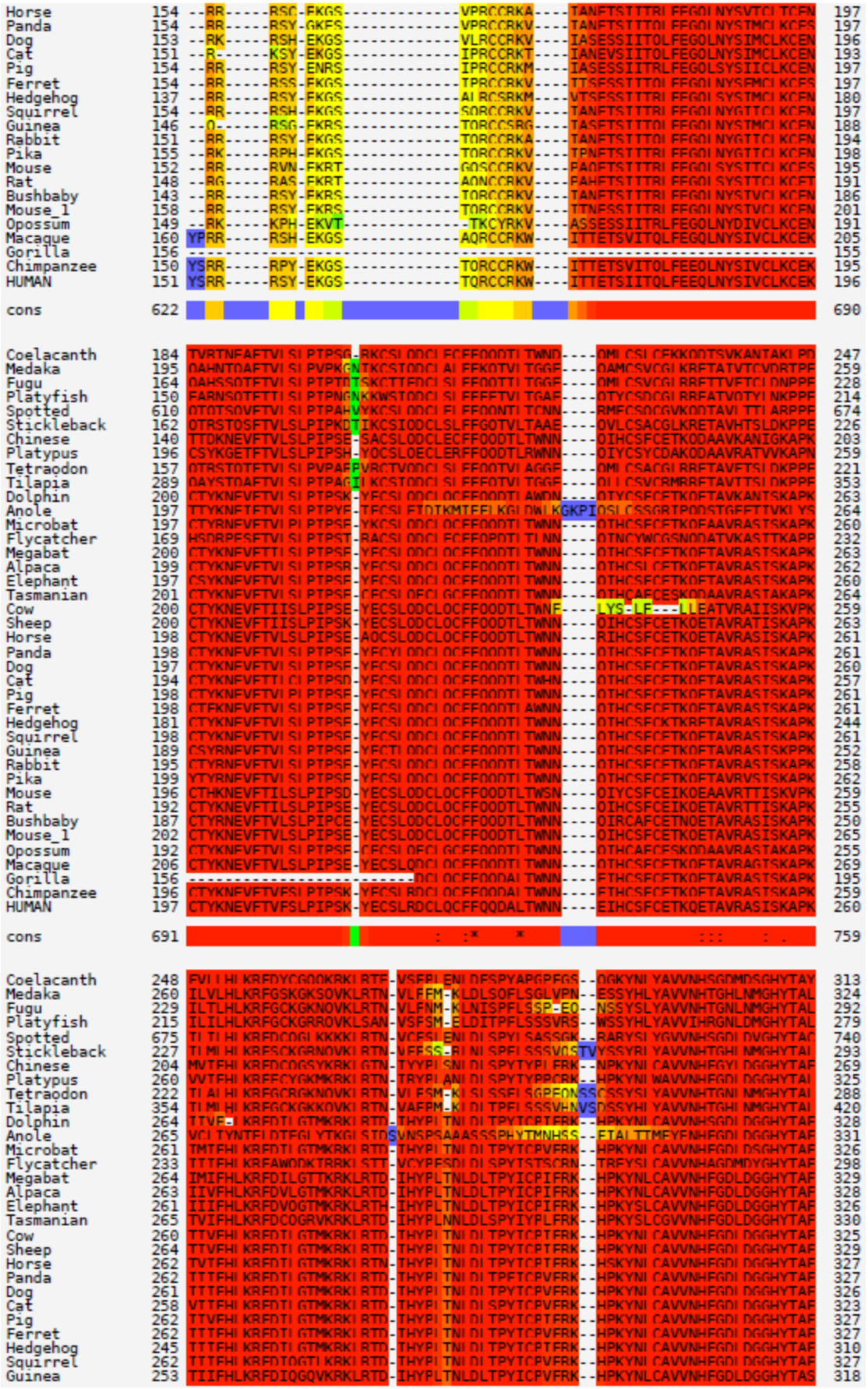
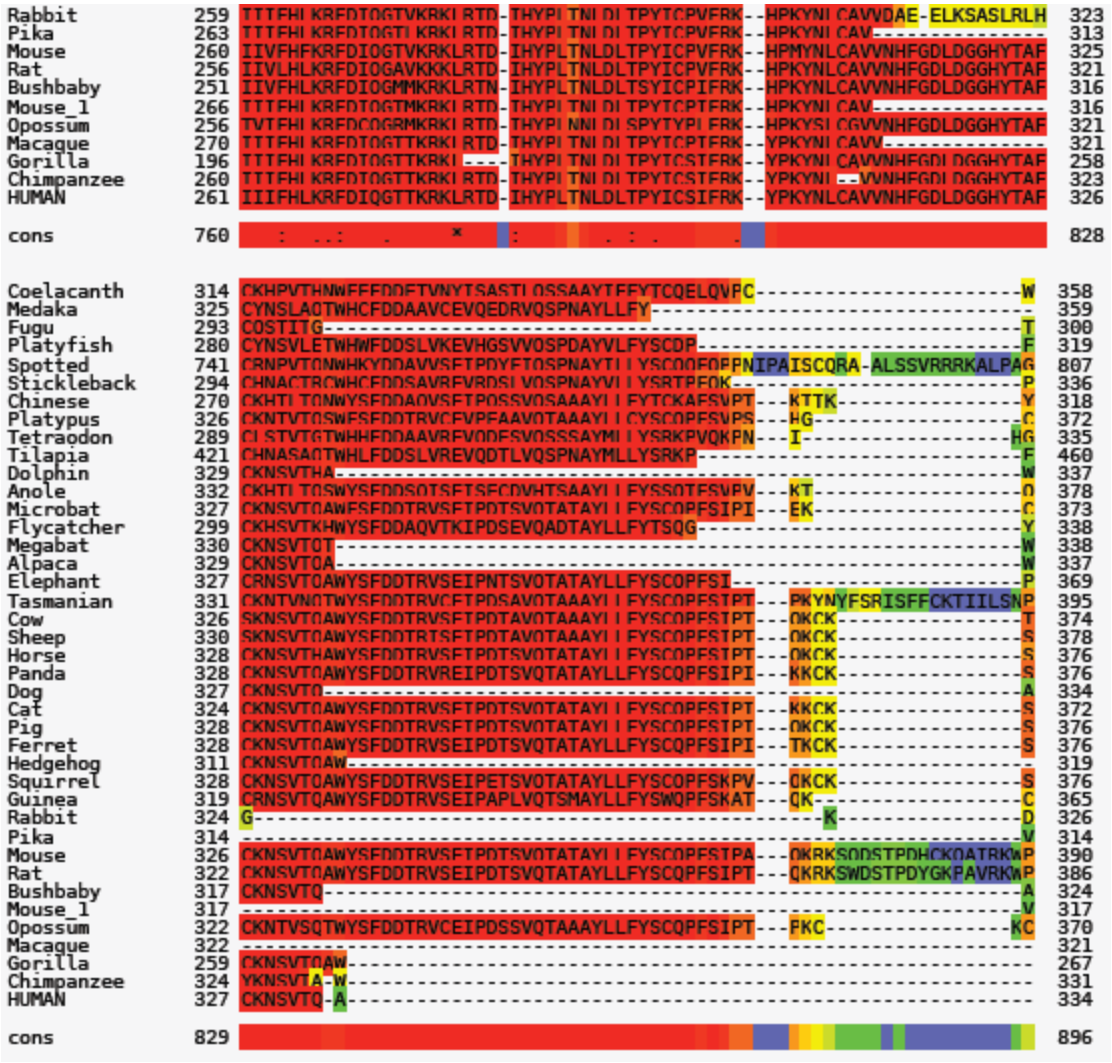


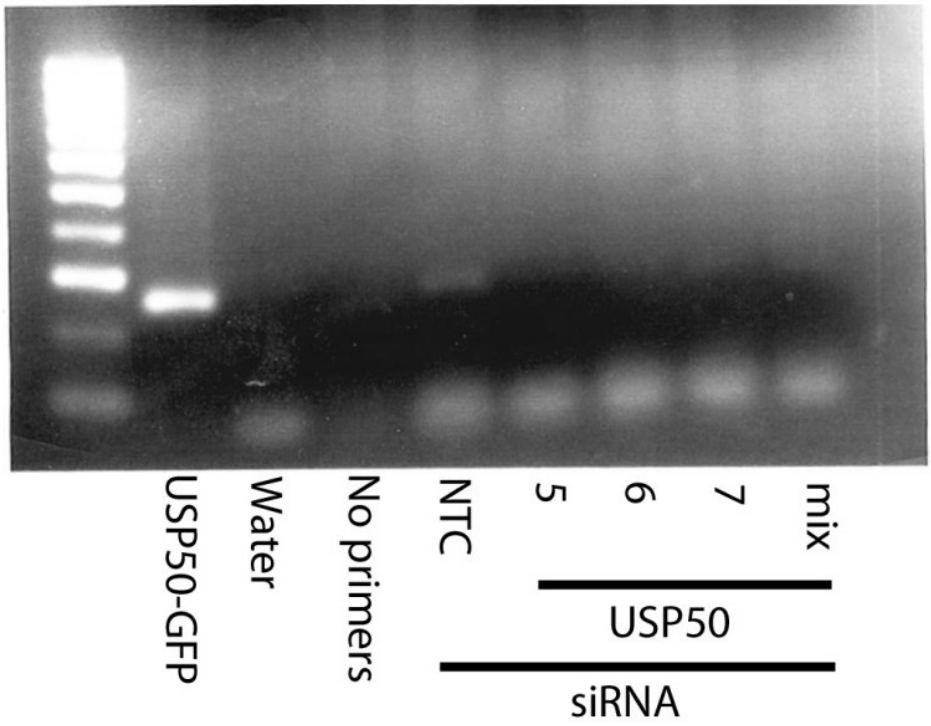




A4 Alignment of USP50 amino acid sequences across 40 species. T-coffee alignment of USP50 amino acid sequences from 40 species. Conservation of amino acids was calculated as well as the probability of the alignment being correct, represented by the colours.

A5 USP50 cDNA after USP50 siRNA treatment

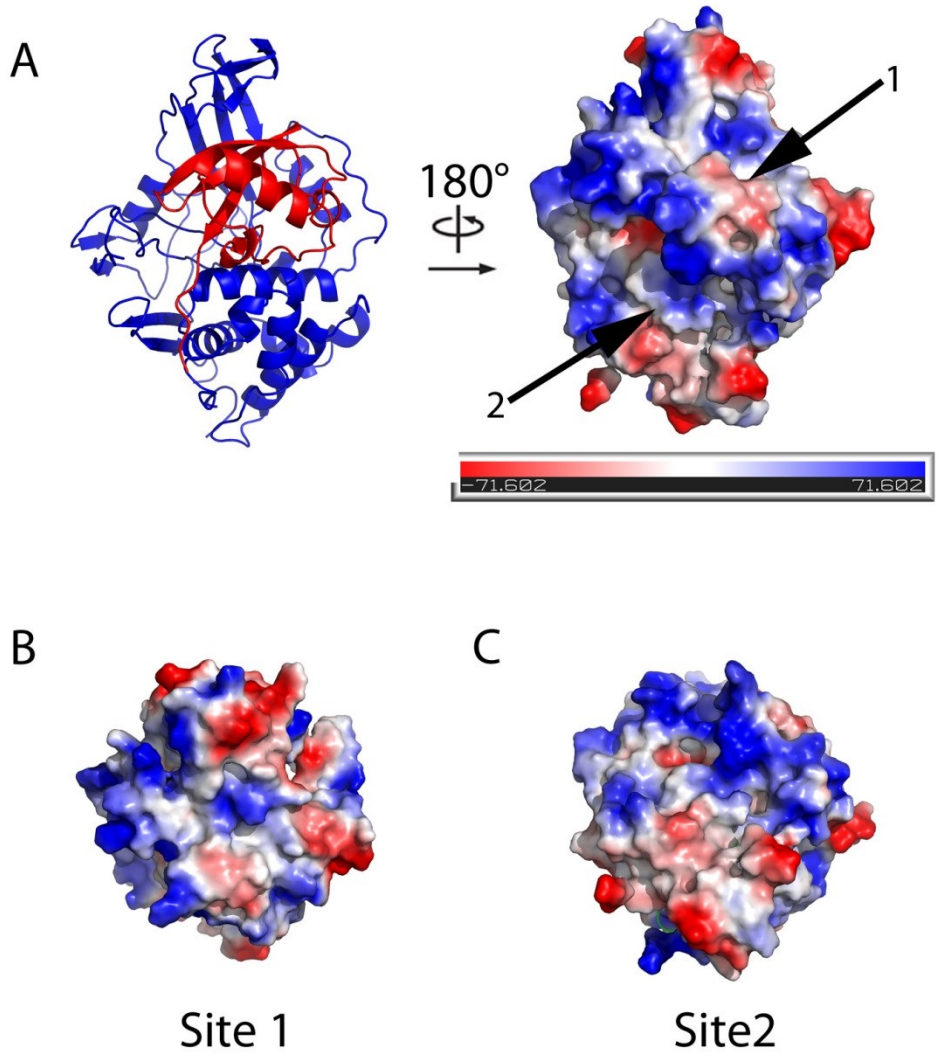
Cells were plated and transfected with NTC or UP50 siRNA for 72 hours before mRNA was extracted. The mRNA was converted into cDNA and then amplified using USP50 specific primers. DNA was run on a 1% agarose gel containing Ethidium bromide. DNA was visualised using UV light and imaged using GeneSnap by Syngene system.



A5 USP50 cDNA is reduced upon USP50 siRNA treatment. USP50-GFP plasmid was used as a positive control to confirm the reaction was working. Water replaced cDNA as a negative control or no primers were included to check for non-specific bands. cDNA from cells treated with NTC or USP50 5, 6, 7 or a mix of all siRNAs and run on a gel.

A6 Hydrophobic residues of USP50 on surface behind binding domain

The electrostatic potentials of the amino acids in USP50 bound to Ub were calculated using Pymol. On the side of USP50 that does not bind Ub, there were two patches of uncharged amino acids, these may represent binding sites for other proteins. Uncharged amino acids are represented in white.



A6 Potential binding domains of USP50. **A)** USP50 bound to Ub ribbon model was rotated 180° and the surface electrostatic potential of the amino acids calculated using Pymol. Two patches of uncharged molecules were identified. **B)** View of patch 1 potential binding site. **C)** View of patch 2 potential binding domain.

A7 Putative phosphorylation sites of USP50

PhosphoSitePlus bioinformatics searches indicates three potential phosphorylation sites in human USP50. Each site was seen in on mass spectrometry study.

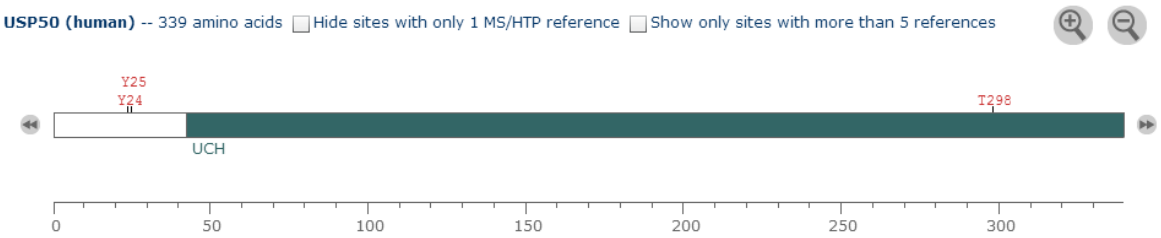


Fig A7 The putative phosphorylation sites of USP50. PhosphoSitePlus putative phosphorylation sites of human USP50. Three potential phosphorylation sites have been identified

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