Investigating the regulation of ZUP1, a novel deubiquitinating enzyme involved in DNA repair, by coupled monoubiquitination



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March 2021

This thesis is submitted to the Department of Biochemistry, University of Oxford, in fulfilment of the requirements for the Degree of Master of Science by Research in Biochemistry. This thesis is entirely my own work and describes my own research except where otherwise indicated.

Acknowledgment

I would like to express my gratitude to my supervisor, Dr Ian Gibbs-Seymour, for his continued support and guidance. His patience, extensive knowledge and insight were crucial throughout the completion of this work. I would also like to thank the postdoctoral researchers of the Gibbs-Seymour group, Dr Ben Foster and Dr Martin Attwood, for their helpful comments and recommendations on this thesis. I would further like to thank Dr Martin Attwood, as my primary contact in the laboratory, for his calming presence and generosity with his time.

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Abstract

Ubiquitination, a major post-translational modification, is reversed through the action of a large family of specialised proteases termed deubiquitinating enzymes (DUBs). Due to their involvement in key cellular pathways, such as inflammation, the cell cycle and DNA repair, as well as their potential as targets for therapeutic intervention, DUBs are critical to investigate. ZUP1 is a recently discovered DUB with cleavage specificity for lysine-63 conjugated polyubiquitin chains and has been proposed to have a role in DNA repair and genome stability maintenance via deubiquitination of the Replication Protein A complex. The regulation of DUBs is not well understood, but it has been suggested that ZUP1 undergoes a modification termed coupled monoubiquitination. Coupled monoubiquitination is a process whereby ubiquitin-binding proteins are monoubiquitinated themselves, which effectively abolishes their ubiquitin binding in trans, providing an inhibitory control mechanism. Here, by mutating two of the ubiquitin-binding domains of ZUP1, I show that ZUP1 monoubiquitination is dependent on the integrity of its ubiquitin binding domains, indicating that ZUP1 undergoes coupled monoubiquitination. Moreover, loss of monoubiquitination leads to a reduction in binding to another deubiquitinating enzyme involved in DNA repair, USP11 and its binding partner TCEAL1, an as of yet uncharacterised DNA repair protein. Together, I provide evidence that ZUP1 is regulated by coupled monoubiquitination which sets the foundation to further dissect the functional importance of this regulation in cells and to its enzymatic activity.

1. Introduction

1.1 Ubiquitin Signalling

Ubiquitination is a reversible post-translational modification (PTM) whereby ubiquitin (Ub), a 76 amino acid polypeptide, is covalently attached to lysine (K) residues of target proteins (Pickart & Eddins, 2004). This PTM serves a variety of functions, including proteasomal degradation, endocytosis and other cellular signalling events. Seven internal lysine residues of Ub (K6, K11, K27, K29, K33, K48, K63), as well as an N-terminal methionine (M1) allow the covalent conjugation of ubiquitin to another ubiquitin molecule, giving rise to polyubiquitin chains of varying linkages that greatly diversify the ubiquitin code (Yau & Rape, 2016) (Figure 1). Substrate proteins can therefore be monoubiquitinated, multi-monoubiquitinated, or polyubiquitinated. More recently, heterologous chains consisting of different linkages have also been studied (Yau et al., 2017) (Figure 1). Distinct linkage types of polyUb chains serve as signals for different functional outcomes for the substrate protein, including protein localisation, protein-protein interactions and proteasomal degradation. For instance, K48-linked polyUb chains mainly serve to target substrate proteins for proteasomal degradation, while K63-linked polyUb chains are thought to regulate cellular signalling events, including endocytosis, innate immunity pathways and DNA repair (Yau & Rape, 2016) (Figure 1).

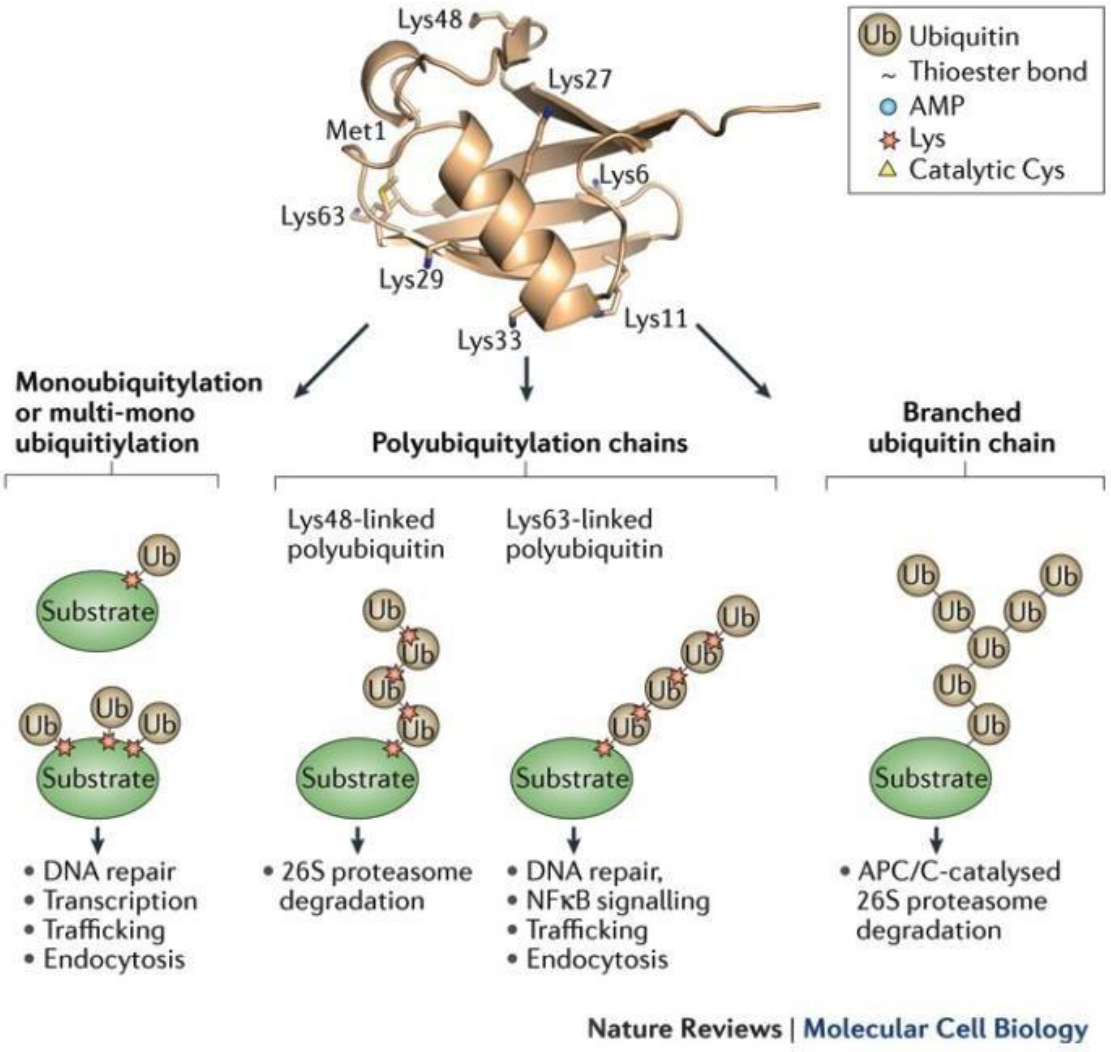


Figure 1. Ubiquitin is a 76 amino acid polypeptide with seven internal lysine residues that allow the formation of polyubiquitin chains of varying linkages. Different linkage types signal for different functional outcomes, regulating proteasomal degradation of the target protein, subcellular location and interaction with other proteins. Figure adapted from Buetow & Huang, 2016.

Proteins are ubiquitinated by the successive action of three enzymes termed E1 (ubiquitin-activating), E2 (ubiquitin-conjugating) and E3 (ubiquitin ligase) (Pickart & Eddins, 2004). Following the ATP-dependent formation of a covalent thioester bond between the E1 catalytic cysteine and the C terminal di-glycine motif of ubiquitin, ubiquitin is transferred from the E1 to E2, again attached to the E2 catalytic cysteine, forming an E2-ubiquitin thioester complex. Finally, an E3 enzyme binds the E2 with the attached ubiquitin moiety and the substrate to catalyse the formation of an isopeptide bond between the ε-amino group of a lysine residue side chain of the substrate and the C terminal carboxyl of ubiquitin (Buetow & Huang, 2016) (Figure 2A).

In order to maintain cellular homeostasis of ubiquitin, ubiquitin needs to be removed and recycled from substrates. This is achieved by several families of deubiquitinating enzymes (DUBs), which catalytically cleave ubiquitin from lysine residues of target proteins. The human genome encodes roughly 100 DUBs, which are classified into seven different families, six of which belong to thiol proteases that contain a catalytic cysteine residue, and one family which functions as Zn^{2+} metalloproteases (Clague et al., 2019) (Figure 2B). Most DUBs catalyse a proteolytic reaction between the C terminal glycine of ubiquitin and a lysine ε-amino group. In thiol protease DUBs, this is achieved by a catalytic diad or triad, consisting of the catalytic cysteine, a nearby histidine residue and sometimes an asparagine or aspartate residue (Komander et al., 2009). Aided by histidine, the cysteine performs a nucleophilic attack on the isopeptide bond between the carboxy group of the C terminal glycine of ubiquitin and the ε-amino group of a lysine residue (Komander et al., 2009). DUBs have diverse functions, from processing newly synthesised ubiquitin chains, to rescuing proteins targeted for degradation and regulating protein complex formation and protein-protein interactions through the removal of ubiquitin chains (Clague et al., 2019) (Figure 2A). While the majority of DUBs are not selective and are able of cleaving various types of polyubiquitin

chains (Faesen et al., 2011), some endo-DUBs that cleave within chains prefer certain linkages over others, allowing them to function in pathways associated with these ubiquitin chains.

The role of ubiquitin signalling in DNA repair and tumourigenesis has been increasingly recognised. When a cell encounters DNA damage or replication stress, damage sensors will recruit and activate cellular machinery that are part of the DNA damage response (DDR) to regulate DNA repair, cell cycle progression and cellular survival (Kiwerska & Szyfter, 2019). Unrepaired DNA lesions can lead to oncogenic transformation, making the DDR a key focus of the study of cancer progression and treatment. It is therefore unsurprising that DDR-associated ubiquitination is often deregulated in many cancers (Brinkmann et al., 2015). Many DUBs are involved in the DDR, often controlling the recruitment of DNA repair factors to damage sites and limiting the spread of damage-associated ubiquitin signalling (Le et al., 2019). This ranges from deubiquitination of FANCD2 by USP1 in the Fanconi Anaemia pathway of interstrand crosslink repair (Cohn et al., 2007) to the formation of a tripartite DUB complex of USP7, USP9x and OTUD4, enabling deubiquitination of the oxidative demethylase ALKBH3, thereby promoting the removal of methyl groups from DNA (Zhao et al., 2015), to a variety of DUBs that are responsible for deubiquitination of histones following the generation of double-stranded breaks, including the metalloprotease POH1 and USP11 (Le et al., 2019). Owing to this, as well as the involvement of many DUBs in other therapeutically relevant pathways including stem cell renewal, immune- or receptor tyrosine kinase signalling, DUBs have been increasingly realised as targets for the development of therapeutic intervention (Harrigan et al., 2018).

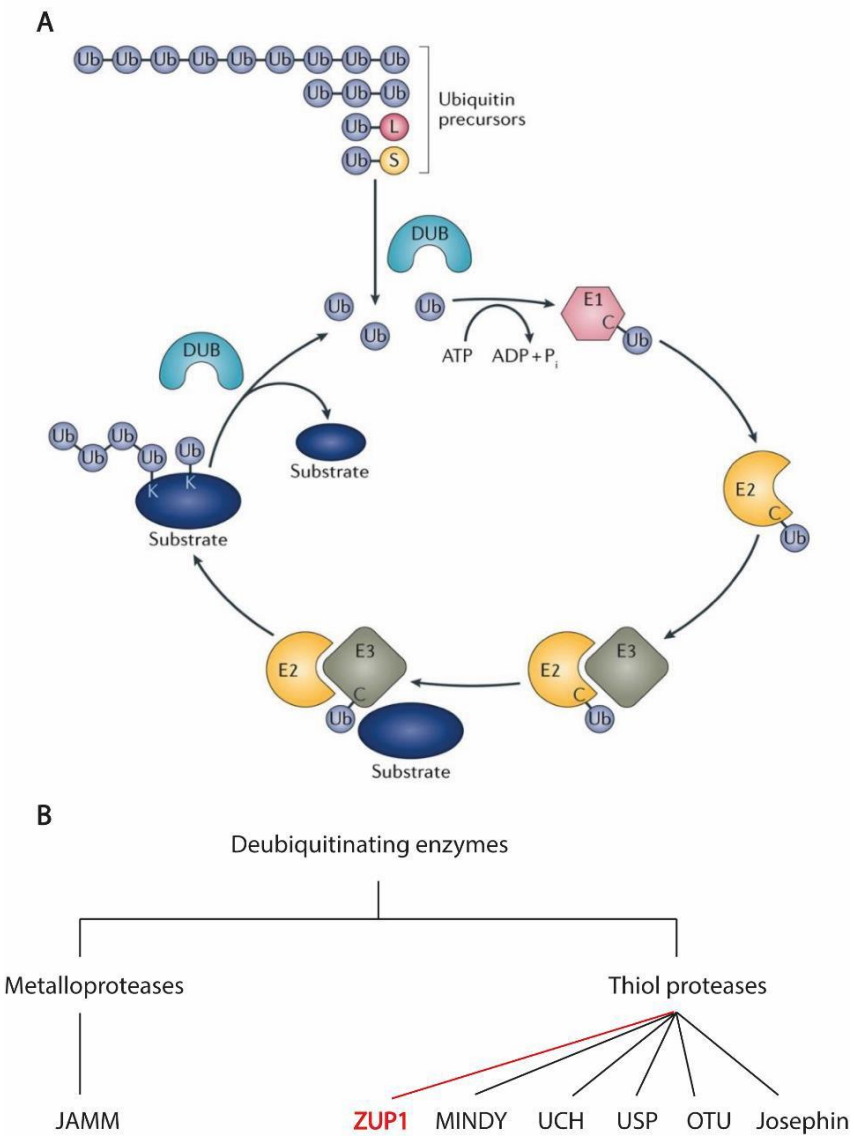


Figure 2. (**A**) Schematic diagram of key stages of the ubiquitination cycle. E1 enzymes activate ubiquitin, dependent on ATP, and ubiquitin is transferred to an E2 conjugating enzyme. Finally, ubiquitin is covalently attached to a lysine residue of a target protein through the help of an E3 ligase. DUBs are able to process ubiquitin precursors and to remove ubiquitin conjugated to proteins. Figure adapted from Harrigan et al. (2018). **(B)** Diagram showing the different DUB families, including ZUP1.

1.2 The ZUP1 DUB class

Recently, several independent research groups identified ZUP1 (Zinc finger containing Ub Peptidase 1), previously known as ZUFSP (Zinc Finger with UFM1-specific peptidase domain protein) as a novel DUB with no homology to other known DUBs, forming the only member of a seventh DUB class (Haahr et al., 2018; Hermanns et al., 2018; Hewings et al., 2018; Kwasna et al., 2018). ZUP1 contains a cysteine protease domain, four N-terminal C2H2 zinc fingers (ZnFs), and a Motif Interacting with Ubiquitin (MIU) (Figure 3A and Figure 3B). All four studies also found that ZUP1 has specificity for recognition and cleavage of K63-linked ubiquitin, with preference for long polyUb chains (Figure 3C). This specificity is conferred by the presence of three distinct ubiquitin-binding domains, only one of which, the Motif Interacting with Ubiquitin (MIU) has been previously annotated (Penengo et al., 2006). A helical arm protruding from the catalytic domain, termed ZUP1 helical arm (ZHA) was found to form the distal ubiquitin binding site (S1), with the proximal ubiquitin binding site (S1') likely formed by the fourth ZnF, which was identified as an atypical ubiquitin-binding zinc finger (UBZ) that is required in conferring cleavage activity to the ZUP1 catalytic domain (Kwasna et al., 2018) (Figure 3B). Interestingly, the MIU was not found to be critical for ubiquitin binding, but is likely to form the binding site of a third ubiquitin moiety in the chain (S2) (Kwasna et al., 2018).

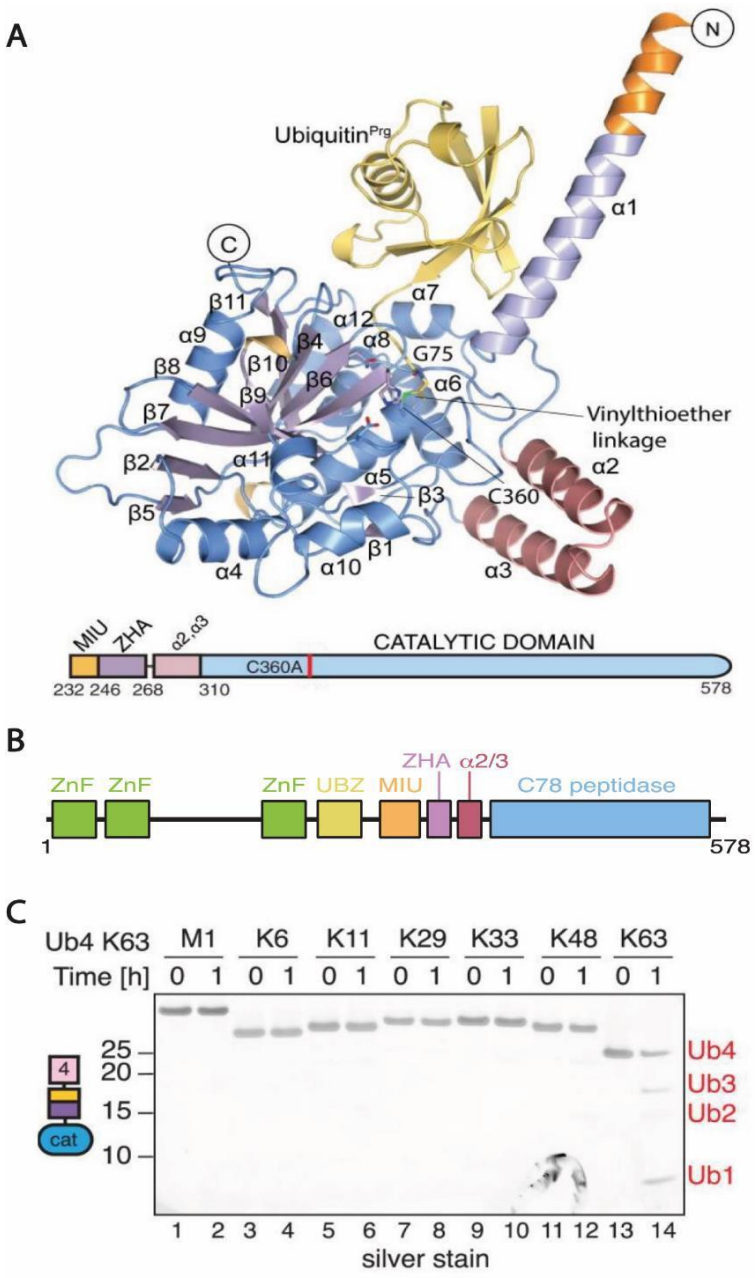


Figure 3. ZUP1 is a novel deubiquitinating enzyme with exquisite specificity towards lysine 63-linked polyubiquitin that contains three different ubiquitin-binding domains and is possibly regulated by coupled monoubiquitination. (**A**) Crystal structure of ZUP1, comprising the catalytic core domain in addition to the Motif Interacting with Ubiquitin (MIU) domain (Kwasna et al., 2018). (**B**) Schematic diagram of ZUP1 domains: UBZ – Ubiquitin binding ZnF, ZHA – ZUP1 Helical Arm. (**C**) DUB assay of the ZUP1 catalytic domain together with MIU and UBZ domains, testing cleavage of different tetraubiquitin chain types (Kwasna et al., 2018).

ZUP1 was found to be important for genome stability maintenance and evidence suggests it is involved in replication-coupled DNA repair. General involvement of ZUP1 in the DDR was indicated by its recruitment to laser-generated DNA lesions (Haahr et al., 2018; Hermanns et al., 2018; Kwasna et al., 2018). An increase in spontaneous DNA damage upon knockdown of ZUP1 in cancer cells was highest during G1/S phases (Kwasna et al., 2018), and ZUP1 was further shown to be recruited to stalled replication forks (Haahr et al., 2018). Moreover, ZUP1's role in DNA repair appears to be linked to its ability to cleave K63-linked ubiquitin chains. Recruitment of ZUP1 to DNA damage sites is dependent on both its catalytic activity as well as the presence of K63-linked ubiquitin chains at DNA lesions (Haahr et al., 2018; Kwasna et al., 2018), with loss of ZUP1 linked to an increase in K63-chains at stalled replication forks (Haahr et al., 2018). Interestingly, ZUP1 was identified as an interacting protein of the major single-stranded DNA (ssDNA) protein Replication Protein A (RPA) (Tkáč et al., 2016), which is involved in DNA replication and replication-stress induced DNA repair (Chen & Wold, 2014). RPA binds exposed ssDNA following replication fork stalling, protecting it from nucleolytic degradation and serves as a platform for the recruitment of further DDR factors, including the S-phase master checkpoint kinase ATR (Vassin et al., 2009), with inhibition and loss of RPA leading to increased replication stress that can potentially be exploited in cancer treatment (Dueva & Iliakis, 2020; Gudjonsson et al., 2012; VanderVere-Carozza et al., 2020). The interaction of ZUP1 with RPA was confirmed by all four groups who initially identified ZUP1 as a novel DUB, and there is some evidence to suggest that ZUP1 deubiquitinates RPA in cells. RPA is known to be modified by K63-linked ubiquitin following replication stress, which promotes restart of stalled forks and homologous recombination (Maréchal et al., 2014; Elia et al., 2015). ZUP1 has been shown to co-localise with RPA at DNA lesions (Haahr et al., 2018), with loss of ZUP1 leading to an increase in K63-ubiquitination of RPA under replication stress (Hewings et al., 2018). ZUP1 was further shown to interact with a host of other DNA repair proteins (Kwasna et al., 2018), among which the DUB USP11 and the E3 ligase UBR5,

as well as the uncharacterised protein TCEAL1 were most highly enriched (Hermanns et al., 2018). USP11 plays a role in NF-kappa-B activation (Sun et al., 2010) and in transcriptional repression through association with the Polycomb Repressive Complex 1 (PRC1)(Maertens et al., 2010). It has been implicated in the repair of double-stranded DNA breaks (Wiltshire et al., 2010) and was reported to deubiquitinate γH2AX among other histones following DNA damage (Ting et al., 2019). Meanwhile, UBR5 has been shown to regulate S-Phase and G2/M DNA damage checkpoints through controlling CHK2 levels (Munoz et al., 2007), while it suppresses the excessive spread of histone ubiquitination following DNA damage by negatively regulating levels of the E3 ligase RNF168 (Gudjonsson et al., 2012; Cipolla et al., 2019).

Taken together, these findings suggest that ZUP1 regulates DNA repair and genome stability by specifically deubiquitinating K63-ubiquitinated targets at sites of DNA damage and replication stress, with one of the major substrates likely to be ubiquitinated RPA.

1.3 Coupled monoubiquitination

Binding to ubiquitin and recognition of ubiquitinated proteins is often mediated by conserved ubiquitin binding modules, or ubiquitin-binding domains (UBDs) of ubiquitin receptors. Proteins containing these UBDs are frequently monoubiquitinated themselves, in a process termed coupled monoubiquitination that is dependent on the presence and integrity of the UBD, but not on the presence of lysine residues within the UBD. Coupled monoubiquitination has been observed in several endocytic proteins, as well as DNA repair pathways, and negatively regulates ubiquitin binding ability by engaging the UBD in intramolecular interaction with monoubiquitin, causing autoinhibition (Hoeller et al., 2006).

The majority of known UBDs interact with ubiquitin by contacting the hydrophobic patch on the surface of ubiquitin formed by Ile44, surrounded by Leu8, Val70 and other hydrophobic residues (Randles & Walters, 2012) (Figure 4). Most common among the UBDs are alpha-

helical motifs, including the ubiquitin interacting motif (UIMs), motif interacting with ubiquitin (MIU), double-sided UIM (DUIM), ubiquitin-associated domain (UBA), with another prominent group formed by Ub-binding zinc fingers (ZnFs) (Figure 4). Well-classified ubiquitin-binding zinc fingers include the Npl4 ZnF (NZF), the A20 ZnF as well as the ubiquitin-binding ZnF (UBZ) domain of Y-family DNA polymerases (Bienko et al., 2005). The presence of several UBDs within one protein is not uncommon, as most single UBDs do not bind ubiquitin, especially monoubiquitin, with high affinity (Randles & Walters, 2012)

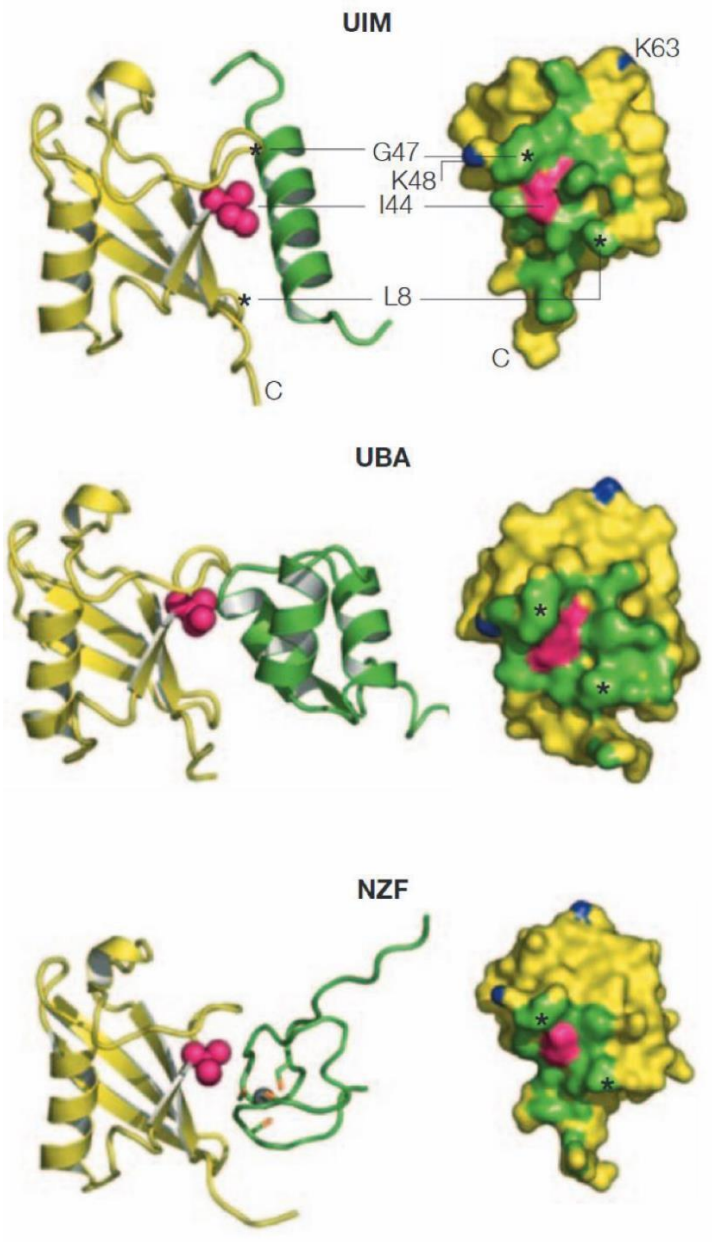


Figure 4. Different ubiquitin-binding domains contact the hydrophobic Ile44 surface of ubiquitin. Three different UBD complex structures are shown, structures of which were determined using either X-ray crystallography or NMR spectroscopy. On the left, ubiquitin is shown in yellow, the UBD appears in green and the Ile44 residue is represented by a red, spacefill representation. On the right, ubiquitin is again shown in yellow, while the green represents the surfaces in contact with the UBD, and the Ile44 side chain is represented in red. Lys48 and Lys63 amino groups are shown in blue. The UIM (ubiquitin-interacting motif) shown represents the UIM of Vps27 (Protein Data Bank: 1Q0W), the UBA (ubiquitin-associated domain) stems from Dsk2p (Protein Data Bank: 1WR1), while the NZF represents the Npl4 zinc finger domain. Figure adapted from Hicke et al., 2005.

1.3.1 Functional consequences of coupled monoubiquitination

Coupled monoubiquitination has been observed in several proteins and is generally thought to serve as an inhibitory regulatory mechanism. Several examples of the role of this modification stem from endocytosis, where it was first discovered. To avoid hyperstimulation of the cell, activated receptor tyrosine kinases (RTKs) are ubiquitinated, leading to their sorting into endocytic vesicles destined for the lysosome, a process that requires ubiquitin-binding adaptor proteins. Early studies on the topic found that the endocytic adaptor proteins Eps15 and eps15R were monoubiquitinated following epidermal growth factor receptor (EGFR) activation in a manner that requires the second UIM (Klapisz et al., 2002; Polo et al., 2002). This UIM-dependent monoubiquitination prevented interaction of Eps15 with exogenous ubiquitin as well as polyubiquitinated, ligand-bound EGFR in endocytic vesicles (Hoeller et al., 2006), effectively delaying its internalisation and degradation (Fallon et al., 2006). Similarly, suppressors of T-cell receptor signalling 1 and 2 (Sts1 and Sts2) interact with ubiquitinated RTKs through N-terminal UBA domains and negatively regulate their endocytic degradation (Hoeller et al., 2006). Monoubiquitination of Sts1 and Sts2 depends on conserved UBA residues

and the expression of mutant chimeras mimicking permanent coupled monoubiquitination leads to a marked decrease in EGFR levels, indicating interference with the ability to block EGFR degradation (Hoeller et al., 2006).

Rabex-5, a guanine exchange factor (GEF) of Rab5 and E3 ubiquitin ligase is recruited to the plasma membrane upon EGFR activation. Rabex-5 contains a UIM with the critical residues appearing in reverse orientation (Penengo et al., 2006), making it the first protein in which the MIU was identified. Monoubiquitination of Rabex-5 is MIU-dependent, and while wild-type Rabex-5 was found to be membrane-bound, a ubiquitin-Rabex-5 chimera was predominantly located in the cytosol (Mattera & Bonifacino, 2008)**,** with the authors proposing that Rabex-5 is deubiquitinated upon recruitment to endosomes, enabling interaction with ubiquitinated cargo, followed by renewed monoubiquitination and dissociation from endosomes after cargo delivery (Mattera & Bonifacino, 2008).

Importantly, coupled monoubiquitination has also been found to regulate protein-protein interactions in DNA repair pathways. Monoubiquitination of the translesion synthesis polymerase Pol η depends on its ubiquitin-binding zinc finger (UBZ) (Bienko et al., 2005), and was shown to prevent its effective recruitment to stalled replication forks (Figure 5A, i), where it interacts with monoubiquitinated DNA clamp PCNA, enabling bypass translesion synthesis (Bienko et al., 2010). Furthermore, it was shown that the lysine residues targeted by monoubiquitination are situated in the PCNA interacting surface (PIR) of Pol η, engaging both the UBZ and the PIR domains upon monoubiquitination, thereby making them unavailable for PCNA interaction (Bienko et al., 2010). Finally, following treatment of cells with DNA damaging agents that induce translesion synthesis (UV irradiation, cisplatin, methyl methanesulfonate (MMS)), a decrease in monoubiquitinated Pol η was observed (Figure 5A, ii), increasing the fraction of unmodified Pol η capable of binding to PCNA (Bienko et al., 2010).

Another example of coupled monoubiquitination can be found in the DNA-protein crosslink (DPC) protease SPRTN (alternatively known as DVC1). Recruitment of SPRTN to stalled replisomes is negatively regulated by UBZ-dependent monoubiquitination (Mosbech et al., 2012)(Figure 5B, i). Monoubiquitination of SPRTN is reduced upon induction of DPCs following formaldehyde treatment (Figure 5B, ii), corresponding to a simultaneous re-localisation of the SPRTN pool to chromatin, where it is known to interact with ubiquitinated PCNA (Stingele et al., 2016; Centore et al., 2012; Machida et al., 2012).

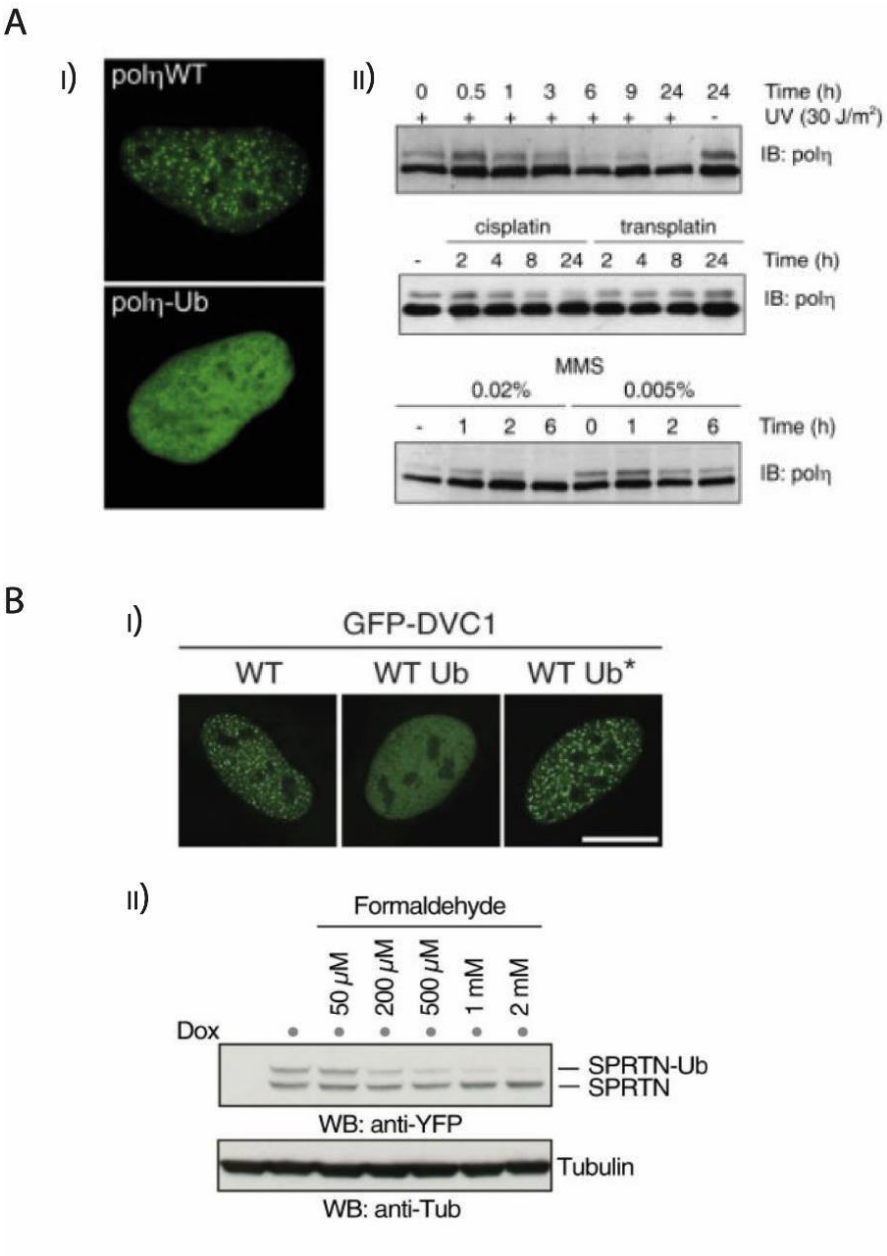


Figure 5. Coupled monoubiquitination in DNA repair. (A) Coupled monoubiquitination of Pol η. **(i)** Wild-type EGFP- Pol η and an EGFP- Pol η ubiquitin chimera (EGFP- Pol η -Ub) expressed in MRC5 cells, showing that the chimera was found at replication foci less frequently than the wild-type. **(ii)** Immunoblots of Pol η after treatment of MRC5 cells with UV, cisplatin, transplatin or MMS. Figures adapted from Bienko et al. (2010). **(B)** Coupled monoubiquitination of SPRTN (DVC1). **(i)** Wild-type GFP-SPRTN (WT), a GFP-SPRTN ubiquitin chimera (WT-Ub) and a GFP-SPRTN ubiquitin chimera in which Ile44 is mutated to alanine (WT Ub*) expressed in U2OS cells, showing that the Ub-chimera is not recruited to UV light-induced nuclear foci. Figure adapted from Mosbech et al. (2012) **(ii)** Treatment of Doxycycline-inducible YFP-SPRTN-Strep HeLa Flp-In TRex cells with increasing doses of Formaldehyde, showing Formaldehyde-dependent decrease in SPRTN monoubiquitination. Figure adapted from Stingele et al. (2016).

1.3.2 Coupled monoubiquitination of deubiquitinating enzymes

Numerous DUBs have been reported to be ubiquitinated, with limited information on the functional significance of this modification (Haq & Ramakrishna, 2017). One known example is Ataxin-3, a DUB involved in ubiquitin-dependent protein quality control and cytoskeletal regulation, monoubiquitination of which enhances its catalytic activity towards polyubiquitin chains (Todi et al., 2009). Similarly, monoubiquitination of JosD1 increases its catalytic activity and promotes localisation to the plasma membrane (Seki et al., 2013). In these cases, however, monoubiquitination is not dependent on the UBDs of Ataxin-3 or JosD1, so the inhibitory effect of coupled monoubiquitination would not be expected.

Importantly, there are reports of regulation of DUBs in the form of coupled monoubiquitination. UCH-L1, exclusively expressed in neurons and testis/ovary, mutations of which have been linked to Parkinson's disease (Lowe et al., 1990; Osaka et al., 2003). Neuronal UCH-L1 is

monoubiquitinated at lysine residues near the active site cysteine residue, which inhibits its ubiquitin binding ability (Meray & Lansbury, 2007). Loss-of-function mutations of residues required for ubiquitin binding nearly abolished monoubiquitination, indicating that the modification is coupled to the integrity of the ubiquitin binding site (Meray & Lansbury, 2007).

Another potential example comes from the muscle isoform of USP25 (USP25m), which is thought to have a role in muscular differentiation and function (Bosch-Comas et al., 2006). Monoubiquitination of USP25m was found to be enhanced by the presence of functional UIM domains (UIM1 and UIM2) and a UBA domain (Denuc et al., 2009), possibly indicating coupled monoubiquitination. Monoubiquitination was found to occur at K99, a residue previously shown to be targeted by SUMOylation, which caused a decrease in ubiquitin binding and the catalytic activity of USP25m (Meulmeester et al., 2008). Interestingly, the authors suggested that USP25m monoubiquitination at K99 had the opposite effect, enhancing catalytic activity, proposing a dual regulation of USP25m at K99. This would provide the first example of coupled monoubiquitination not having an inhibitory effect on the host protein. The hypothesis was based on the observation that a USP25m K99R mutant led to impaired rescue of its ubiquitinated target MyBPC1 from proteasomal degradation (Denuc et al., 2009). However, there are some issues with this experiment. Since both ubiquitin and SUMO are attached to lysine residues, and lysine to arginine mutations are widely used to abolish both modifications, the K99R mutation would effectively eliminate the preferential site for both. Aware of this, the authors surmised that if the K99R mutation prevented SUMOylation alone, which was previously shown to have an inhibitory effect on USP25 (Meulmeester et al., 2008), loss of SUMOylation would have led to increased DUB activity of USP25m K99R, instead of the observed decrease. However, precisely because the mutation abolishes both modifications, it is not possible to determine loss of which had the observed effect of increasing catalytic activity, or to assume that this effect is due to a loss of monoubiquitin. The study that showed

the K99 SUMOylation-dependent decrease of USP25m activity did not do so by introducing a K99R mutation, but rather by generating a USP25m-SUMO fusion construct (Meulmeester et al., 2008). The same approach would have to be taken to unequivocally compare the effect of each modification on the activity of USP25m, by generating USP25m-ubiquitin and USP25m-SUMO fusion constructs and testing their ability to cleave polyubiquitin chains and to rescue ubiquitinated substrates from degradation.

Finally, both UCH-L1 and USP25m are capable of auto-deubiquitination. This is indicated by an increase in the monoubiquitinated fraction of both enzymes following mutation of the catalytic cysteine residue (Meray & Lansbury, 2007; Denuc et al., 2009). In UCH-L1, this was further confirmed by analysing the monoubiquitination status of purified wild-type and catalytic-dead mutant UCH-L1 at different time points, which showed that catalytically inactive UCH-L1 remained in its monoubiquitinated state throughout, while the deubiquitinated fraction of wild-type UCH-L1 progressively increased (Meray & Lansbury, 2007). These findings suggest that coupled monoubiquitination of DUBs can be removed in an auto-regulatory loop, not requiring other DUBs to do so, or at least provide a base mechanism that maintains balance between ubiquitinated and deubiquitinated states.

1.3.3 Mechanism of coupled monoubiquitination

Since UBDs were found to signal for ubiquitination without being ubiquitinated themselves, with the UIM of eps15 shown to be sufficient for ubiquitination of a chimeric glutathione-S-transferase (GST)-UIM fusion protein (Oldham et al., 2002), it was proposed that they promote ubiquitination by recruiting ubiquitination machinery (Hicke et al., 2005). A few models of ubiquitin transfer were proposed initially, with the two most probable ones being the E3-thiolester model and the E3-isopeptide model (Hicke et al., 2005).

In a series of *in vitro* ubiquitination assays employing various ubiquitin and UIM mutants, the E3-isopeptide model was confirmed using the example of eps15 (Woelk et al., 2006). The authors showed that the UIM of eps15 bound to the ubiquitin molecule covalently attached to Nedd4, a HECT-type E3 ligase, followed by subsequent transfer of the thiol ester linked ubiquitin to eps15, instead of recognising the thiol ester-bound ubiquitin with the UIM (E3 thiolester model). Similarly, the RING-type E3 ligase Parkin which contains a ubiquitin-like domain (Ubl), is also able to cause eps15 monoubiquitination through a Ubl-UIM interaction (Fallon et al., 2006)**.** Both Nedd4 and Parkin were shown to monoubiquitinate eps15 following EGF stimulation of cells, thereby negatively regulating interaction of eps15 and EGFR (Polo et al., 2002; Fallon et al., 2006; Woelk et al., 2006).

While the E3-isopeptide model was shown to be true, at least in the case of UIM-containing proteins, it is unlikely to be the only mechanism for coupled monoubiquitination, even for eps15. Monoubiquitination of UBD-containing proteins occurs upon overexpression of ubiquitin, but does not require overexpression of E3 ligases (Bienko et al., 2005; Hoeller et al., 2006).

Interestingly, it has been shown that the presence of UBDs allows for E3-independent ubiquitination. Monoubiquitination of UBD-containing purified proteins *in vitro* was possible in the presence of E1/ATP and E2 enzymes despite the lack of an E3 ligase (Hoeller et al., 2007)**.** A ubiquitin mutant (I44A) linked to the E2 enzyme via a thiol ester bond was not conjugated to UBD-containing chimeric proteins, and UBD integrity was shown to be necessary and sufficient for ubiquitination by E2 enzymes. The same study also found that knockdown of Nedd4 and Parkin did not significantly affect monoubiquitination of eps15. Meanwhile, the significant reduction in Sts2 monoubiquitination upon knockdown of two different E2 enzymes could be rescued by the expression of an E3-binding-deficient E2 mutant (Hoeller et al., 2007).

Since ubiquitin could be conjugated to TLS pol η without any E3 ligases, it was concluded that ubiquitination occurred by binding of pol η to E2-ubiquitin (Bienko et al., 2010).

Thus, two different mechanisms of coupled monoubiquitination have been shown, one in which the UBD of a protein recognises ubiquitin covalently attached to a E3 ligase (or a Ubl), leading to the transfer of the thiol ester-bonded ubiquitin, and another in which UBDs-containing proteins enable ligase activity in *cis* by recruiting Ub-carrying E2 enzymes. It has been proposed that these mechanisms could serve different functions, with cis-ligase activity potentially maintaining homeostasis of the ubiquitin-binding ability of UBD-containing proteins, while E3-mediated ubiquitination could act in response to cellular signals (Hoeller & Dikic, 2010).

1.3.4 Coupled monoubiquitination of ZUP1

Given the importance for ZUP1 in the cellular response to replication stress, ZUP1 activity is likely strictly regulated. Interestingly, on immunoblots of ZUP1, a higher molecular weight form can be seen above the predominant ZUP1 signal, likely representing a PTM (Figure 5). Modification of proteins with PTMs resulting in increased molecular mass (M_r) is represented by a mobility shift in SDS-PAGE corresponding to the molecular weight of the added modification. Monoubiquitination of proteins appears as a mobility shift on SDS-PAGE gels corresponding to the molecular mass of a single ubiquitin, which equals roughly 8.6 kDa. While at this point it cannot be excluded that the modification is another PTM, such as phosphorylation or acetylation, the higher-molecular weight fraction of ZUP1 corresponds to an increase of roughly the molecular mass of ubiquitin compared to unmodified ZUP1, making monoubiquitination the most likely PTM in question (Figure 6). Importantly, unpublished data by the Gibbs-Seymour group showed that upon mutation of the MIU, this top band disappeared (Figure 6), suggesting that ZUP1 might be regulated by coupled monoubiquitination.

On the basis of this finding, I therefore set out to begin to understand whether ZUP1 is regulated by coupled monoubiquitination and how it might impact ZUP1 function.

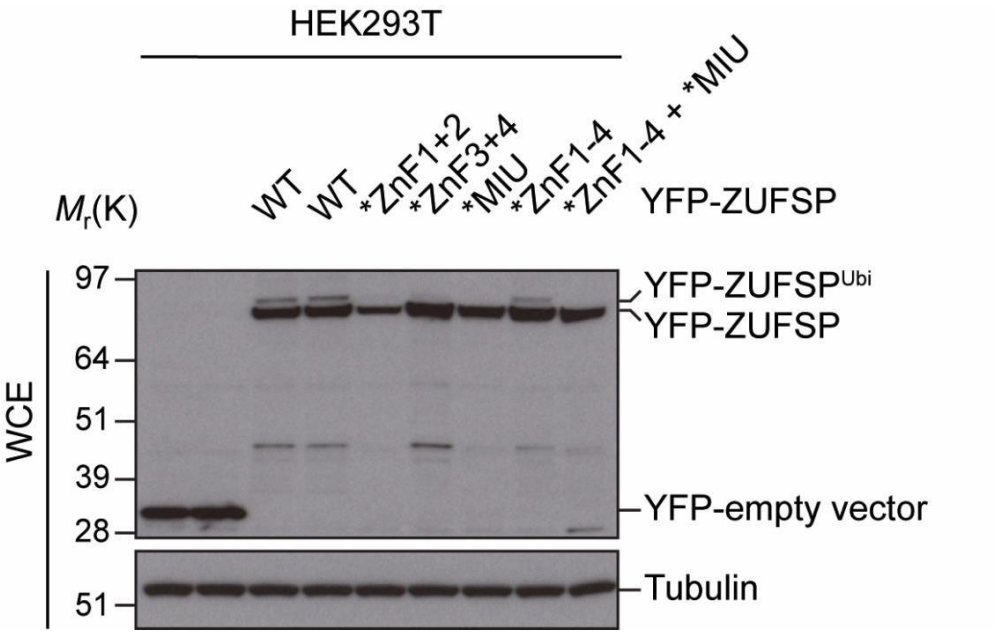


Figure 6. Monoubiquitination of ZUP1 is abolished upon mutation of the MIU domain. Unpublished data from the Gibbs-Seymour group. HEK293T cells were transfected with the indicated wild-type and mutant YFP-tagged ZUP1 constructs, and an anti-GFP immunoblot was performed on the whole-cell extract (WCE).

2. Materials and Methods

2.1 Method Details

Cell culture:

HEK293T and U-2 OS cells were cultured in DMEM (Sigma) containing 10% fetal bovine serum (FBS) at 37ºC, with 5% CO_2. To generate Doxycycline-inducible HEK293 cells, HEK293 Flp-In TRex cells stably expressing TetR were transduced with pcDNA5/FRT/TO, pcDNA5/FRT/TO ZUP1 WT or pcDNA5/FRT/TO ZUP1 C360A and selected in 15ug/ml Blasticidin (Thermo Fisher Scientific) and 100ug/ml Hygromycin (Thermo Fisher Scientific). Screening of clones was performed by treating cells with Doxycycline (Sigma Aldrich) at 1/1000 dilution, followed by anti-FLAG and anti-ZUP1 immunoblotting.

Transient DNA transfections were performed using TransIT®-LT1 (Mirus), with only half of the transfection reagent and DNA used that was recommended by the manufacturer. For instance, for a 10cm dish containing HEK293T cells at 50% confluency, 7.5µl DNA (1µg/µl stock) and 22.5µl transfection reagent were used. Reverse siRNA transfections were performed using Lipofectamine RNAiMAX (Thermo Fisher Scientific), with siUSP11 and siTCEAL1 used at a final concentration of 50nM, and siUBR5 at a final concentration of 5nM.

For DNA damage experiments, the following drug concentrations were used: Hydroxyurea (2mM, Sigma-Aldrich), Camptothecin (1µM, Sigma-Aldrich), Mitomycin C (1µM, Sigma-Aldrich), Neocarzinostatin (100ng/ml, Sigma-Aldrich), 4-Nitroquinoline 1-oxide (1µg/ml, Sigma-Aldrich), 16% Methanol-free Formaldehyde (1mM, Thermo Fisher Scientific).

FLAG co-immunoprecipitation:

Cells were lysed in Triton X-100 buffer (50mM Tris-HCl pH 8, 1% Triton X-100, 100mM NaCl) and roughly 800µl of clarified lysate was incubated with 200µl ANTI-FLAG® M2 Affinity Gel for 30min at 4ºC while rotating. Beads were washed five times with Triton X-100

buffer and eluted with 4X NuPAGE LDS Sample buffer (+10% DTT) and heated for 10min at 70°C.

Immunoblotting:

Cells were washed three times with ice-cold PBS/Tween (PBS/T) and lysed either in Triton X-100 buffer (50mM Tris-HCl pH 8, 1% Triton X-100, 100mM NaCl) or EBC buffer (150mM NaCl, 50mM Tris pH 7.5, 1mM EDTA, 0.5% Np50) supplemented with additives (5mM NEM, 1mM DTT, 1mM PIC, 1mM NaF, 10mM β-glycerophosphate, 0.1mM Sodium Orthovanadate). Samples were then lysed while rotating at 4°C for 20 min and collected by centrifugation. Protein concentration was determined using the Bradford Dye Assay (Bio-Rad) and samples were prepared by adding 4X NuPAGE LDS Sample buffer (Thermo Fisher Scientific) and boiled for 5min at 95°C.

Membranes were blocked in 5% milk in 1X PBS/Tween (PBS/T) for 1h and incubated in primary antibody in 5% milk in 1X PBS/T overnight. Membranes probed for phospho-protein were blocked with 5% milk in TBS/T and primary antibody was incubated in 5% BSA instead.

Molecular Biology:

Full-length ZUP1 cDNA was cloned into the pcDNA5/FRT/TO/FLAG vector (modified from Thermo Fisher Scientific) to generate FLAG-ZUP1 using the NEBuilder HiFi DNA Assembly Master Mix (NEB). All point mutations (K276R, K301R, K522/525R, K534R, C360A, A237G, N204A/Y205A, I207A/L208A, N204A/Y205A + I207A/L208A) were introduced into the FLAG-ZUP1 background using the Q5 Site-directed Mutagenesis Kit (New England Biolabs). Primers were designed using NEBaseChanger (https://nebasechanger.neb.com/) and polymerase chain reactions (PCR) were performed using the Q5 High-Fidelity DNA Polymerase at the recommended annealing temperature. All constructs generated were analysed by restriction digest and confirmed by Sanger sequencing.

2.2 Materials

Antibodies:

Antibody	Source	Identifier
Mouse anti-FLAG-HRP	Sigma-Aldrich	RRID: AB_439702, Cat# A8592-.2MG
Mouse anti-GFP	Abcam	RRID: AB_303395, Cat# ab290
Rabbit anti-RPA70	Cell Signalling Technology	RRID: AB_2180506, Cat# 2267S
Rabbit anti-RPA32	Bethyl	RRID: AB_185548, Cat# A300-244A
Rabbit anti-RPA32 pS4/S8	Bethyl	RRID: AB_210547, Cat# A300-245A
Rabbit anti-RPA14	Novus Biologicals	RRID: AB_11037634, Cat# NBP1-87141
Sheep anti-SPRTN	MRC PPU	Cat# S061D
Mouse anti-TCEAL1	Santa Cruz Biotechnology	Cat# sc-393621
Rabbit anti-TCEAL1	Proteintech	Cat# 11993-1-AP20ul
Rabbit anti-UBR5	Bethyl	RRID: AB_2779312, Cat# A300-573A-T
Rabbit anti-UBR5	Cell Signalling Technology	RRID: AB_2799679, Cat# 65344S
Mouse anti-USP11	Santa Cruz Biotechnology	RRID: AB_108438863, Cat# sc-365528
Rabbit anti-USP11	Bethyl	RRID: AB_1211380, Cat# A301-613A-M
Mouse anti-Ubiquitin	Thermo Fisher Scientific	RRID: AB_837154, Cat# 14-6078-82
Rabbit anti-Ubiquitin Lys63	Sigma-Aldrich	RRID: AB_1587580, Cat# 05-1308
Mouse anti-Vinculin	Sigma-Aldrich	RRID: AB_10603627, Cat# V9264-200UL
Mouse anti-ZUP1	Custom IGS (DC Biosciences)	N/A
Rabbit anti-ZUP1	MyBioSource	Cat# MBS2560365
Donkey anti-Mouse	Jackson ImmunoResearch	RRID: AB_23340770, Cat# 715-035-150-JIR-0.5ml
Donkey anti-Rabbit	Jackson ImmunoResearch	RRID: AB_10015282, Cat# 711-0350152-JIR-0.5ml

Donkey anti-Sheep	Jackson ImmunoResearch	RRID: AB_2340710, Cat# 713-035-147-JIR-0.5ml

Bacterial strains for cloning:

Bacterial strain	Source	Identifier
5-alpha competent *E.coli*	NEB	Cat# C2987H

Chemicals and peptides:

Chemicals and peptides	Source	Identifier
ANTI-FLAG® M2 Affinity Gel	Sigma-Aldrich	Cat# A2220-5ML
Camptothecin	Sigma-Aldrich	Cat# C9911-100MG
Hydroxyurea	Sigma-Aldrich	Cat# H8627-1G
Mitomycin C	Sigma-Aldrich	Cat# M4287-2MG
Neocarzinostatin	Sigma-Aldrich	Cat# N9162-100UG
4-Nitroquinoline 1-oxide	Sigma-Aldrich	Cat# N8141-250MG
Formaldehyde	Thermo Fisher Scientific	Cat# 28906
Doxycycline	Sigma Aldrich	Cat# D9891-1G
Blasticidin	Thermo Fisher Scientific	Cat# A1113903
Hygromycin	Thermo Fisher Scientific	Cat# 10687010
TransIT®-LT1 Transfection Reagent	Mirus	Cat# MIR 2300
Lipofectamine™ RNAiMAX Transfection Reagent	Thermofisher Scientific	Cat# 13778075

Experimental models: Cell lines

Cell lines	Source	Identifier
293 Flp-In T-Rex	Thermo Fisher Scientific	Cat# R78007
293T	ATCC	Cat# ATCC CRL-3216
U-2 OS	ATCC	Cat# ATCC HTB-96
U-2 OS ZUP1 WT	IGS Group	N/A
U-2 OS ZUP1 KO	IGS Group	N/A

Oligonucleotides:

Primers	Source	Identifier
ZUP1 K276R FWD: 5' - GGAGGATACAgACAACAACAAC – 3' REV: 5' – AGAATTATCTAAACCATATTGTC – 3'	This project	N/A
ZUP1 K301R FWD: 5' – CATAGGAGAAgAGCTGATATGATG – 3' REV: 5' – AAATTCAGATGGAGGCATTC – 3'	This project	N/A
ZUP1 K522/K525R FWD: 5' – ttaagGCAAGACATAGAGGCTAG – 3' REV: 5' – taatcTCTGCATTTCTCGAGAAG – 3'	This project	N/A
ZUP1 K534R: FWD: 5' – AGCAGTCTCAgGCAACTTCGG – 3' REV: 5' – AGCCTCTATGTCTTGCTTTAATAATTTC – 3'	This project	N/A
ZUP1 A237G: FWD: 5' - TCTACAATTGGGTCACCAGCTTC – 3' REV: 5' - TCACCAGAACACTGGACTC – 3'	This project	N/A
ZUP1 N204A/Y205A FWD: 5' – CATATGTACAgctgccCATATTCTTCAGGAACATGTTGACTTG – 3' REV: 5' – AGCCCACACATAGGACAA – 3'	This project	N/A
ZUP1 I207A/L208A:		

FWD: 5’ – AAATTACCATgctgctCAGGAACATGTTGACTTG – 3’ REV: 5’ – GTACATATGAGCCCACAC -3’	This project	N/A
ZUP1 N204A/Y205A + I207A/L208A: FWD: 5’ – TGCTGCTCAGGAACATGTTGACTTGCATTTG – 3’ REV: 5’ – TGGGCAGCTGTACATATGAGCCCACACATAG – 3’	This project	N/A

siRNA	Source	Identifier
siCTRL (ON-TARGETplus Non-targeting Control Pool)	Dharmacon	Cat# D-001810-10-20
siTCEAL1 (SMARTpool: ON-TARGETplus)	Dharmacon	Cat# L-019850-00-0005
siUBR5 (SMARTpool: ON-TARGETplus)	Dharmacon	Cat# L-007189-00-0005
siUSP11 (SMARTpool: ON-TARGETplus)	Dharmacon	Cat# L-006063-00-0005

Recombinant DNA

Plasmids generated at a date prior to the start of this project are denoted with IGS, standing for Dr Ian Gibbs-Seymour, while plasmids generated by myself since the start of this project are denoted by KS, standing for Kira Schuetzenhofer.

Recombinant DNA	Source	Identifier
pcDNA5/FRT/TO/FLAG	IGS	N/A
pcDNA5/FRT/TO/FLAG ZUP1 WT	This project (KS)	N/A
pcDNA5/FRT/TO/FLAG ZUP1 K276R	This project (KS)	N/A
pcDNA5/FRT/TO/FLAG ZUP1 K301R	This project (KS)	N/A
pcDNA5/FRT/TO/FLAG ZUP1 K522/525R	This project (KS)	N/A
pcDNA5/FRT/TO/FLAG ZUP1 K534R	This project (KS)	N/A
pcDNA5/FRT/TO/FLAG ZUP1 A237G	This project (KS)	N/A

pcDNA5/FRT/TO/FLAG ZUP1 C360A	This project (KS)	N/A
pcDNA5/FRT/TO/FLAG ZUP1 N204A/Y205A	This project (KS)	N/A
pcDNA5/FRT/TO/FLAG ZUP1 I207A/L208A	This project (KS)	N/A
pcDNA5/FRT/TO/FLAG ZUP1 N204A/Y205A I207A L208A	This project (KS)	N/A
pcDNA5/FRT/TO/FLAG ZUP1 A237G N204A/Y205A	This project (KS)	N/A
pcDNA5/FRT/TO/FLAG ZUP1 A237G I207A/L208A	This project (KS)	N/A
pcDNA5/FRT/TO/FLAG ZUP1 A237G N204A/Y205A I207A/L208A	This project (KS)	N/A
pcDNA5/FRT/TO/eGFP pcDNA5/FRT/TO/eGFP ZUP1 WT	IGS	N/A
pDEST/YFP ZUP1 A237G	IGS	N/A
pDEST/YFP ZUP1 ZnF1+2: C4S/C7S (ZnF1) C31S/C34S (ZnF2)	IGS	N/A
pDEST/YFP ZUP1 ZnF3+4: C156S/C159S (ZnF3) C195S/C198S (ZnF4)	IGS	N/A
pDEST/YFP ZUP1 ZnF1-4: C4S/C7S (ZnF1) C31S/C34S (ZnF2) C156S/C159S (ZnF3) C195S/C198S (ZnF4)	IGS	N/A
pDEST/YFP ZUP1 A237G ZnF1-4: C4S/C7S (ZnF1) C31S/C34S (ZnF2) C156S/C159S (ZnF3) C195S/C198S (ZnF4)	IGS	N/A

3. Results

3.1 Identifying a loss-of-function mutation in the ZUP1 MIU domain

Due to the presence of two different UBDs, both of which have previously been shown to support coupled monoubiquitination (Bienko et al., 2005; Penengo et al., 2006), either of them could serve as the required UBD. Unpublished data from the Gibbs-Seymour group showed that upon mutation of the MIU, the top band was lost in immunoblots of ZUP1, indicating a possible loss of monoubiquitination. An initial goal of this project was to confirm this finding, using FLAG-tagged ZUP1*MIU mutant constructs. The MIU is a reverse orientation UIM, first identified in Rabex-5 (Penengo et al., 2006). UIMs consist of a sequence of about 20 amino acids, with N and C terminal conserved acidic patches surrounding a central invariable alanine residue and a conserved leucine residue, with the consensus sequence *X-**Ac**-**Ac**-**Ac**-**Ac**-Φ-X-X-**Ala**-X-X-X-**Ser**-X-X-**Ac**-X-X-X-X*, *Φ* denoting a large hydrophobic residue, often leucine, *Ac* denoting acidic residues and *X* less conserved residues (Hofmann & Falquet, 2001). A structural analysis of the yeast endosomal sorting protein Vps27 UIM showed that the hydrophobic patch comprising central Leucine, Isoleucine and Alanine residues on the helical surface directly engaged the Leu8-Ile44-Val70 hydrophobic patch on the surface of ubiquitin (Swanson et al., 2003).

In a series of mutagenesis experiments intended to identify the relative contributions of conserved UIM sequence residues to ubiquitin binding affinity, Fisher et al. (2003) found that a single mutation changing the central alanine to a glycine significantly reduced ubiquitin binding of the UIM tested. Glycine substitutions are often used to “break” helices, as the presence of only a single alpha carbon in the glycine side chain allows it greater conformational flexibility than any other amino acid, thereby destabilising the structure of the alpha helix in which it is placed. Mutation of the critical, central alanine residue in Rabex-5 to glycine (A58G) effectively reduced coupled monoubiquitination of Rabex-5 (Penengo et al.,

2006). The equivalent critical residue in ZUP1 is Ala237, and following the generation of FLAG-tagged wild-type ZUP1 constructs, site-directed mutagenesis (SDM) was employed to induce the A237G mutation to abolish ubiquitin-binding ability of the MIU.

3.2 Identifying lysine residues to abolish monoubiquitination

A complementary approach to UBD mutagenesis is the identification of the lysine residue(s) targeted by ubiquitination, mutation of which should lead to loss of monoubiquitination. Coupled monoubiquitination depends on single lysine residues in some instances, including Sts2 (K202) and USP25m (K99) (Hoeller et al., 2006; Denuc et al., 2009). In order to identify lysine residues that could serve as ubiquitination sites, a search of ZUP1 on PhosphoSitePlus (https://www.phosphosite.org/proteinAction.action?id=1287615&showAllSites=true) was performed (Figure 7A).

PhosphoSitePlus is a curated platform that compiles data from high- and low throughput studies that provides information on how frequently certain PTMs have been reported at distinct sites. Out of several lysine residues, K276, K301, K522, K525 and K534 were chosen for further analysis (Figure 7A). This was partly because they appeared to have greater surface exposure than other potential residues, which is the greatest determinant of whether lysine residues are selected for ubiquitination (Danielsen et al., 2011), as can be seen in a view of ZUP1 (Figure 7B). For instance, while K318 had a similar number of reports of ubiquitination as K301 (Figure 7A), its side chain is not surface exposed as those of the residues chosen for mutation (Figure 7B)

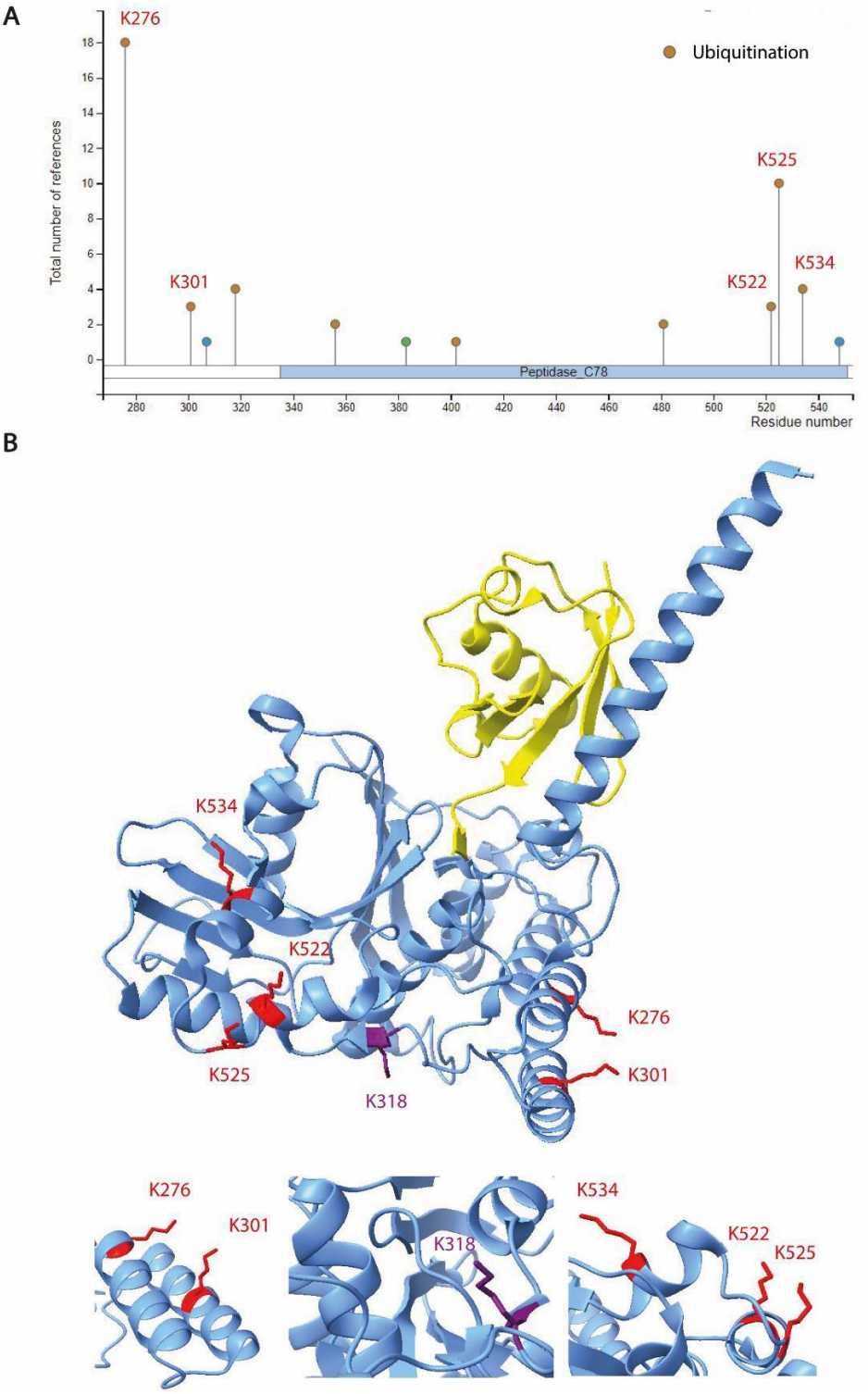

A
K276
Ubiquitination
K525
K534
K301
K522
Peptidase_C78
Total number of references
Residue number
B
K534
K522
K276
K525
K318
K301
K276
K301
K318
K534
K522
K525

Figure 7. Identifying lysine residues for mutation. (**A**) Modified snapshot taken from PhosphoSitePlus/ZUFSP showing ubiquitination sites. The y-axis refers to the total number of reports of a certain post-translational modification from both high- and low-throughput data sources. Lysine residues chosen for further analysis are indicated in red. Orange dots mark ubiquitination sites. (**B**) View of the crystal structure of ZUP1 using Chimera X (Protein Data Bank ID: 6FGE), shown in blue, in complex with ubiquitin, which is shown in yellow. Side chains of the residues chosen for mutation are indicated in red, while the Lys318 side chain is shown in purple.

3.3 Analysis of monoubiquitination of MIU, lysine and catalytic dead mutants

HEK293T cells were transfected with FLAG-tagged ZUP1 WT, the MIU mutant ZUP1*A237G and the lysine mutants ZUP1*K276R, ZUP1*K301R, ZUP1*K522/525R, and ZUP1*K534R. Moreover, to test whether catalytic activity of ZUP1 could affect monoubiquitination, SDM was used to generate the catalytic dead mutant ZUP1*C360A. Catalytic activity could affect ZUP1 monoubiquitination, either by auto-deubiquitinating or by promoting interaction with other DUBs or E3 ubiquitin ligases. While the replacement of the catalytic cysteine with alanine does abolish catalytic activity, it does not interfere with ubiquitin binding ability, making it a viable option to screen for changes in ubiquitin binding (Morrow et al., 2018).

Whole-cell extracts were separated by SDS-Page and immunoblotting was performed (Figure 8). The immunoblot failed to show a loss of the top band in any of the lysine mutants, potentially indicating that neither of the residues chosen serve as the monoubiquitination site, or alternatively, that monoubiquitination does not depend on a single lysine residue. Similarly, the ZUP1*C360A mutant did not show convincing changes in the strength of the top band signal. An increase in the monoubiquitin signal could have indicated that ZUP1 is capable of

deubiquitinating itself, as was observed for UCH-L1 and USP25m (Osaka et al., 2003; Denuc et al., 2009). It must be acknowledged, however, that the cysteine to alanine mutation might not be ideal for investigating the effect of loss of catalytic activity on ZUP1 monoubiquitination precisely because it enhances ubiquitin binding ability (Morrow et al., 2018), potentially leading to an increase in the monoubiquitination signal that is not reflective of the catalytic activity per se. Future studies could avoid this issue by employing a C360R mutant, as substitutions with arginine have been shown to inactivate DUB activity and decrease affinity for ubiquitin (Morrow et al., 2018).

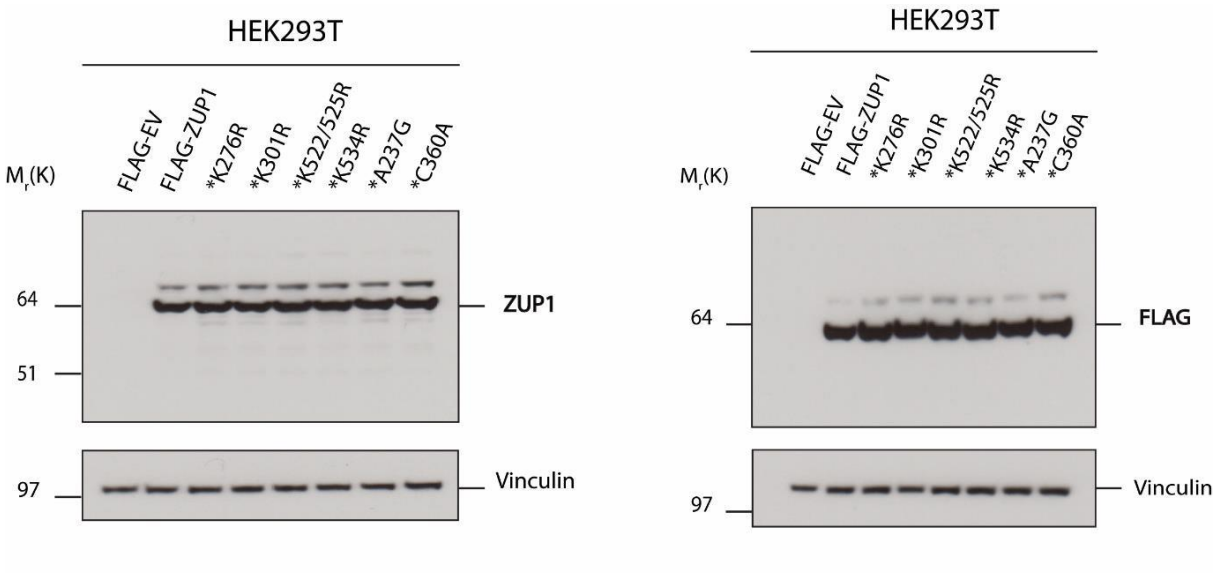


Figure 8. Testing monoubiquitination of lysine, MIU and C360A mutants. Monoubiquitination does not depend on the lysine residues tested and is not affected by mutation of the MIU or loss of the catalytic activity. HEK293T cells were transfected with FLAG-tagged empty vector and the indicated FLAG-tagged ZUP1 wild-type and mutant constructs.

Surprisingly, the ZUP1*MIU mutant retained its coupled monoubiquitination, contradicting earlier unpublished findings (Figure 6). This discrepancy in findings might be explained by differences in the localisation of FLAG-ZUP1 and YFP-ZUP1 constructs based on the tag, which could be tested in the future by using immunofluorescence microscopy. Another potential key difference between the two experiments was the use of different lysis buffers. Whereas in the initial experiment EBC buffer was used, in this experiment Triton X-100 buffer was employed for lysis. An important step in preserving the ubiquitination state of proteins of interest is to include DUB inhibitors in the lysis buffer. While both buffers contained N-ethylmaleimide (NEM), which as an inhibitor of cysteine proteases alkylates the catalytic cysteine of DUBs, the EBC buffer additionally contains ethylenediaminetetraacetic acid (EDTA), which chelates heavy metal ions and thereby inhibits DUBs functioning as metalloproteinases (Emmerich & Cohen, 2015).

Moreover, while the A237G mutation was used for MIU mutants throughout, the constructs used were different, using YFP-tagged ZUP1 instead of FLAG-ZUP1. In order to confirm the initially observed loss of the top band, HEK293T cells were transfected with the previously used YFP-ZUP1 and YFP-ZUP1*A237G constructs and lysed in either EBC or Triton X-100 buffer (Figure 9). Another YFP-ZUP1 construct with the MIU mutation and additional point mutations in all four zinc fingers was analysed as well, to see whether the loss of monoubiquitin observed in unpublished findings by the Gibbs-Seymour group was reproducible (Figure 6). One set of HEK293T cells was lysed in sample buffer without any DUB inhibitors as a further control (Figure 9). It appears that independently of whether EBC or Triton X-100 buffer was used for lysis, monoubiquitination is reduced in the YFP-ZUP1*MIU mutant, while it appears to be lost in the YFP-ZUP1*MIU+ZnF1-4 mutant (Figure 9). This is similar to the findings of Bienko et al. (2010), who also tested monoubiquitination of Pol η under different lysis conditions (+/-NEM) and found no difference.

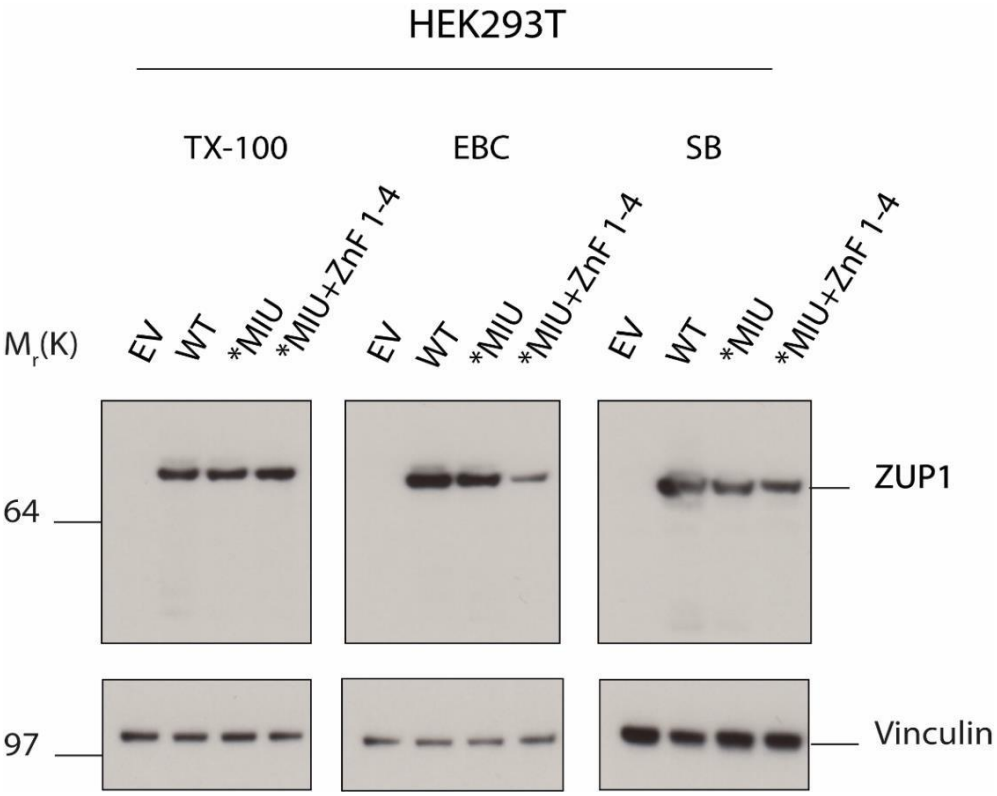


Figure 9. Testing monoubiquitination of ZUP1 in different lysis conditions and with different mutations. HEK293T cells were transfected with YFP-tagged empty-vector, YFP-tagged ZUP1 wild-type, *MIU and *MIU + ZnF1-4 constructs. Cells were lysed under different conditions, either in Triton X-100 (TX-100), EBC or sample buffer (SB).

3.4 Identification of ZUP1 UBZ residues for mutagenesis

Given that ZnF4, otherwise known as UBZ, is responsible for the ubiquitin binding ability of ZUP1, but not any of the other zinc fingers (Kwasna et al., 2018), the next aim was to establish whether the loss of monoubiquitination observed in the YFP-ZUP1*MIU+ZnF1-4 mutant was due to mutations in the UBZ. Moreover, since there were mutations in the zinc fingers as well as the MIU, another question to answer was whether the observed loss was due to loss-of-function mutations in the UBZ alone, or whether mutations in both the MIU and UBZ were required to abolish monoubiquitination.

In order to achieve this, several mutations intended to disrupt ubiquitin binding were introduced into the UBZ. The UBZ of ZUP1 is a C2H2 zinc finger with a β-β-α fold, similar to the first UBZ identified in TLS pol η (Figure 10A) (Bienko et al., 2005; Kwasna et al., 2018). In the pol η UBZ, Zn^{2+} is sandwiched between the α-helix and the β-strands through coordination by conserved β-strand cysteines and α-helical histidine residues (Bomar et al., 2007). Interestingly, UBZ domains have been identified in several proteins involved in DNA repair, and in some cases have shown to confer K63-linkage specificity to the host protein. For instance, recruitment of SLX4, a scaffold for DNA repair nucleases involved in ICL repair, to K63-linked chains at DNA damage sites requires one of its UBZ domains (Lachaud et al., 2014), the UBZ of the Fanconi Anaemia core complex protein FAAP20 mediates its recruitment to K63-ubiquitinated H2A (Yan et al., 2012), and the E3 ubiquitin ligase Rad18 involved in TLS is recruited to K63-ubiquitinated targets at DNA damage sites (Thach et al., 2015). Mutation of the critical Zn^{2+}-coordinating cysteine residues to alanine was previously employed by Haahr et al. (2018), as well as in our group's pDEST-YFP-ZUP1 ZnF 1-4

mutant constructs (Figures 6 and 9), but we sought to mutate other conserved residues required to contact the hydrophobic ubiquitin patch, as opposed to disrupting the domain's function as a zinc finger. The UBZ of ZUP1 is atypical, with low sequence similarity to other well-defined UBZ domains, such as that of pol η or FAAP20 (Kwasna et al., 2018). Interaction of the UBZ domains of pol η and FAAP20 with the conserved surface surrounding Ile44 of ubiquitin depends mainly on hydrophobic and some charged residues situated on the α-helix (Bomar et al., 2007; Toma et al., 2015). Following the identification of several residues potentially important for ubiquitin binding based on position and conservation across multiple species (Figure 10A and 10B), these residues were mutated to alanine and tested for ubiquitin binding (Figure 10C) (Kwasna et al., 2018). Out of the residues tested, the N204A/Y205A and I207A/L208A mutations caused a loss of binding to polyubiquitin (Figure 10C) (Kwasna et al., 2018). Therefore, mutation of these residues was chosen to generate ZUP1 UBZ mutants.

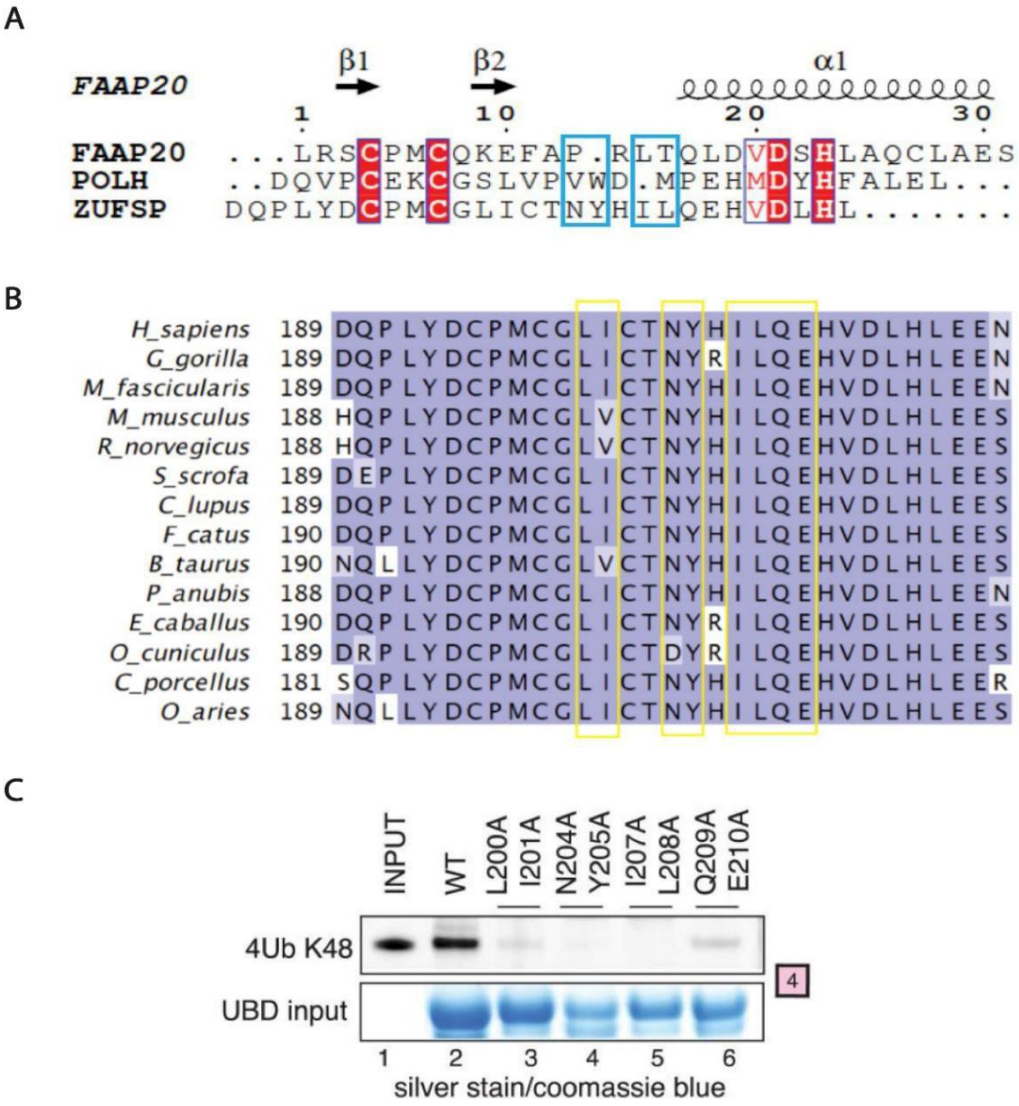


Figure 10. UBZ residues chosen for mutation. (**A**) UBZ sequences from FAAP20, Pol η and ZUP1 are shown in an ESPRIPT sequence alignment, with secondary structure elements of FAAP20 UBZ shown. Residues shown in red are fully conserved. ZUP1 UBZ residues found to be important for ubiquitin binding are highlighted in blue rectangles. (**B**) Multiple sequence alignment of ZUP1 UBZ (residues 189-219) from different species. The residues highlighted in yellow were mutated to alanine and the mutants were tested for ubiquitin binding. (**C**) HALO-tagged ZUP1 UBZ wild-type and mutant domains were tested for their binding to K48-linked tetraubiquitin. All figures are adapted from Kwasna et al. (2018).

3.5 Analysis of the monoubiquitination status of MIU, UBZ and MIU + UBZ mutants

Once all the constructs had been generated, HEK293T cells were transfected with FLAG-tagged ZUP1 WT, as well as all the various ZUP1*MIU, ZUP1*UBZ and ZUP1*MIU + UBZ mutants (Figure 11A) and cells were lysed either in Triton-X100 buffer or EBC buffer. Immunoblotting revealed that there does not appear to be a difference based on which buffer the sample was lysed in (Figure 11B). While mutations in the MIU or UBZ alone reduce monoubiquitination, with increasing mutational load in the UBZ leading to a greater decrease, the MIU + UBZ mutations have the greatest effect. The strongest decrease occurs in the construct containing all possible mutations, indicating that monoubiquitination of ZUP1 depends on the integrity of both its MIU and UBZ domains.

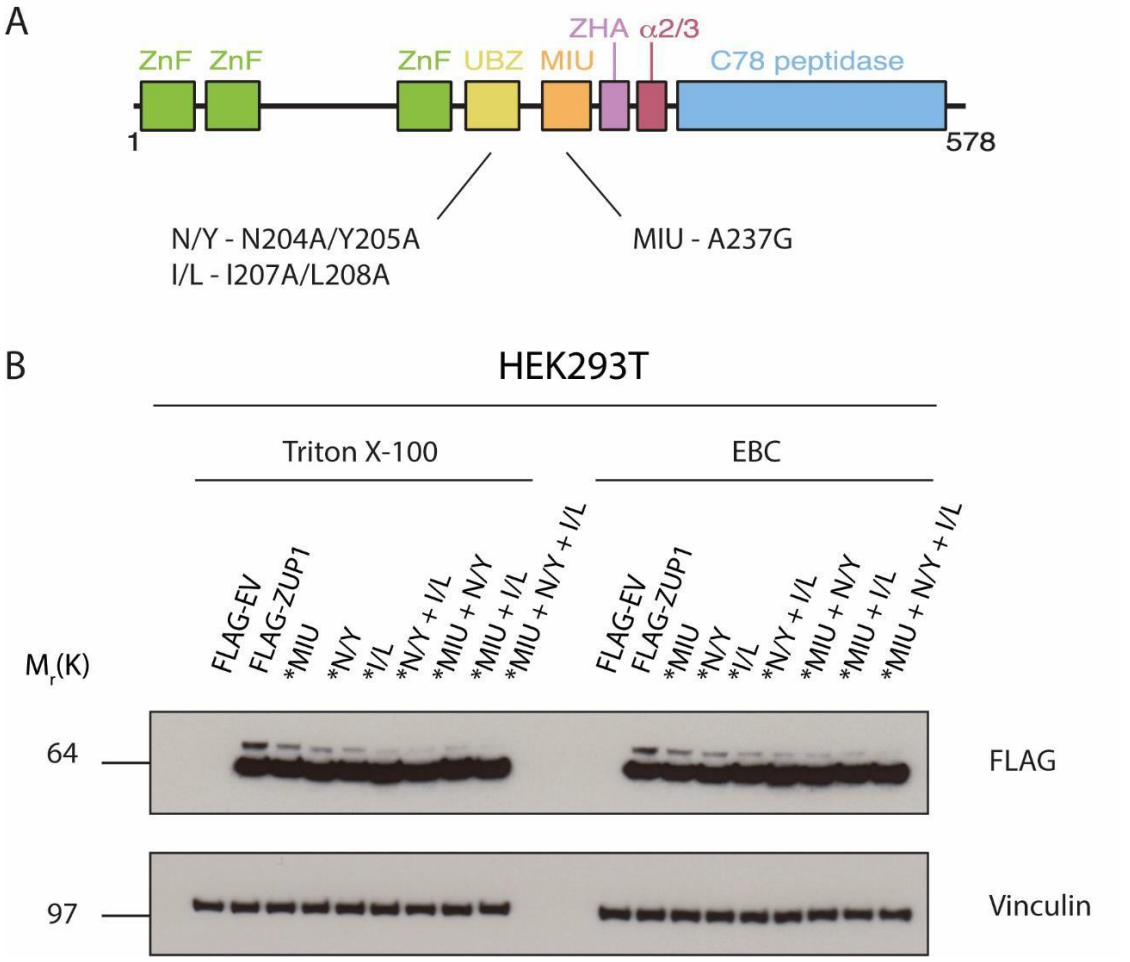


Figure 11. Testing monoubiquitination of ZUP1 MIU and MIU + UBZ mutants. Monoubiquitination of ZUP1 decreases upon mutation of conserved residues in the motif interacting with ubiquitin (MIU) and the ubiquitin-binding zinc finger (UBZ). (**A**) Schematic diagram of ZUP1 domains, showing the position of the UBZ and MIU domains and mutations that were introduced. (**B**) HEK293T cells were transfected with FLAG-tagged empty vector and the indicated FLAG-tagged ZUP1 wild-type and mutant constructs, and were lysed in either Triton X-100 or EBC buffer. Abbreviations: N/Y – N204A/Y205A, I/L – I207A/L208A, MIU – A237G.

In order to further test the function of the various mutant constructs, cleavage activity assays and localisation studies could be performed, thereby eliminating any confounding factors potentially affecting monoubiquitination.

3.6 Confirming coupled monoubiquitination of ZUP1

While all the signs hint at ZUP1 being modified by monoubiquitination, it has not been directly confirmed. There are several possible ways of doing so, with one of the most widely employed experimental methods is transfection of cells with ubiquitin and the protein of interest, followed by pull-down under denaturing conditions of either and subsequent immunoblotting (Bienko et al., 2005; Hoeller et al., 2006). An experiment of this kind was planned, with transfection of HEK293T cells with His-tagged ubiquitin alone or in combination with FLAG-ZUP1, followed by Ni^{2+}-NTA purification under denaturing conditions and immunoblotting for either endogenous ZUP1 or FLAG. However, due to limited time, execution of this experiment was not possible. Instead, an immunoblot for ubiquitin was performed on FLAG-IP samples from HEK293T cells transiently transfected with FLAG-ZUP1 and on previous samples from a FLAG-IP in order to compare potential differences in ubiquitin binding between wild-type

ZUP1 and the ZUP1*A237G + N204A/Y205A + I207A/L208A mutant. Since these samples were not lysed under denaturing conditions, the immunoblot also shows non-covalent interactions with ubiquitin which may be via associated proteins or free ubiquitin chains.

As can be seen in Figure 12, no clear reduction in the ubiquitination signal at the expected molecular weight (around 64kDa) can be observed. This experiment is not ideal to prove covalent binding of monoubiquitin to ZUP1, and further experiments are required to definitively establish that ZUP1 is modified by coupled monoubiquitination. Moreover, the anti-FLAG immunoblot shows that while monoubiquitination does decrease in the mutant, it is not completely lost, confirming that mutations in both the MIU and the UBZ are not entirely sufficient to abolish monoubiquitination. However, the overall reduction in the binding to ubiquitin of the FLAG-ZUP1* A237G + N204A/Y205A +I207A/L208A mutant shows that the UBZ and MIU mutations were effective in disrupting ubiquitin binding ability (Figure 12). Additionally, the decrease in the (K63-linked) ubiquitin signal in the UBD-mutant might indicate that ZUP1 is in fact modified by (K63-linked) polyubiquitin, dependent on the integrity of its UBDs (Figure 12).

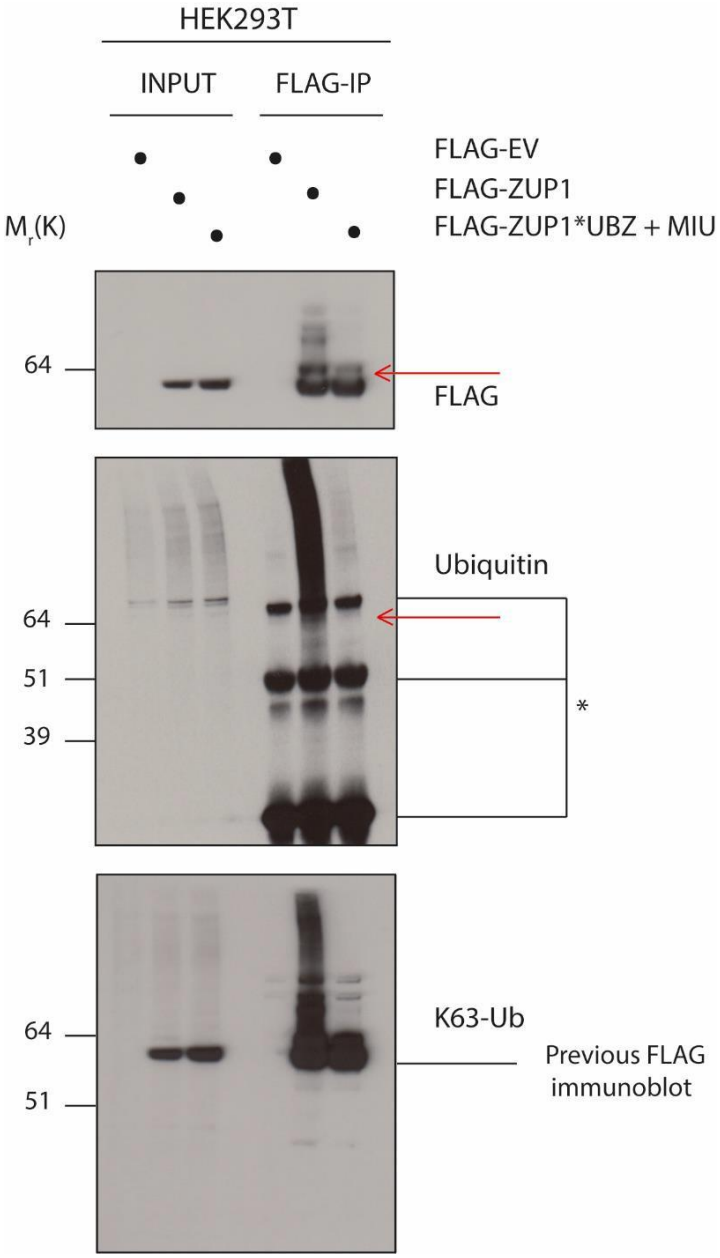


Figure 12. Analysing ubiquitin binding of the FLAG-ZUP1* A237G + N204A/Y205A + I207A/L208A mutant. HEK293T cells were transfected with FLAG-EV, FLAG-ZUP1 and FLAG-ZUP1*A237G + N204A/Y205A +I207A/L208A and a FLAG-IP was performed. The red arrows point at the position of monoubiquitinated ZUP1 around 64kDa. * Indicated signals reflect mouse immunoglobulin of the anti-FLAG M2 affinity gel used for immunoprecipitation that was recognised by the mouse secondary antibody used. Following immunoprecipitation for FLAG, the membrane was stripped and was re-blotted with K63-ubiquitin, explaining the remaining FLAG signal.

Interestingly, the shift in mobility of ZUP1 can also be observed in 293 Flp-In T-REx cells which stably incorporate FLAG-ZUP1 or FLAG-ZUP1*C360A (Figure 13). In the 293 Flp-In T-Rex cell line, the gene of interest is under the control of a promoter containing a tetracycline response element (TRE), allowing expression only upon treatment of cells with tetracycline or doxycycline (Yao et al., 1998; Thermo Fisher Scientific/Flp-In System). These cell lines were initially generated to decrease the amount of time spent on transient transfections and to improve the homogeneity of expression across different immunoblotting and immunoprecipitation experiments, but due to time restraints they were never utilised. However, initial immunoblotting to test for expression of different clonal populations shows that the mobility shift of ZUP1 persists (Figure 13). Monoubiquitination can therefore be seen in endogenous ZUP1, transiently expressed YFP-ZUP1 and FLAG-ZUP1 as well as following doxycycline-induced expression in cells constitutively incorporating the constructs. The persistence of this modification across many different constructs indicates that it likely serves a biological function.

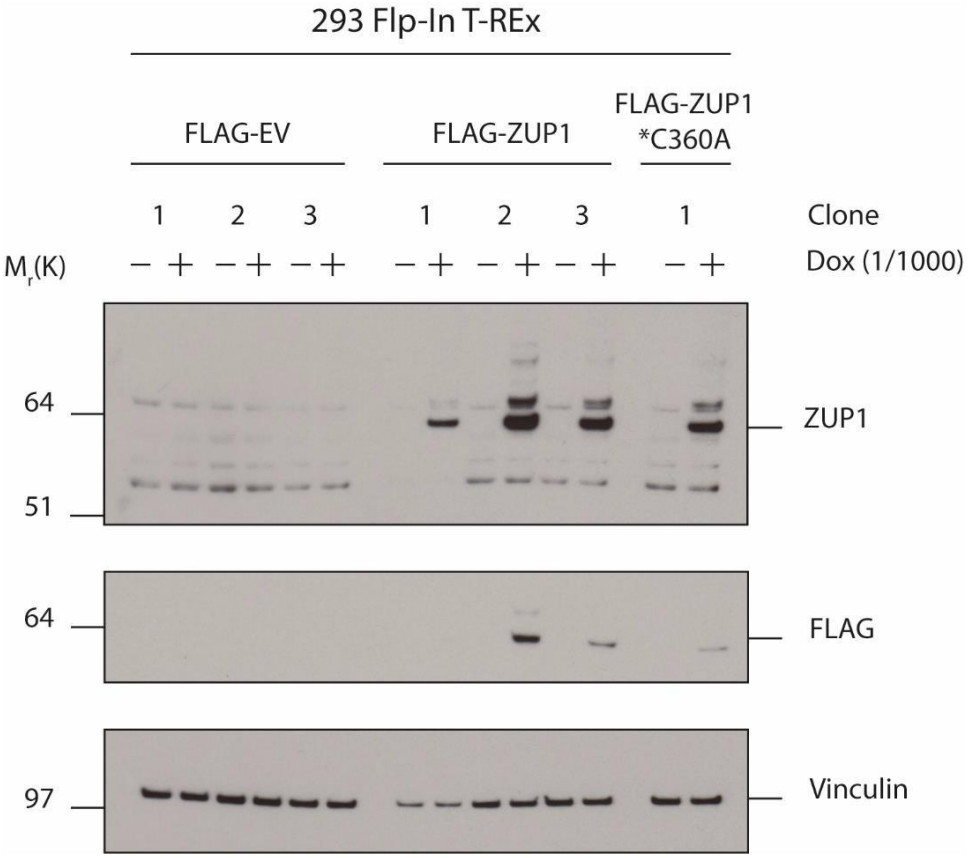


Figure 13. Monoubiquitination persists in 293 Flp-In T-Rex cells stably expressing FLAG-ZUP1 and FLAG-ZUP1*C360A. The day prior to harvesting, cells were treated with Doxycycline (1/1000) to induce expression of FLAG-ZUP1 and FLAG-ZUP1*C360A. Immunoblot of lysates from 293 Flp-In T-Rex cells derived from different clones stably expressing FLAG empty vector, FLAG-ZUP1 or FLAG-ZUP1*C360A.

3.7 Effect of ZUP1 monoubiquitination on interaction with known interacting proteins

The ZUP1 interactome has been studied by two independent groups, with a number of proteins involved in DNA replication or DNA repair identified by each (Hermanns et al., 2018; Kwasna et al., 2018). Figure 14 shows the different proteins identified by each group, with UBR5 and all three RPA subunits identified by both groups. The interaction between RPA and ZUP1 has been suggested previously, from a proteomic investigation of RPA complex interactors (Tkáč et al., 2016), and has since been confirmed a number of times using a variety of methods (Haahr et al., 2018; Hermanns et al., 2018; Hewings et al., 2018; Kwasna et al., 2018). Apart from RPA, the proteins most consistently identified as interacting with ZUP1 are USP11, TCEAL1 and UBR5 (Hermanns et al., 2018; Hewings et al., 2018; Huttlin et al., 2015; Huttlin et al., 2017; Kwasna et al., 2018). While interaction of ZUP1 with RPA and UBR5 was suggested to be dependent on DNA, interaction with USP11 and TCEAL1 was independent of chromatin (Hermanns et al., 2018).

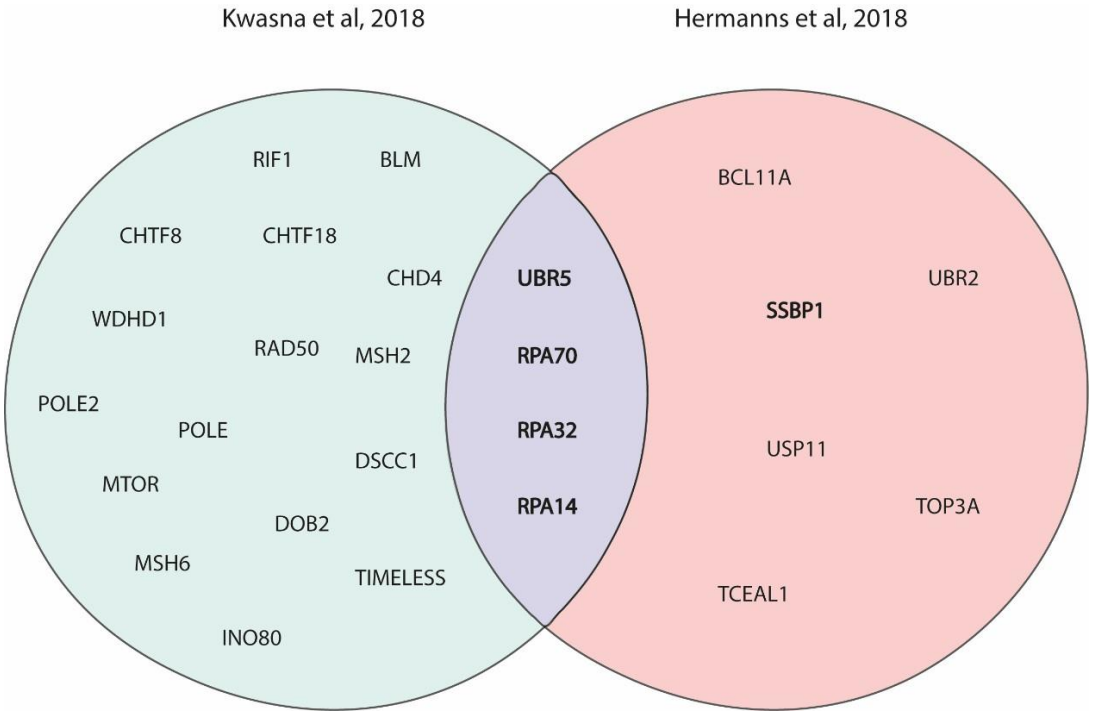


Figure 14. Proteins involved in DNA replication or repair found to interact with ZUP1 by two independent studies. Only the three RPA subunits and UBR5 were identified by both. Proteins that are bold signify interactions that are dependent on DNA (Hermanns et al., 2018). The three RPA subunits were not identified by mass spectrometry in the Kwasna et al. study, but the interaction was confirmed using immunoblotting.

Some of the differences in the top hits between the two studies could be explained by the respective experimental setups. For instance, after observing that ZUP1 was mainly localised in the nucleus, Kwasna et al. (2018) immunoprecipitated endogenous ZUP1 from nuclear fractions of HEK293 cells only, while Hermanns et al. (2018) immunopurified stably expressed FLAG-tagged ZUP1 from Flp-In T-Rex 293 cells under tetracycline control from both cytosolic and nuclear fractions. Differences in localisation, as well as overexpression and the addition of a tag might therefore play a role in the different outcomes. Both studies used the MaxQuant proteomics software to analyse their datasets (albeit different versions), and Kwasna et al. (2018) further used the DAVID bioinformatics software to determine functional classification of identified genes. Owing to the fact that ZUP1's role in DNA repair was only established relatively recently, not many studies have focused on genetic interactions with other proteins or on establishing common functional pathways. A previous genome-scale pooled shRNA screen investigating negative genetic interactions across isogenic cancer cell lines (HCT116) included ZUP1, but while negative interactions with BLM helicase and Mus18 endonuclease, both involved in replication-coupled DNA repair (Zhang et al., 2005), were observed and reported (http://www.genomernai.org/v17/genedetails/221302), they were insignificant (Vizeacoumar et al., 2013; http://www.genomernai.org/v17/genedetails/221302).

3.7.1 Background on RPA, USP11, TCEAL1 and UBR5

RPA the major eukaryotic ssDNA binding protein, essential for DNA replication, DNA repair and recombination. It is formed of a heterotrimeric complex comprised of RPA1 (70kDa), RPA2 (32kDa) and RPA3 (14kDa) (Wold, 1997). RPA binds ssDNA with high affinity, forming a stable complex with roughly 30 nucleotides (Chen & Wold, 2014), thereby protecting it from endonucleases and maintaining its unwound state. Its importance is indicated by its involvement in each of the main DNA repair pathways, DNA double strand (DSB) repair, nucleotide excision repair (NER), base excision repair (BER) and DNA mismatch repair (MMR) (Wold, 1997). RPA is known to be hyperphosphorylated following replication stress and DNA damage by checkpoint kinases, including DNA-PK (DNA-dependent protein kinase), ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related), which promotes stabilisation of stalled replication forks, DNA repair and replication checkpoint arrest (Liu et al., 2012). More recently it was found that RPA is also modified by K63-linked polyubiquitin chains in response to stalling of replication forks and DNA damage (Maréchal et al., 2014; Elia et al., 2015). K63-ubiquitination of RPA has been proposed to occur in a feed-forward loop with phosphorylation, whereby ATR-driven phosphorylation promotes K63-ubiquitylation and vice versa (Maréchal et al., 2014) (Dubois et al., 2017). Given the exquisite specificity of ZUP1 for K63-linked chains, its interaction with RPA and its importance in genome stability, it is very likely that ZUP1 is the DUB responsible for RPA deubiquitination.

USP11 is also a DUB (~110kDa) belonging to the ubiquitin-specific protease (USP) subfamily of thiol-protease DUBs and is involved in various cellular pathways, including viral RNA replication, TGFβ signalling and DNA repair. USP11 has been shown to deubiquitinate H2AK119ub and H2BK120ub, thereby promoting efficient DNA repair and genome stability (Ting et al., 2019). Furthermore, USP11 counteracts RNF8/RNF168 by deubiquitinating γH2AX, (Yu et al., 2016), and a role for USP11 in homologous double-stranded break (DSB)

repair has been found as well, as loss of catalytically active USP11 led to increased cellular sensitivity to PARP inhibitors and impaired DSB repair, as well as increased spontaneous DNA damage (Wiltshire et al., 2010). USP11 also deubiquitinates and thereby stabilises the cyclin-dependent kinase inhibitor p21, maintaining proper control over cell cycle progression (Deng et al., 2018). Interactions between DUBs are rare, but USP11 has in fact been reported to interact with other DUBs, such as USP7 (Sowa et al., 2009; Haq & Ramakrishna, 2017). Given the earlier observation that ZUP1 does not appear to be capable of auto-deubiquitination, it would require another DUB to do so, which would make USP11 an attractive candidate.

TCEAL1 (transcription elongation factor A like 1) is an uncharacterised protein, which as a member of the transcription elongation factor A (SII)-like gene family is predicted to function in transcriptional regulation. Since it has been identified as an interactor of USP11 (*Usp11 Protein (Mouse) - STRING Interaction Network*), USP11 and TCEAL1 most likely function in a complex.

UBR5, also known as EDD1, is a large (300kDa) HECT-type E3 ubiquitin ligase that mainly generates K48-linked polyubiquitin chains and is involved in inflammatory as well as DNA repair pathways and has been found to be mutated or overexpressed in breast, ovarian and colon cancer (O'Brien et al., 2008; Shearer et al., 2015). UBR5 also functions to regulate the DDR by negatively regulating levels of RNF168, an E3 ubiquitin ligase that adds K63-linked ubiquitin to histones H2A and H2AX, thereby preventing excessive spread of ubiquitinated chromatin at DNA damage sites, which in turn has been shown to abnormal and toxic activation of TLS pol η (Gudjonsson et al., 2012; Cipolla et al., 2019).

Interestingly, UBR5 was also found that to promote proteasomal degradation of the proapoptotic factor TXNIP by adding K48-linked ubiquitin chains to pre-existing K63-linked chains, resulting in branched K48/K63 chains (Ohtake et al., 2018). Considering this, and the fact that interaction of ZUP1 with both UBR5 and RPA depends on DNA, it is possible that

ZUP1 and UBR5 constitute opposing forces regulating the stability of RPA at stalled replication forks. While no direct interaction between RPA and UBR5 has been shown, knock-down of UBR5 has been shown to lead to an increase in RPA-ssDNA foci (Cipolla et al., 2019).

Another possibility is that UBR5 functions as the E3 ubiquitin ligase responsible for monoubiquitination of ZUP1, in accordance with the E3-isopeptide model (Woelk et al., 2006). UBR5 does not contain a Ubl domain, but there have been reports of ubiquitination (PhosphositePlus®/UBR5). If UBR5 were to promote ubiquitination of ZUP1 via the proposed mechanism, interaction between ZUP1 and UBR5 might be expected to increase following loss of monoubiquitin.

3.7.2 Testing antibodies for USP11, TCEAL1 and UBR5

First, in order to test the interaction of ZUP1 with USP11, TCEAL1 and UBR5, the specificity of the antibodies needed to be tested as they had not been previously used by any members of the Gibbs-Seymour group. For each protein, two different antibodies were available, one monoclonal and polyclonal. To test the specificity of each antibody for the respective protein, 293T cells were treated either with non-targeting control siRNA or siRNA targeting either USP11, TCEAL1 or UBR5. Immunoblotting was then performed using the different antibodies at various concentrations in the range recommended by the manufacturers. Results can be seen in Figure 15. Since UBR5 knockdown turned out to be very toxic, as has been observed in other studies (Maatsura et al., 2017; Cipolla et al., 2019), a lower concentration of siRNA had to be used (5nM siUBR5 instead of 50nM for siUSP11 and siTCEAL1), explaining the remaining signal in the knockdown lane. The polyclonal antibodies for USP11 and TCEAL1 and the monoclonal antibody for UBR5 were chosen for further use. Since the monoclonal TCEAL1 antibody was tested at a later date compared to the other antibodies, and it was clearly visible that the polyclonal antibody was superior, no loading control was performed for this antibody.

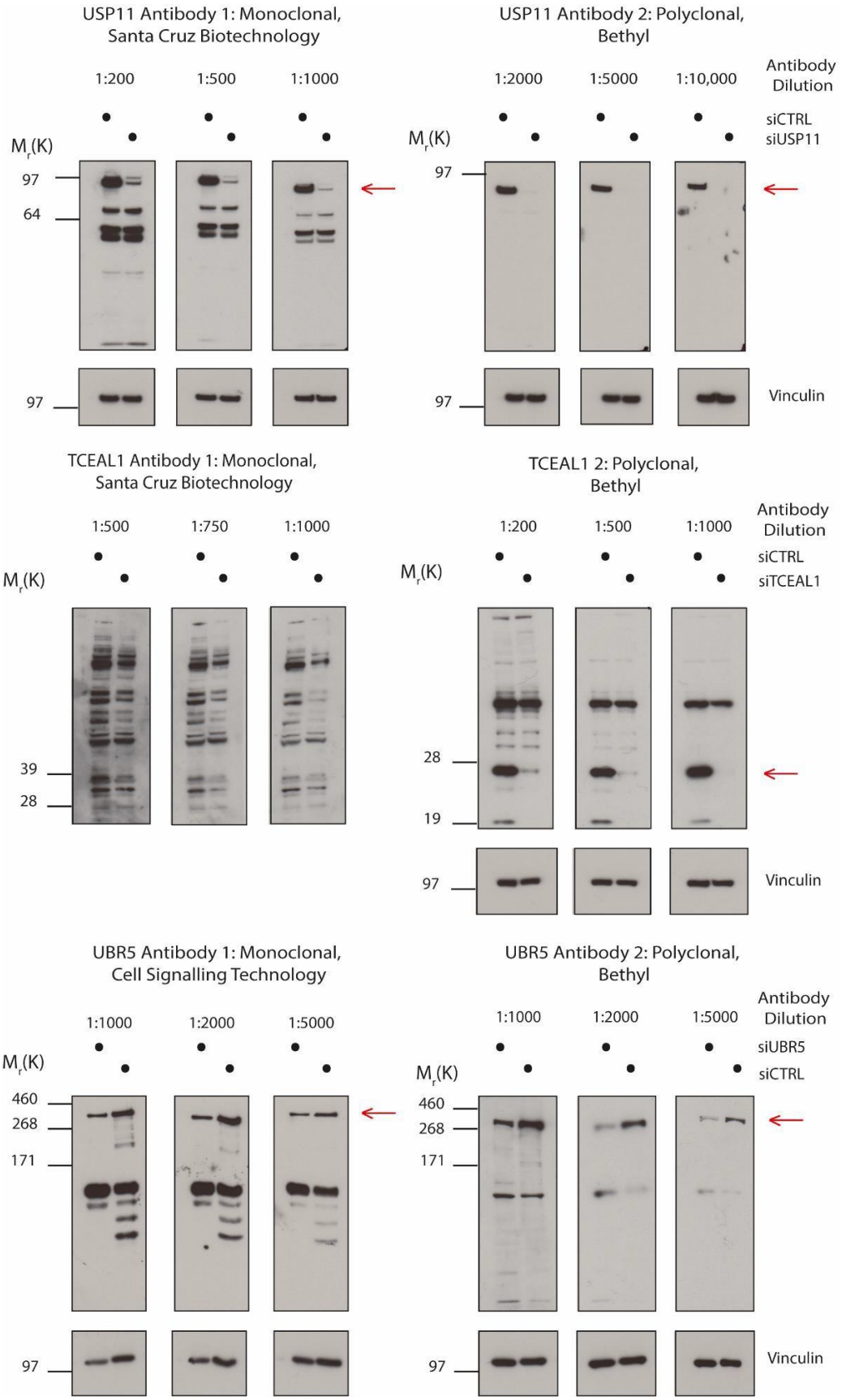

USP11 Antibody 1: Monoclonal, Santa Cruz Biotechnology
1:200
1:500
1:1000
Mr(K)
97
64
97
USP11 Antibody 2: Polyclonal, Bethyl
1:2000
1:5000
1:10,000
Antibody Dilution
siCTRL
siUSP11
Mr(K)
97
97
Vinculin
TCEAL1 Antibody 1: Monoclonal, Santa Cruz Biotechnology
1:500
1:750
1:1000
Mr(K)
39
28
TCEAL1 2: Polyclonal, Bethyl
1:200
1:500
1:1000
Antibody Dilution
siCTRL
siTCEAL1
Mr(K)
28
19
97
Vinculin
UBR5 Antibody 1: Monoclonal, Cell Signalling Technology
1:1000
1:2000
1:5000
Mr(K)
460
268
171
97
UBR5 Antibody 2: Polyclonal, Bethyl
1:1000
1:2000
1:5000
Antibody Dilution
siUBR5
siCTRL
Mr(K)
460
268
171
97
Vinculin

Figure 15. Testing specificity of USP11, TCEAL1 and UBR5 antibodies. HEK293T cells were transfected either with siCTRL or siRNA targeting USP11, TCEAL1 (50nM) or UBR5 (5nM), and the lysate was immunoblotted with either a monoclonal or polyclonal antibody at varying concentrations.

3.7.3 Analysis of ZUP1* MIU and UBZ mutant interactions with RPA, USP11, TCEAL1 and UBR5

In order to establish how ZUP1 monoubiquitination affected binding to these confirmed interaction proteins, HEK293T cells were transfected with FLAG-ZUP1 WT and FLAG-ZUP1*A237G + N204A/Y205A + I207A/L208A, the mutant with the greatest loss of monoubiquitin (Figure 11). This was done on two separate occasions, and co-immunoprecipitations were performed by pulling down FLAG-tagged wild-type or mutant ZUP1, and an immunoblot for the proteins of interest was performed (Figure 16A and 16B). The apparent signal in Figure 16B to the right of the lane representing pulled-down FLAG-ZUP1*MIU + N/Y + I/L is due the well contents spilling over.

In accordance with earlier findings, the FLAG-blot shows the expected decrease in the top band of the MIU + UBZ mutant. Moreover, the mobility shifts observed in the FLAG-IP indicate that ZUP1 is modified by further PTMs. This could reflect polyubiquitination or multiple monoubiquitination, given the presence of multiple lysine residues in ZUP1. While there did seem to be slight changes in the interaction of the monoubiquitination mutant with all three of the RPA subunits, they were not consistent across the two experiments (Figure 16A and 16B). However, this experiment shows that interaction of ZUP1 with the RPA subunits does not depend on ZUP1's ubiquitin binding ability. While there did seem to be a decrease in the interaction of the MIU + UBZ mutant with UBR5 in the first experiment, the same stark decrease was not reproducible in the second experiment (Figure 16B).

There was, however, a consistent decrease in the interaction of the ZUP1*MIU+UBZ mutant with USP11 (Figure 16A and 16B). This decrease can also be observed for TCEAL1, supporting the hypothesis that it functions in complex with USP11.

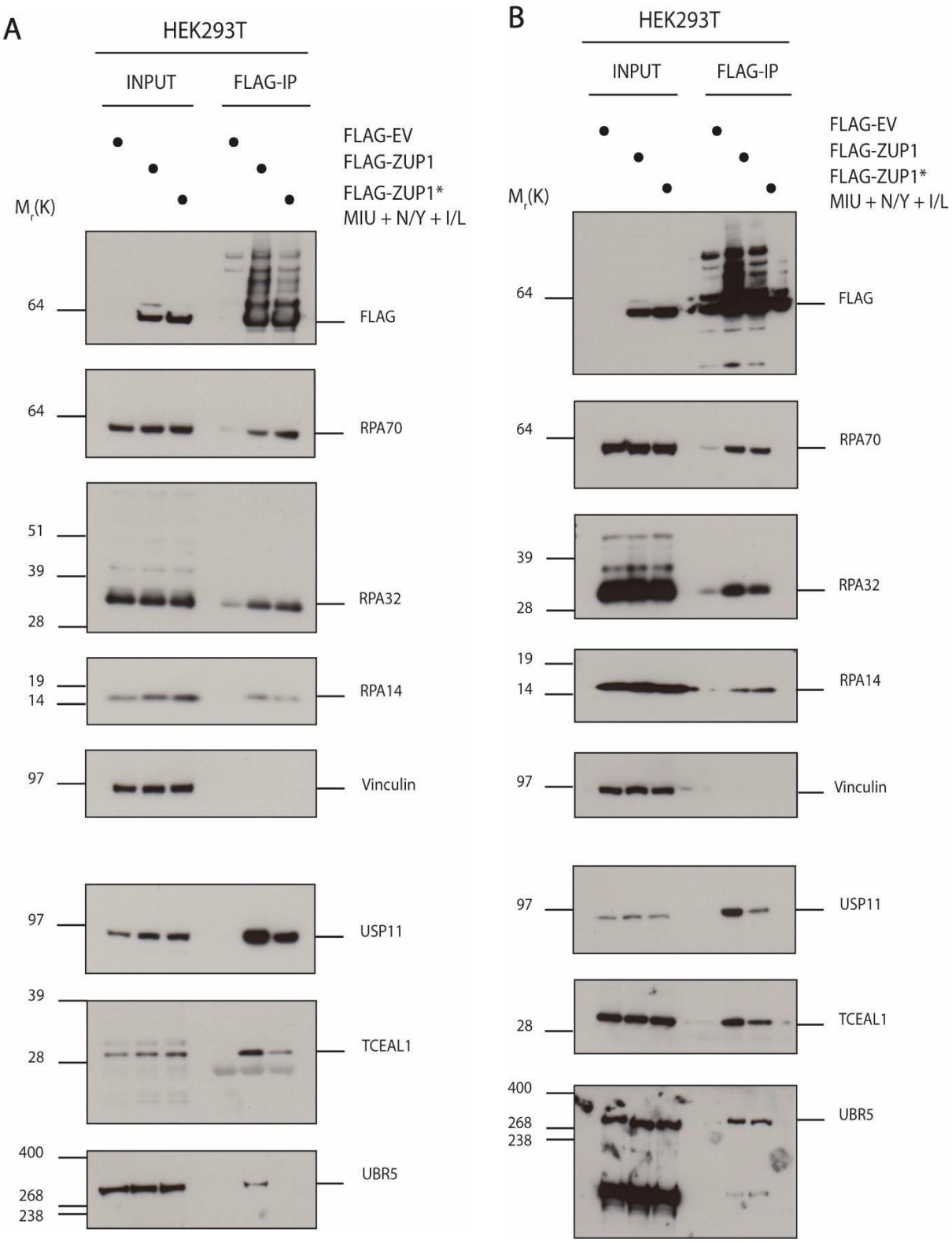


Figure 16. Testing interaction of the ZUP1 *MIU + UBZ mutant with RPA, USP11, TCEAL1 and UBR5. Loss of ZUP1 monoubiquitination decreases interaction with USP11 and TCEAL1. HEK293T cells were transfected with FLAG-tagged empty vector, wild-type ZUP1 and ZUP1*A237G + N204A/Y205A + I207A/L208A at two independent occasions (**A** and **B**). FLAG co-immunoprecipitations were performed followed by immunoblotting for RPA subunits, USP11, TCEAL1 and UBR5. Abbrevations: MIU = A237G, N/Y – N204A/Y205A, I/L = I207A/L208A.

3.8 Effect of DNA damage on ZUP1 monoubiquitination

Given the importance of coupled monoubiquitination in regulating the function of its host protein, this modification itself must be tightly regulated. This has been observed for other proteins undergoing coupled monoubiquitination, in which monoubiquitination is affected by distinct cellular signals. As ZUP1 has been shown to be important for DNA repair and genome stability maintenance, it is possible that ZUP1 monoubiquitination is affected by the induction of DNA damage.

3.8.1 The impact of DNA damage on coupled monoubiquitination of other DNA repair proteins

Upon replication fork stalling, PCNA is monoubiquitinated (Hoege et al., 2002), increasing the affinity of translesion synthesis (TLS) polymerases to PCNA, which replace high-fidelity replicative DNA polymerases and promote lesion bypass (Kannouche et al., 2004). In accordance with this, pol η was found to be deubiquitinated following exposure of cells to UV irradiation, cisplatin and methyl methanesulfonate (MMS) (Bienko et al., 2010), all of which lead to DNA damage in the form of bulky DNA adducts that stall replication forks and activate TLS (Wit et al., 2015; Rocha et al., 2018). This likely leads to an increase in the pool of pol η capable of interacting with PCNA, thereby promoting TLS. Deubiquitination of pol η was not

observed following treatment with hydroxyurea (HU), which causes stalling of replication forks but does not induce DNA damage. Similarly, SPRTN is deubiquitinated following formaldehyde treatment, which causes the formation of DNA-protein crosslinks, supporting SPRTN's role as a DPC protease (Stingele et al., 2016). SPRTN monoubiquitination is not affected by treatment of cells with other DNA damaging agents, such as UV-irradiation, aphidicolin or ionizing radiation (Stingele et al., 2016).

Taken together, this suggests that changes in coupled monoubiquitination of DNA repair proteins occur only in response to treatment that activate pathways which involve and require the function of the DNA repair protein.

3.8.2 Analysis of effect of DNA damage on ZUP1 monoubiquitination

While there is strong evidence for an involvement of ZUP1 in replication-coupled DNA repair, a broader repertoire of genotoxic stresses hasn't been examined for their impact on ZUP1 ubiquitination status. In order to see whether ZUP1 monoubiquitination is affected by DNA damage, HEK293T cells in mid-log phase were treated with different DNA damaging agents over various time courses followed by immunoblotting for ZUP1 to detect any changes in the higher molecular weight band. Cells were treated with HU, Camptothecin (CPT), Mitomycin C (MMC), Neocarzinostatin (NCS), 4-Nitroquinoline 1-oxide (4NQO) and formaldehyde (FA). Both HU and CPT cause replication fork stalling, HU by depleting the deoxyribonucleotide pool and CPT by inhibiting topoisomerase I and trapping it on DNA (Koç et al., 2004; Xu & Her, 2015), which can result in single-stranded and double-stranded DNA breaks. Mitomycin C induces the formation of inter-strand crosslinks, which are repaired by a host of DNA repair pathways, including nucleotide-excision repair, TLS, homologous recombination and the Fanconi Anaemia (FA) pathway (Hashimoto et al., 2016). NCS is an ionising radiation mimetic that produces reactive oxygen species (ROS), leading to generation of DSBs (Kang et al., 2012).

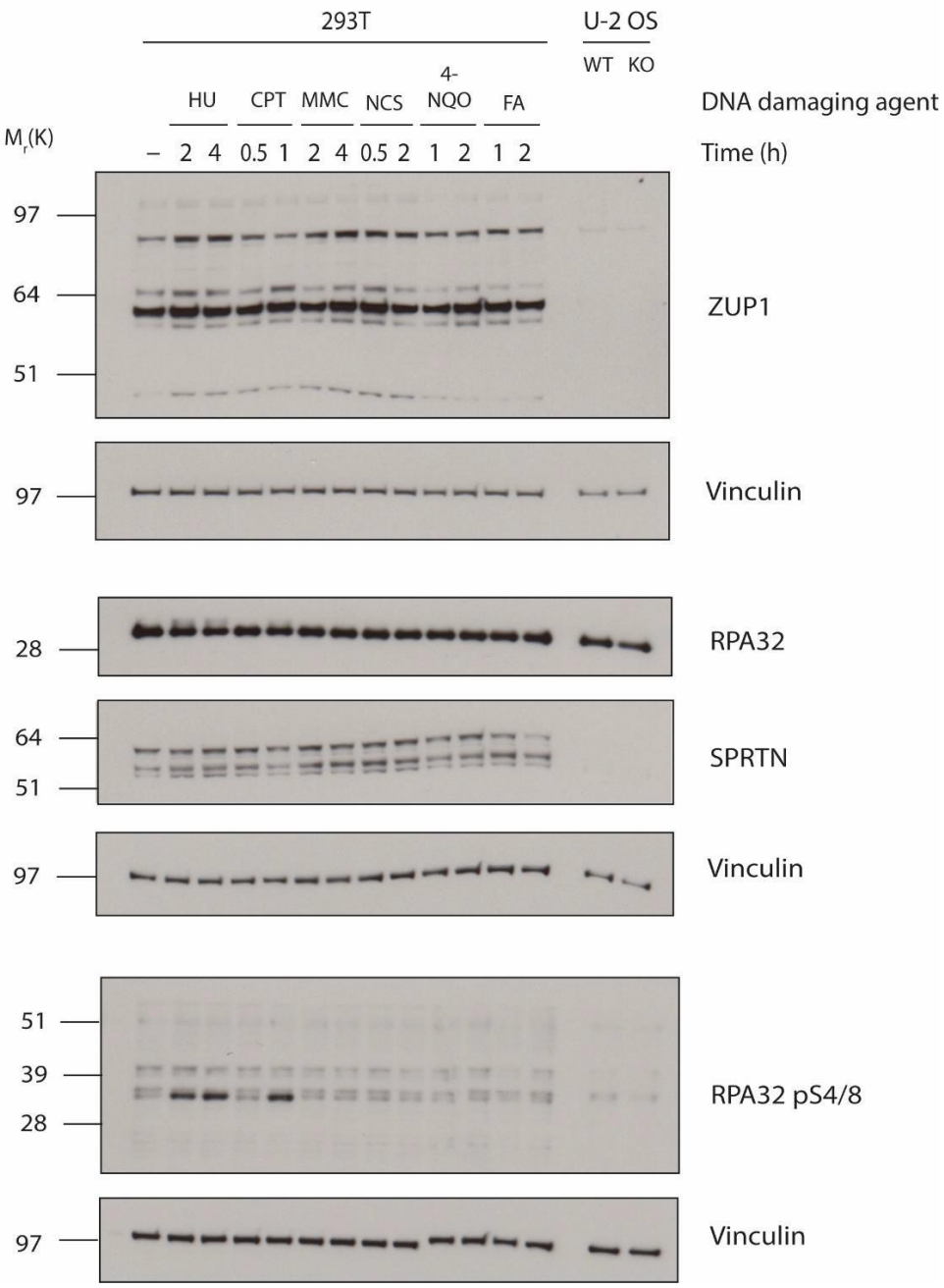
4NQO is a UV-radiation mimetic that induces generation of bulky DNA adducts and chromosomal aberrations (Wang et al., 2016). FA induces the generation of DNA protein crosslinks, activating NER and HR pathways, as well as a proteolytic pathway involving SPRTN (H. Zhang et al., 2020).

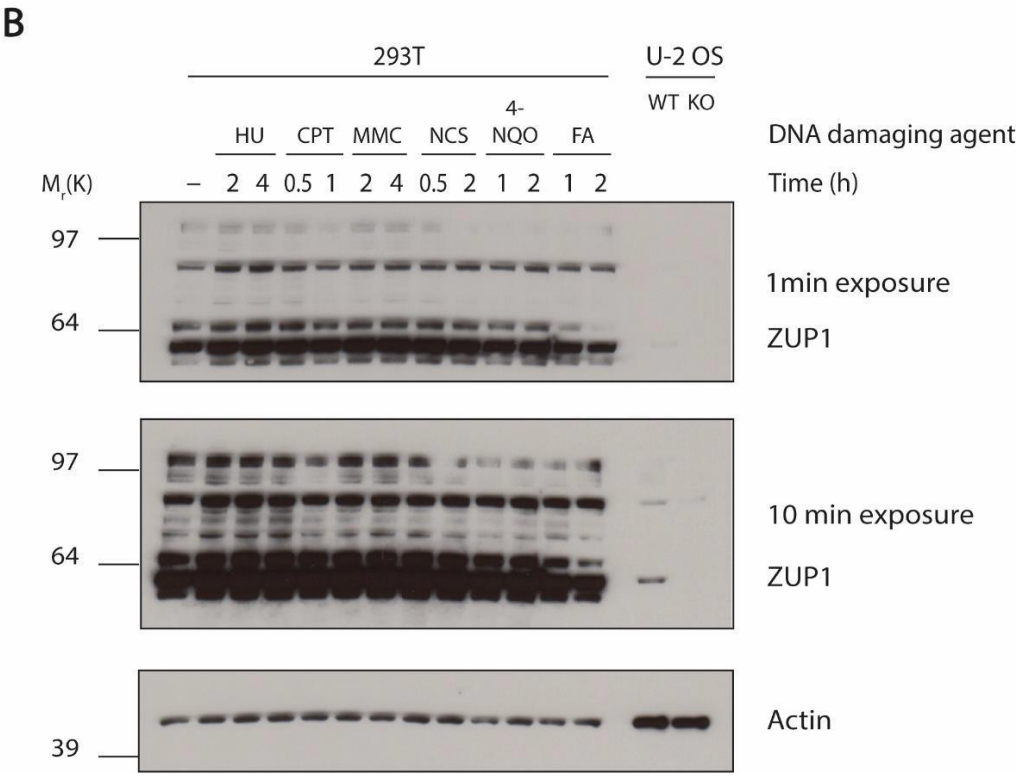
Immunoblots of total RPA32 and RPA32 phosphorylated at S4/8 (pS4/8) were included as a control (Figure 17A and 17C). RPA32 hyperphosphorylation is a biochemical marker of replication-stress, and RPA32 pS4/8 in particular is a signal that promotes repair of DSBs (Liu et al., 2012). Similarly, an immunoblot of SPRTN was shown to assess whether treatment with FA was effective. Cell lysates from a wild-type or ZUP1 knockout U2OS cell line were included in order to determine the specificity of the observed bands.

As expected, treatment of HEK293T cells with HU and CPT led to RPA hyperphosphorylation (Figure 17A and 17C), indicating that treatment with the drugs was effective. However, no phosphorylation of RPA32 was observed in response to treatment with MMC, NCS or 4NQO, contrary to what would have been expected (Liaw et al., 2011; Elia et al., 2015), indicating that treatment with these drugs was not effective. Agreeing with earlier findings, a decrease in the higher molecular weight band of SPRTN, reflecting monoubiquitinated SPRTN, can be observed following treatment with FA (Figure 16A and 16C), but not in response to any other drugs. Since the U2OS protein yield was comparatively low, only a longer exposure of the film managed to show which of the bands recognised by the ZUP1 antibody is specific to ZUP1 (Figure 17B, 10min exposure). From the immunoblot of the first experiment (Figure 17A), it appears that there is a slight increase in ZUP1 monoubiquitination following treatment with HU and CPT that is congruent with RPA phosphorylation. Moreover, monoubiquitination seems to slightly decrease following treatment with FA (Figure 17A). In order to ensure that any changes observed were not due to differences in loading, potentially indicated by differences in the signal of the non-specific

band seen in the ZUP1 immunoblot (around 80-90kDa), the samples were probed once more (Figure 17B). No striking changes were found between the first and the second blot. A second experiment was performed, with cells treated with MMC for 2h and 6h, instead of 2h and 4h. Results are shown in Figure 17C. Protein yield of the second experiment (Figure 17C) was much lower than the first time, explaining the low ZUP1 signal. Overnight exposure of the ECL film did not further improve the signal. Again, treatment with HU, CPT and FA seemed to be effective, but no clear changes in monoubiquitination of ZUP1 can be seen. While there appears to be a general decrease in the total ZUP1 signal following FA treatment following the second experiment (Figure 17C) that does not correspond to a decrease in the loading control, the cross-reacting higher molecular weight bands are decreased as well, making it difficult to conclude anything about the effect of FA on total ZUP1 levels.

A


293T
U-2 OS
WT KO
HU CPT MMC NCS 4-NQO FA
DNA damaging agent
M_r(K)
– 2 4 0.5 1 2 4 0.5 2 1 2 1 2
Time (h)
97
64
51
ZUP1
97
Vinculin
28
RPA32
64
51
SPRTN
97
Vinculin
51
39
28
RPA32 pS4/8
97
Vinculin


B
293T
U-2 OS
WT KO
HU
CPT
MMC
NCS
4-NQO
FA
DNA damaging agent
M_r(K)
– 2 4 0.5 1 2 4 0.5 2 1 2 1 2
Time (h)
97
64
1min exposure
ZUP1
97
64
10 min exposure
ZUP1
39
Actin

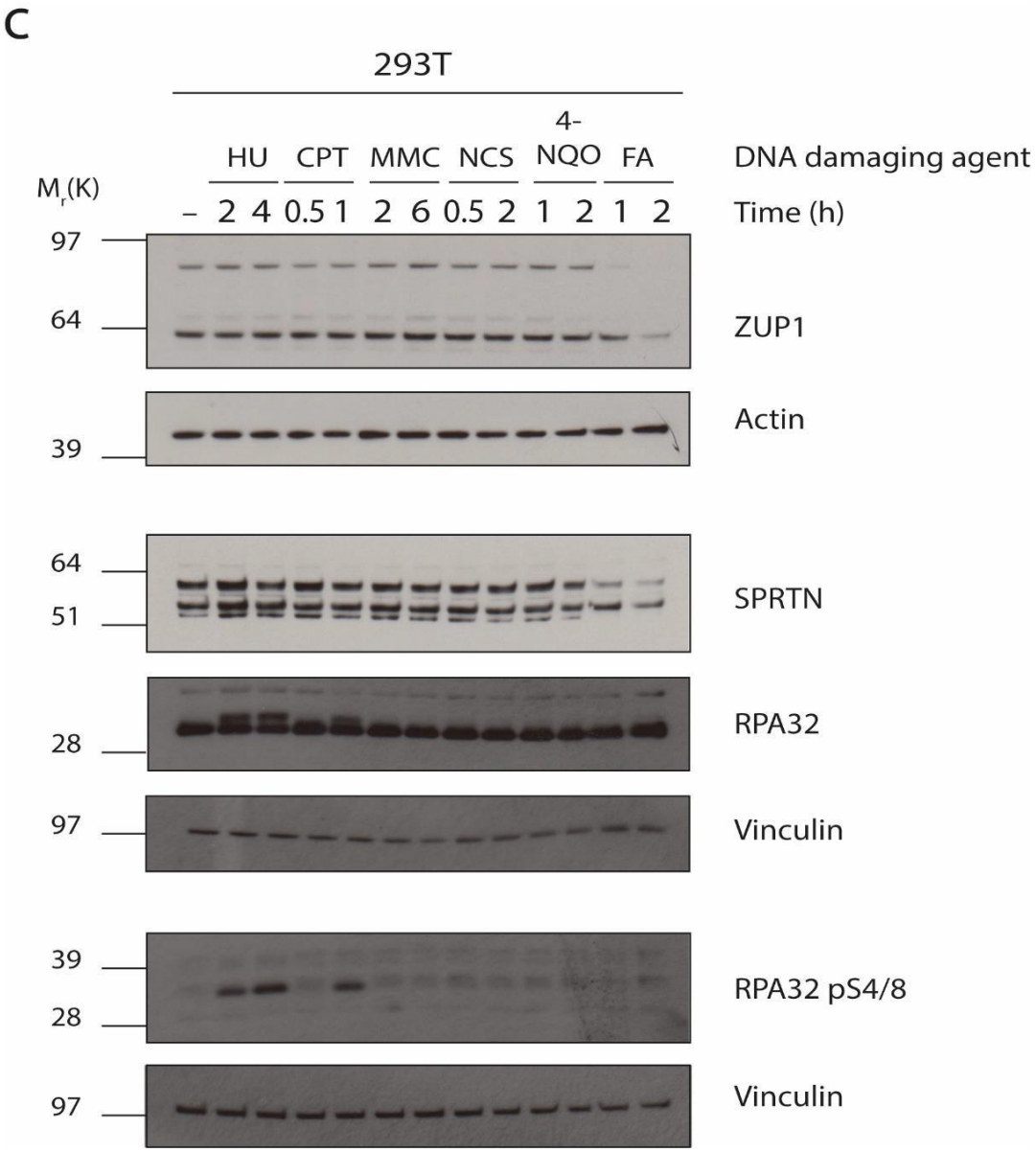


Figure 17. Testing the effect of different DNA damaging agents on ZUP1 monoubiquitination. HEK293T cells were treated with the indicated DNA damaging agents for differing time periods, and the lysate was analysed by anti-ZUP1, anti-SPRTN and anti-RPA32 immunoblots. (**A**) First experiment. (**B**) Samples of the first experiment were analysed again using anti-ZUP1 antibody. A longer exposure was included to show U2OS immunoblots. (B) Second experiment. Abbreviations: HU- Hydroxyurea, CPT – Camptothecin, MMC – Mitomycin C, NCS – Neocarzinostatin, 4NQO – 4-Nitroquinoline 1-oxide, FA – Formaldehyde.

4. Discussion

The aim of this project was to determine whether ZUP1 is regulated by coupled monoubiquitination and which UBD(s) and lysine residue(s) the modification depends on. Another objective was to investigate whether and how monoubiquitination of ZUP1 affected its interaction with known interacting proteins RPA, USP11, TCEAL1 and UBR5. Finally, the effect of different DNA damaging agents on ZUP1 monoubiquitination was analysed.

The biggest caveat of this study is that covalent interaction between ZUP1 and monoubiquitin was not definitively proven. Future studies will need to be conducted, for instance using co-transfections of His-ubiquitin alone, or together with FLAG-ZUP1 or FLAG-ZUP1*MIU + UBZ constructs, followed by Ni^{2+}-NTA purification for His under denaturing conditions to abolish all non-covalent binding, followed by immunoblotting for endogenous ZUP1 or FLAG. This will allow the comparison of the ubiquitin signal reflecting covalently bound ubiquitin between wild-type ZUP1 and the MIU + UBZ mutant, allowing the detection of a reduction in the UBD mutant.

Monoubiquitination of ZUP1 appears to depend on the integrity of its UBDs. While the introduction of loss-of-function mutations in either the MIU or the UBZ causes a decrease in the ubiquitin signal, the loss was much more pronounced when both domains are mutated. Interestingly, for most proteins reported to undergo coupled monoubiquitination, monoubiquitination appeared to depend on only one of several UBDs. For instance, Eps15 monoubiquitination only depends on one of its two UIM domains (Polo et al., 2002), and despite the presence of both an MIU and a ubiquitin-binding zinc finger (RUZ) in Rabex-5, monoubiquitination requires only the MIU (Penengo et al., 2006). The only other example of the need for several UBDs for coupled monoubiquitination comes from USP25m, where deletion of multiple UBDs leads to greater loss of ubiquitination (Denuc et al., 2009). An important factor here might be that while the MIU of ZUP1 contains conserved residues typical

of an MIU, it does not confer activity to the catalytic domain (Kwasna al., 2018). As it is thought that the MIU may form the S2 binding site, preferring K63-linked ubiquitin, it might not be essential for binding to monoubiquitin in *cis*. Interestingly, the ZHA was identified as a third, unique UBD of ZUP1 that forms the distal ubiquitin binding site, making extensive contacts with the Ile44-centered hydrophobic patch of ubiquitin (Kwasna et al., 2018). In the future, it would be interesting to see whether mutation of the conserved hydrophobic ZHA residues affects ZUP1 monoubiquitination.

None of the different lysine mutants tested, K276R, K301R, K522/525R, K534R, affected ZUP1 monoubiquitination. This is not necessarily surprising, as monoubiquitination might not depend on a single lysine residue, but rather flexibly attach to a different available lysine residue if the preferred site is mutated. According to PhosphoSitePlus, K276 is the predominant ubiquitination site, but other lysine residues have also been reported to be ubiquitinated. Therefore, introducing multiple simultaneous lysine to arginine mutations in ZUP1 might abolish ubiquitination. The same was shown for TLS pol η, where simultaneous mutation of four different lysine residues was required to completely abolish monoubiquitination (Bienko et al., 2010). However, when the same approach was taken for SPRTN, as mutation of single lysine residues did not affect monoubiquitination, a mutant containing ten different lysine to arginine mutations was very unstable and showed low expression (Stingele et al., 2016), suggesting that there are potential issues with the introduction of too many mutations. Nevertheless, it is very likely that mutation of several ZUP1 lysine residues at once, especially if they are clustered closely together, will result in a reduction of monoubiquitination.

The identification of such a mutant is very valuable, as this will allow examination of the effect of monoubiquitination loss without a simultaneous impairment of the overall ubiquitin binding ability, as is the case for the MIU + UBZ mutant. While the MIU + UBZ mutant is very useful,

some of the changes observed using this mutant might be due to the loss of ubiquitin binding, rather than loss of monoubiquitination per se.

Interestingly, further higher molecular weight bands could be observed in immunoblots of immunoprecipitated FLAG-ZUP1, which decrease upon mutation of the MIU and the UBZ domains. Moreover, mutation of the UBZ and the MIU also led to a visible decrease in the binding of ZUP1 to (K63-linked) ubiquitin. It can therefore at this point not be excluded that ZUP1 is modified by (K63-linked) polyubiquitin. This opens up the possibility that the observed monoubiquitination of ZUP1 is not the "original" modification, but rather what remains of a lengthier polyubiquitin chain that has been trimmed. Assays that test the effectiveness of various DUBs against (poly)ubiquitinated ZUP1 could help to establish whether this is the case. Similarly, ZUP1 could be probed for other PTMs by for instance employing phosphatase assays. Ultimately, mass spectrometric analysis of ZUP1 would be the most useful tool in order to establish the nature and extent of its modification.

Perhaps surprisingly, no consistent change was observed in the interaction of the ZUP1 MIU and UBZ mutant with any of the three RPA subunits, as might have been expected if coupled monoubiquitination of ZUP1 inhibited its interaction with its ubiquitinated target RPA. However, the fact that the MIU and UBZ mutant interacts with RPA does show that interaction is not dependent on the ubiquitin binding ability of ZUP1. Since RPA ubiquitination is triggered by replication stress and DNA damage, it would be interesting to repeat this experiment following treatment of cells with certain DNA damaging agents, in order to see whether an increase in ubiquitination has any effect on the interaction.

A consistent decrease in binding of the ZUP1 MIU and UBZ mutant to USP11 and TCEAL1 was found. One possible explanation for this could be that USP11 recognises monoubiquitinated ZUP1 as a target for deubiquitination. Like other USP DUBs, USP11 has a UBL domain inserted into its catalytic domain which is in close proximity to the ubiquitin

binding Fingers subdomain, itself nested within the catalytic domain, as well as an N-terminal "domain present in USPs" (DUSP) and a second UBL domain (Zhu et al., 2007). If binding between USP11 and ZUP1 was dependent on the interaction between the ZUP1 UBDs and USP11 UBLs, the observed decrease in binding upon loss of monoubiquitination would be surprising, since binding of ubiquitin in *cis* of ZUP1 would be expected to prevent interaction with any UBL, thereby promoting interaction of unmodified ZUP1 with USP11.

However, a structural analysis of the N-terminal DUSP and UBL domains of USP11 found that the UBL residues corresponding to the hydrophobic surface residues of ubiquitin important in UBD binding are either poorly conserved or buried and inaccessible (Harper et al., 2014). Moreover, using deletion constructs, the authors showed that neither the presence of the N-terminal UBL or the UBL within the catalytic domain were required for, or significantly affected cleavage of a ubiquitinated model substrate, nor did they affect substrate specificity, as has been suggested for other USP family DUBs (Faesen et al., 2011; Harper et al., 2014). Given the redundance of either for catalytic activity and the poor conservation of the N terminal UBL, it is unlikely that either of the UBL domains affects interaction of USP11 with ubiquitinated target proteins, allowing unhindered interaction of monoubiquitinated ZUP1 with USP11.

The theory that USP11 serves as a DUB to deubiquitinate ZUP1 is attractive, particularly because ZUP1 does not appear to deubiquitinate itself. The field of "dubbing" DUBs is relatively new and unexplored, as is the field of DUB regulation in general (Haq & Ramakrishna, 2017), but it opens interesting opportunities for therapeutic intervention. Thiol protease DUBs in particular serve as attractive drug targets due to the presence of a defined catalytic cysteine residue which reacts with a wide range of electrophilic compounds (D'Arcy et al., 2015), and the potential of affecting the function of one DUB through the modulation of activity of another could provide an intriguing extension of this concept.

Future experiments could involve testing whether loss of USP11, for instance through knockdown, affects monoubiquitination (or polyubiquitination) of ZUP1, and to test whether any observed change is dependent on the catalytic activity of USP11. Similarly, it would be interesting to see how knockdown of TCEAL1 affects ZUP1 monoubiquitination, with emphasis on whether any observed effect is dependent on interaction between TCEAL1 and USP11. At this point it cannot be excluded that ZUP1 is in fact responsible for deubiquitination of USP11, which has been shown to be ubiquitinated (PhosphoSitePlus/USP11).

While there seemed to be a loss of interaction between the ZUP1 MIU + UBZ mutant and the E3 ubiquitin ligase UBR5 in the first experiment, this was not observed a second time. A consistent decrease in interaction of the UBD mutant with UBR5 could have indicated that binding depends on the overall ubiquitin binding ability of ZUP1, or its coupled monoubiquitination. In order to test whether UBR5 functions as an E3 ubiquitin ligase for ZUP1, it would be interesting to test whether loss of UBR5, for example by knockdown, would affect ZUP1 monoubiquitination at all. On the other hand, one could test whether interaction of ZUP1 and UBR5 depends on binding of K63-ubiquitin at DNA damage sites, which would support the possibility that ZUP1 counteracts UBR5-mediated generation of branched K48-K63 chains. Cells could be treated with siRNA targeting UBC13, the E2 conjugating enzyme responsible for generating K63-linked polyubiquitin at DNA lesions (Hodge et al., 2016), followed by testing interaction of ZUP1 with UBR5 in the nucleus. In general, repetition of the FLAG co-IP experiments with an effective, multiple lysine-to-arginine mutant would be useful to better determine how coupled monoubiquitination of ZUP1 affects binding to interacting proteins.

No striking or consistent changes of ZUP1 monoubiquitination following DNA damage were observed. There was a possible decrease in the fraction of monoubiquitinated ZUP1 following treatment with formaldehyde, and a slight increase in response to treatment with HU and CPT,

but these findings would need to be confirmed by further experiments to draw any conclusions. More repeats of the experiment could include adjustments to dosage and time ranges over which the drugs are administered, since many of the drugs tested were not effective. Furthermore, investigating the recruitment of a ZUP1 multiple lysine-to-arginine mutant lacking monoubiquitination to DNA damage sites generated by UV-laser microirradiation would be very interesting.

Nevertheless, it needs to be taken into consideration that monoubiquitination of ZUP1 might not necessarily regulate its function in all the pathways it is involved in. For instance, while monoubiquitination of SPRTN is only abolished following treatment with formaldehyde, and not in response to UV irradiation, recruitment of SPRTN to chromatin occurs in response to both formaldehyde and UV irradiation (Centore et al., 2012; Stingele et al., 2016). Moreover, ZUP1 does not only interact with DNA repair proteins; it was further shown to bind an abundance of other proteins, many of which are involved in lysosomal transport (Kwasna et al., 2018). It could therefore be possible that coupled monoubiquitination is important for the regulation of ZUP1 activity only in some DNA repair pathways, or not at all in its response to the DDR, but rather other processes it is involved in.

Further investigation into the role of coupled monoubiquitination in the regulation of ZUP1 will no doubt provide interesting insights into how this novel DUB functions in DNA damage response, as well as other pathways.

5. References

Bienko, M., Green, C. M., Crosetto, N., Rudolf, F., Zapart, G., Coull, B., Kannouche, P., Wider, G., Peter, M., Lehmann, A. R., Hofmann, K., & Dikic, I. (2005). Biochemistry: Ubiquitin-binding domains in Y-family polymerases regulate translesion synthesis. *Science*, *310*(5755), 1821–1824. https://doi.org/10.1126/science.1120615

Bienko, M., Green, C. M., Sabbioneda, S., Crosetto, N., Matic, I., Hibbert, R. G., Begovic, T., Niimi, A., Mann, M., Lehmann, A. R., & Dikic, I. (2010). Regulation of Translesion Synthesis DNA Polymerase η by Monoubiquitination. *Molecular Cell*. https://doi.org/10.1016/j.molcel.2009.12.039

Bomar, M. G., Pai, M. T., Tzeng, S. R., Li, S. S. C., & Zhou, P. (2007). Structure of the ubiquitin-binding zinc finger domain of human DNA Y-polymerase η. *EMBO Reports*, *8*(3), 247–251. https://doi.org/10.1038/sj.embor.7400901

Bosch-Comas, A., Lindsten, K., Gonzàlez-Duarte, R., Masucci, M. G., & Marfany, G. (2006). The ubiquitin-specific protease USP25 interacts with three sarcomeric proteins. *Cellular and Molecular Life Sciences*, *63*(6), 723–734. https://doi.org/10.1007/s00018-005-5533-1

Brinkmann, K., Schell, M., Hoppe, T., & Kashkar, H. (2015). Regulation of the DNA damage response by ubiquitin conjugation. In *Frontiers in Genetics* (Vol. 6, Issue MAR, p. 98). Frontiers Media S.A. https://doi.org/10.3389/fgene.2015.00098

Buetow, L., & Huang, D. T. (2016). Structural insights into the catalysis and regulation of E3 ubiquitin ligases. In *Nature Reviews Molecular Cell Biology* (Vol. 17, Issue 10, pp. 626–642). Nature Publishing Group. https://doi.org/10.1038/nrm.2016.91

Centore, R. C., Yazinski, S. A., Tse, A., & Zou, L. (2012). Spartan/C1orf124, a Reader of PCNA Ubiquitylation and a Regulator of UV-Induced DNA Damage Response. *Molecular Cell*, *46*(5), 625–635. https://doi.org/10.1016/j.molcel.2012.05.020

Chen, R., & Wold, M. S. (2014). Replication protein A: Single-stranded DNA's first responder: Dynamic DNA-interactions allow replication protein A to direct single-strand DNA intermediates into different pathways for synthesis or repair Prospects & Overviews R. Chen and M. S. Wold. *BioEssays*, *36*(12), 1156–1161. https://doi.org/10.1002/bies.201400107

Cipolla, L., Bertoletti, F., Maffia, A., Liang, C.-C., Lehmann, A. R., Cohn, M. A., & Sabbioneda, S. (2019). UBR5 interacts with the replication fork and protects DNA replication from DNA polymerase toxicity. *Nucleic Acids Research*, *47*(21), 11268–11283. https://doi.org/10.1093/nar/gkz824

Clague, M. J., Urbé, S., & Komander, D. (2019). Breaking the chains: deubiquitylating enzyme specificity begets function. In *Nature Reviews Molecular Cell Biology* (Vol. 20, Issue 6, pp. 338–352). Nature Publishing Group. https://doi.org/10.1038/s41580-019-0099-1

Cohn, M. A., Kowal, P., Yang, K., Haas, W., Huang, T. T., Gygi, S. P., & D'Andrea, A. D. (2007). A UAF1-Containing Multisubunit Protein Complex Regulates the Fanconi Anemia Pathway. *Molecular Cell*, *28*(5), 786–797. https://doi.org/10.1016/j.molcel.2007.09.031

D'Arcy, P., Wang, X., & Linder, S. (2015). Deubiquitinase inhibition as a cancer therapeutic

strategy. In *Pharmacology and Therapeutics* (Vol. 147, pp. 32–54). Elsevier Inc. https://doi.org/10.1016/j.pharmthera.2014.11.002

Danielsen, J. M. R., Sylvestersen, K. B., Bekker-Jensen, S., Szklarczyk, D., Poulsen, J. W., Horn, H., Jensen, L. J., Mailand, N., & Nielsen, M. L. (2011). Mass spectrometric analysis of lysine ubiquitylation reveals promiscuity at site level. *Molecular and Cellular Proteomics*, *10*(3), 1–12. https://doi.org/10.1074/mcp.M110.003590

Deng, T., Yan, G., Song, X., Xie, L., Zhou, Y., Li, J., Hu, X., Li, Z., Hu, J., Zhang, Y., Zhang, H., Sun, Y., Feng, P., Wei, D., Hu, B., Liu, J., Tan, W., & Ye, M. (2018). Deubiquitylation and stabilization of p21 by USP11 is critical for cell-cycle progression and DNA damage responses. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(18), 4678–4683. https://doi.org/10.1073/pnas.1714938115

Denuc, A., Bosch-Comas, A., Gonzàlez-Duarte, R., & Marfany, G. (2009). The UBA-UIM Domains of the USP25 Regulate the Enzyme Ubiquitination State and Modulate Substrate Recognition. *PLoS ONE*, *4*(5), e5571. https://doi.org/10.1371/journal.pone.0005571

Dubois, J. C., Yates, M., Gaudreau-Lapierre, A., Clément, G., Cappadocia, L., Gaudreau, L., Zou, L., & Maréchal, A. (2017). A phosphorylation-and-ubiquitylation circuitry driving ATR activation and homologous recombination. *Nucleic Acids Research*, *45*(15), 8859–8872. https://doi.org/10.1093/nar/gkx571

Dueva, R., & Iliakis, G. (2020). Replication protein A: a multifunctional protein with roles in DNA replication, repair and beyond. *NAR Cancer*, *2*(3). https://doi.org/10.1093/narcan/zcaa022

Elia, A. E. H., Wang, D. C., Willis, N. A., Boardman, A. P., Hajdu, I., Adeyemi, R. O., Lowry, E., Gygi, S. P., Scully, R., & Elledge, S. J. (2015). RFWD3-Dependent Ubiquitination of RPA Regulates Repair at Stalled Replication Forks. *Molecular Cell*, *60*(2), 280–293. https://doi.org/10.1016/j.molcel.2015.09.011

Emmerich, C. H., & Cohen, P. (2015). Optimising methods for the preservation, capture and identification of ubiquitin chains and ubiquitylated proteins by immunoblotting. In *Biochemical and Biophysical Research Communications* (Vol. 466, Issue 1, pp. 1–14). Academic Press Inc. https://doi.org/10.1016/j.bbrc.2015.08.109

Faesen, A. C., Luna-Vargas, M. P. A., Geurink, P. P., Clerici, M., Merkx, R., Van Dijk, W. J., Hameed, D. S., El Oualid, F., Ovaa, H., & Sixma, T. K. (2011). The differential modulation of USP activity by internal regulatory domains, interactors and eight ubiquitin chain types. *Chemistry and Biology*, *18*(12), 1550–1561. https://doi.org/10.1016/j.chembiol.2011.10.017

Fallon, L., Bélanger, C. M. L., Corera, A. T., Kontogiannea, M., Regan-Klapisz, E., Moreau, F., Voortman, J., Haber, M., Rouleau, G., Thorarinsdottir, T., Brice, A., van Bergen en Henegouwen, P. M. P., & Fon, E. A. (2006). A regulated interaction with the UIM protein Eps15 implicates parkin in EGF receptor trafficking and PI(3)K-Akt signalling. *Nature Cell Biology*, *8*(8), 834–842. https://doi.org/10.1038/ncb1441

Fisher, R. D., Wang, B., Alam, S. L., Higginson, D. S., Robinson, H., Sundquist, W. I., & Hill, C. P. (2003). Structure and ubiquitin binding of the ubiquitin-interacting motif. *Journal of Biological Chemistry*, *278*(31), 28976–28984. https://doi.org/10.1074/jbc.M302596200

Gudjonsson, T., Altmeyer, M., Savic, V., Toledo, L., Dinant, C., Grøfte, M., Bartkova, J., Poulsen, M., Oka, Y., Bekker-Jensen, S., Mailand, N., Neumann, B., Heriche, J. K., Shearer, R., Saunders, D., Bartek, J., Lukas, J., & Lukas, C. (2012). TRIP12 and UBR5 suppress spreading of chromatin ubiquitylation at damaged chromosomes. *Cell*, *150*(4), 697–709. https://doi.org/10.1016/j.cell.2012.06.039

Haahr, P., Borgermann, N., Guo, X., Typas, D., Achuthankutty, D., Hoffmann, S., Shearer, R., Sixma, T. K., & Mailand, N. (2018). ZUFSP Deubiquitylates K63-Linked Polyubiquitin Chains to Promote Genome Stability. *Molecular Cell*, *70*(1), 165-174.e6. https://doi.org/10.1016/j.molcel.2018.02.024

Haq, S., & Ramakrishna, S. (2017). Deubiquitylation of deubiquitylases. *Open Biology*, *7*(6), 170016. https://doi.org/10.1098/rsob.170016

Harper, S., Gratton, H. E., Cornaciu, I., Oberer, M., Scott, D. J., Emsley, J., & Dreveny, I. (2014). Structure and catalytic regulatory function of ubiquitin specific protease 11 N-terminal and ubiquitin-like domains. *Biochemistry*, *53*(18), 2966–2978. https://doi.org/10.1021/bi500116x

Harrigan, J. A., Jacq, X., Martin, N. M., & Jackson, S. P. (2018). Deubiquitylating enzymes and drug discovery: Emerging opportunities. In *Nature Reviews Drug Discovery* (Vol. 17, Issue 1, pp. 57–77). Nature Publishing Group. https://doi.org/10.1038/nrd.2017.152

Hashimoto, S., Anai, H., & Hanada, K. (2016). Mechanisms of interstrand DNA crosslink repair and human disorders. *Genes and Environment*, *38*(1), 9. https://doi.org/10.1186/s41021-016-0037-9

Hermanns, T., Pichlo, C., Woiwode, I., Klopffleisch, K., Witting, K. F., Ovaa, H., Baumann, U., & Hofmann, K. (2018). A family of unconventional deubiquitinases with modular chain specificity determinants. *Nature Communications*, *9*(1), 1–13. https://doi.org/10.1038/s41467-018-03148-5

Hewings, D. S., Heideker, J., Ma, T. P., Ahyoung, A. P., El Oualid, F., Amore, A., Costakes, G. T., Kirchhofer, D., Brasher, B., Pillow, T., Popovych, N., Maurer, T., Schwerdtfeger, C., Forrest, W. F., Yu, K., Flygare, J., Bogyo, M., & Wertz, I. E. (2018). Reactive-site-centric chemoproteomics identifies a distinct class of deubiquitinase enzymes. *Nature Communications*, *9*(1), 1–17. https://doi.org/10.1038/s41467-018-03511-6

Hicke, L., Schubert, H. L., & Hill, C. P. (2005). Ubiquitin-binding domains. In *Nature Reviews Molecular Cell Biology* (Vol. 6, Issue 8, pp. 610–621). https://doi.org/10.1038/nrm1701

Hodge, C. D., Spyracopoulos, L., & Mark Glover, J. N. (2016). Ubc13: The Lys63 ubiquitin chain building machine. In *Oncotarget* (Vol. 7, Issue 39, pp. 64471–64504). Impact Journals LLC. https://doi.org/10.18632/oncotarget.10948

Hoege, C., Pfander, B., Moldovan, G. L., Pyrowolakis, G., & Jentsch, S. (2002). RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature*, *419*(6903), 135–141. https://doi.org/10.1038/nature00991

Hoeller, D., Crosetto, N., Blagoev, B., Raiborg, C., Tikkanen, R., Wagner, S., Kowanetz, K., Breitling, R., Mann, M., Stenmark, H., & Dikic, I. (2006). Regulation of ubiquitin-binding proteins by monoubiquitination. *Nature Cell Biology*, *8*(2), 163–169. https://doi.org/10.1038/ncb1354

Hoeller, D., & Dikic, I. (2010). Regulation of ubiquitin receptors by coupled

monoubiquitination. *Subcellular Biochemistry*, *54*, 31–40. https://doi.org/10.1007/978-1-4419-6676-6_3

Hoeller, D., Hecker, C. M., Wagner, S., Rogov, V., Dötsch, V., & Dikic, I. (2007). E3-Independent Monoubiquitination of Ubiquitin-Binding Proteins. *Molecular Cell*, *26*(6), 891–898. https://doi.org/10.1016/j.molcel.2007.05.014

Huttlin, E. L., Bruckner, R. J., Paulo, J. A., Cannon, J. R., Ting, L., Baltier, K., Colby, G., Gebreab, F., Gygi, M. P., Parzen, H., Szpyt, J., Tam, S., Zarraga, G., Pontano-Vaites, L., Swarup, S., White, A. E., Schweppe, D. K., Rad, R., Erickson, B. K., … Wade Harper, J. (2017). Architecture of the human interactome defines protein communities and disease networks. *Nature*, *545*(7655), 505–509. https://doi.org/10.1038/nature22366

Huttlin, E. L., Ting, L., Bruckner, R. J., Gebreab, F., Gygi, M. P., Szpyt, J., Tam, S., Zarraga, G., Colby, G., Baltier, K., Dong, R., Guarani, V., Vaites, L. P., Ordureau, A., Rad, R., Erickson, B. K., Wühr, M., Chick, J., Zhai, B., … Gygi, S. P. (2015). The BioPlex Network: A Systematic Exploration of the Human Interactome. *Cell*, *162*(2), 425–440. https://doi.org/10.1016/j.cell.2015.06.043

Kang, M. A., So, E. Y., Simons, A. L., Spitz, D. R., & Ouchi, T. (2012). DNA damage induces reactive oxygen species generation through the H2AX-Nox1/Rac1 pathway. *Cell Death and Disease*, *3*(1), 249. https://doi.org/10.1038/cddis.2011.134

Kannouche, P. L., Wing, J., & Lehmann, A. R. (2004). Interaction of human DNA polymerase η with monoubiquitinated PCNA: A possible mechanism for the polymerase switch in response to DNA damage. *Molecular Cell*, *14*(4), 491–500. https://doi.org/10.1016/S1097-2765(04)00259-X

Kiwerska, K., & Szyfter, K. (2019). DNA repair in cancer initiation, progression, and therapy—a double-edged sword. In *Journal of Applied Genetics* (Vol. 60, Issues 3–4, pp. 329–334). Springer Verlag. https://doi.org/10.1007/s13353-019-00516-9

Klapisz, E., Sorokina, I., Lemeer, S., Pijnenburg, M., Verkleij, A. J., & Van Bergen En Henegouwen, P. M. P. (2002). *A Ubiquitin-interacting Motif (UIM) Is Essential for Eps15 and Eps15R Ubiquitination**. https://doi.org/10.1074/jbc.M203004200

Koç, A., Wheeler, L. J., Mathews, C. K., & Merrill, G. F. (2004). Hydroxyurea Arrests DNA Replication by a Mechanism that Preserves Basal dNTP Pools. *Journal of Biological Chemistry*, *279*(1), 223–230. https://doi.org/10.1074/jbc.M303952200

Komander, D., Clague, M. J., & Urbé, S. (2009). Breaking the chains: Structure and function of the deubiquitinases. In *Nature Reviews Molecular Cell Biology* (Vol. 10, Issue 8, pp. 550–563). Nature Publishing Group. https://doi.org/10.1038/nrm2731

Kwasna, D., Abdul Rehman, S. A., Natarajan, J., Matthews, S., Madden, R., De Cesare, V., Weidlich, S., Virdee, S., Ahel, I., Gibbs-Seymour, I., & Kulathu, Y. (2018). Discovery and Characterization of ZUFSP/ZUP1, a Distinct Deubiquitinase Class Important for Genome Stability. *Molecular Cell*. https://doi.org/10.1016/j.molcel.2018.02.023

Lachaud, C., Castor, D., Hain, K., Muñoz, I., Wilson, J., MacArtney, T. J., Schindler, D., & Rouse, J. (2014). Distinct functional roles for the two SLX4 ubiquitin-binding UBZ domains mutated in Fanconi anemia. *Journal of Cell Science*, *127*(13), 2811–2817. https://doi.org/10.1242/jcs.146167

Le, J., Perez, E., Nemzow, L., & Gong, F. (2019). Role of deubiquitinases in DNA damage response. In *DNA Repair* (Vol. 76, pp. 89–98). Elsevier B.V.

https://doi.org/10.1016/j.dnarep.2019.02.011

Liaw, H., Lee, D., & Myung, K. (2011). DNA-PK-Dependent RPA2 Hyperphosphorylation Facilitates DNA Repair and Suppresses Sister Chromatid Exchange. *PLoS ONE*, *6*(6), e21424. https://doi.org/10.1371/journal.pone.0021424

Liu, S., Opiyo, S. O., Manthey, K., Glanzer, J. G., Ashley, A. K., Amerin, C., Troksa, K., Shrivastav, M., Nickoloff, J. A., & Oakley, G. G. (2012). Distinct roles for DNA-PK, ATM and ATR in RPA phosphorylation and checkpoint activation in response to replication stress. *Nucleic Acids Research*, *40*(21), 10780–10794. https://doi.org/10.1093/nar/gks849

Lowe, J., McDermott, H., Landon, M., Mayer, R. J., & Wilkinson, K. D. (1990). Ubiquitin carboxyl-terminal hydrolase (PGP 9.5) is selectively present in ubiquitinated inclusion bodies characteristic of human neurodegenerative diseases. *The Journal of Pathology*, *161*(2), 153–160. https://doi.org/10.1002/path.1711610210

Machida, Y., Kim, M. S., & Machida, Y. J. (2012). Spartan/C1orf124 is important to prevent UV-induced mutagenesis. *Cell Cycle*, *11*(18), 3395–3402. https://doi.org/10.4161/cc.21694

Maertens, G. N., El Messaoudi-Aubert, S., Elderkin, S., Hiom, K., & Peters, G. (2010). Ubiquitin-specific proteases 7 and 11 modulate Polycomb regulation of the INK4a tumour suppressor. *EMBO Journal*, *29*(15), 2553–2565. https://doi.org/10.1038/emboj.2010.129

Maréchal, A., Li, J. M., Ji, X. Y., Wu, C. S., Yazinski, S. A., Nguyen, H. D., Liu, S., Jiménez, A. E., Jin, J., & Zou, L. (2014). PRP19 Transforms into a Sensor of RPA-ssDNA after DNA Damage and Drives ATR Activation via a Ubiquitin-Mediated Circuitry. *Molecular Cell*, *53*(2), 235–246. https://doi.org/10.1016/j.molcel.2013.11.002

Mattera, R., & Bonifacino, J. S. (2008). Ubiquitin binding and conjugation regulate the recruitment of Rabex-5 to early endosomes. *EMBO Journal*, *27*(19), 2484–2494. https://doi.org/10.1038/emboj.2008.177

Meray, R. K., & Lansbury, P. T. (2007). Reversible monoubiquitination regulates the Parkinson disease-associated ubiquitin hydrolase UCH-L1. *Journal of Biological Chemistry*, *282*(14), 10567–10575. https://doi.org/10.1074/jbc.M611153200

Meulmeester, E., Kunze, M., Hsiao, H. H., Urlaub, H., & Melchior, F. (2008). Mechanism and Consequences for Paralog-Specific Sumoylation of Ubiquitin-Specific Protease 25. *Molecular Cell*, *30*(5), 610–619. https://doi.org/10.1016/j.molcel.2008.03.021

Morrow, M. E., Morgan, M. T., Clerici, M., Growkova, K., Yan, M., Komander, D., Sixma, T. K., Simicek, M., & Wolberger, C. (2018). Active site alanine mutations convert deubiquitinases into high-affinity ubiquitin-binding proteins. *EMBO Reports*, *19*(10). https://doi.org/10.15252/embr.201745680

Mosbech, A., Gibbs-Seymour, I., Kagias, K., Thorslund, T., Beli, P., Povlsen, L., Nielsen, S. V., Smedegaard, S., Sedgwick, G., Lukas, C., Hartmann-Petersen, R., Lukas, J., Choudhary, C., Pocock, R., Bekker-Jensen, S., & Mailand, N. (2012). DVC1 (C1orf124) is a DNA damage-targeting p97 adaptor that promotes ubiquitin-dependent responses to replication blocks. *Nature Structural and Molecular Biology*. https://doi.org/10.1038/nsmb.2395

Munoz, M. A., Saunders, D. N., Henderson, M. J., Clancy, J. L., Russell, A. J., Lehrbach, G.,

Musgrove, E. A., Watts, C. K. W., & Sutherland, R. L. (2007). The E3 ubiquitin ligase EDD regulates S-phase and G2/M DNA damage checkpoints. *Cell Cycle*, *6*(24), 3070–3077. https://doi.org/10.4161/cc.6.24.5021

O'Brien, P. M., Davies, M. J., Scurry, J. P., Smith, A. N., Barton, C. A., Henderson, M. J., Saunders, D. N., Gloss, B. S., Patterson, K. I., Clancy, J. L., Heinzelmann-Schwarz, V. A., Scolyer, R. A., Zeng, Y., Williams, E. D., Scurr, L., DeFazio, A., Quinn, D. I., Watts, C. K. W., Hacker, N. F., … Sutherland, R. L. (2008). The E3 ubiquitin ligase EDD is an adverse prognostic factor for serous epithelial ovarian cancer and modulates cisplatin resistance in vitro. *British Journal of Cancer*, *98*(6), 1085–1093. https://doi.org/10.1038/sj.bjc.6604281

Ohtake, F., Tsuchiya, H., Saeki, Y., & Tanaka, K. (2018). K63 ubiquitylation triggers proteasomal degradation by seeding branched ubiquitin chains. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.1716673115

Oldham, C. E., Mohney, R. P., Miller, S. L. H., Hanes, R. N., & O'bryan, J. P. (2002). The ubiquitin-interacting motifs target the endocytic adaptor protein epsin for ubiquitination. *Current Biology*, *12*(13), 1112–1116. https://doi.org/10.1016/S0960-9822(02)00900-4

Osaka, H., Wang, Y. L., Takada, K., Takizawa, S., Setsuie, R., Li, H., Sato, Y., Nishikawa, K., Sun, Y. J., Sakurai, M., Harada, T., Hara, Y., Kimura, I., Chiba, S., Namikawa, K., Kiyama, H., Noda, M., Aoki, S., & Wada, K. (2003). Ubiquitin carboxy-terminal hydrolase L1 binds to and stabilizes monoubiquitin in neuron. *Human Molecular Genetics*, *12*(16), 1945–1958. https://doi.org/10.1093/hmg/ddg211

Penengo, L., Mapelli, M., Murachelli, A. G., Confalonieri, S., Magri, L., Musacchio, A., Di Fiore, P. P., Polo, S., & Schneider, T. R. (2006). Crystal Structure of the Ubiquitin Binding Domains of Rabex-5 Reveals Two Modes of Interaction with Ubiquitin. *Cell*, *124*(6), 1183–1195. https://doi.org/10.1016/j.cell.2006.02.020

Pickart, C. M., & Eddins, M. J. (2004). Ubiquitin: Structures, functions, mechanisms. In *Biochimica et Biophysica Acta - Molecular Cell Research* (Vol. 1695, Issues 1–3, pp. 55–72). Elsevier. https://doi.org/10.1016/j.bbamcr.2004.09.019

Polo, S., Sigismund, S., Faretta, M., Guidi, M., Capua, M. R., Bossi, G., Chen, H., De Camilli, P., & Di Fiore, P. P. (2002). A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. *Nature*, *416*(6879), 451–455. https://doi.org/10.1038/416451a

Randles, L., & Walters, K. J. (2012). Ubiquitin and its binding domains. *Frontiers in Bioscience*, *17*(6), 2140–2157. https://doi.org/10.2741/4042

Rocha, C. R. R., Silva, M. M., Quinet, A., Cabral-Neto, J. B., & Menck, C. F. M. (2018). DNA repair pathways and cisplatin resistance: An intimate relationship. In *Clinics* (Vol. 73, Issue Suppl 1). Universidade de Sao Paulo. https://doi.org/10.6061/clinics/2018/e478s

Seki, T., Gong, L., Williams, A. J., Sakai, N., Todi, S. V., & Paulson, H. L. (2013). JosD1, a membrane-targeted deubiquitinating enzyme, is activated by ubiquitination and regulates membrane dynamics, cell motility, and endocytosis. *Journal of Biological Chemistry*, *288*(24), 17145–17155. https://doi.org/10.1074/jbc.M113.463406

Shearer, R. F., Iconomou, M., Watts, C. K. W., & Saunders, D. N. (2015). Functional roles of

the E3 Ubiquitin Ligase UBR5 in cancer. In *Molecular Cancer Research* (Vol. 13, Issue 12, pp. 1523–1532). American Association for Cancer Research Inc. https://doi.org/10.1158/1541-7786.MCR-15-0383

Sowa, M. E., Bennett, E. J., Gygi, S. P., & Harper, J. W. (2009). Defining the Human Deubiquitinating Enzyme Interaction Landscape. *Cell*, *138*(2), 389–403. https://doi.org/10.1016/j.cell.2009.04.042

Stingele, J., Bellelli, R., Alte, F., Hewitt, G., Sarek, G., Maslen, S. L., Tsutakawa, S. E., Borg, A., Kjær, S., Tainer, J. A., Skehel, J. M., Groll, M., & Boulton, S. J. (2016). Mechanism and Regulation of DNA-Protein Crosslink Repair by the DNA-Dependent Metalloprotease SPRTN. *Molecular Cell*. https://doi.org/10.1016/j.molcel.2016.09.031

Sun, W., Tan, X., Shi, Y., Xu, G., Mao, R., Gu, X., Fan, Y., Yu, Y., Burlingame, S., Zhang, H., Rednam, S. P., Lu, X., Zhang, T., Fu, S., Cao, G., Qin, J., & Yang, J. (2010). USP11 negatively regulates TNFα-induced NF-κB activation by targeting on IκBα. *Cellular Signalling*, *22*(3), 386–394. https://doi.org/10.1016/j.cellsig.2009.10.008

Swanson, K. A., Kang, R. S., Stamenova, S. D., Hicke, L., & Radhakrishnan, I. (2003). Solution structure of Vps27 UIM-ubiquitin complex important for endosomal sorting and receptor downregulation. *EMBO Journal*, *22*(18), 4597–4606. https://doi.org/10.1093/emboj/cdg471

Thach, T. T., Lee, N., Shin, D., Han, S., Kim, G., Kim, H., & Lee, S. (2015). Molecular determinants of polyubiquitin recognition by continuous ubiquitin-binding domains of Rad18. *Biochemistry*, *54*(12), 2136–2148. https://doi.org/10.1021/bi5012546

Ting, X., Xia, L., Yang, J., He, L., Si, W., Shang, Y., & Sun, L. (2019). USP11 acts as a histone deubiquitinase functioning in chromatin reorganization during DNA repair. *Nucleic Acids Research*, *47*(18), 9721–9740. https://doi.org/10.1093/nar/gkz726

Tkáč, J., Xu, G., Adhikary, H., Young, J. T. F., Gallo, D., Escribano-Díaz, C., Krietsch, J., Orthwein, A., Munro, M., Sol, W., Al-Hakim, A., Lin, Z. Y., Jonkers, J., Borst, P., Brown, G. W., Gingras, A. C., Rottenberg, S., Masson, J. Y., & Durocher, D. (2016). HELB Is a Feedback Inhibitor of DNA End Resection. *Molecular Cell*, *61*(3), 405–418. https://doi.org/10.1016/j.molcel.2015.12.013

Todi, S. V., Winborn, B. J., Scaglione, K. M., Blount, J. R., Travis, S. M., & Paulson, H. L. (2009). Ubiquitination directly enhances activity of the deubiquitinating enzyme ataxin-3. *EMBO Journal*, *28*(4), 372–382. https://doi.org/10.1038/emboj.2008.289

Toma, A., Takahashi, T. S., Sato, Y., Yamagata, A., Goto-Ito, S., Nakada, S., Fukuto, A., Horikoshi, Y., Tashiro, S., & Fukai, S. (2015). Structural basis for ubiquitin recognition by ubiquitin-binding zinc finger of FAAP20. *PLoS ONE*, *10*(3). https://doi.org/10.1371/journal.pone.0120887

Usp11 protein (mouse) - STRING interaction network. (n.d.). Retrieved September 8, 2020, from https://string-db.org/cgi/network.pl?taskId=k8iru7XjvDPw

VanderVere-Carozza, P. S., Pawelczak, K. S., Gavande, N. S., Jalal, S. I., Pollok, K. E., Ekinci, E., Heyza, J., Patrick, S. M., & Turchi, J. J. (2020). Chemical exhaustion of RPA in cancer treatment. In *bioRxiv* (p. 2020.11.30.404640). bioRxiv. https://doi.org/10.1101/2020.11.30.404640

Vassin, V. M., Anantha, R. W., Sokolova, E., Kanner, S., & Borowiec, J. A. (2009). Human RPA phosphorylation by ATR stimulates DNA synthesis and prevents ssDNA

accumulation during DNA-replication stress. *Journal of Cell Science*, *122*(22), 4070–4080. https://doi.org/10.1242/jcs.053702

Vizeacoumar, F. J., Arnold, R., Vizeacoumar, F. S., Chandrashekhar, M., Buzina, A., Young, J. T. F., Kwan, J. H. M., Sayad, A., Mero, P., Lawo, S., Tanaka, H., Brown, K. R., Baryshnikova, A., Mak, A. B., Fedyshyn, Y., Wang, Y., Brito, G. C., Kasimer, D., Makhnevych, T., … Moffat, J. (2013). A negative genetic interaction map in isogenic cancer cell lines reveals cancer cell vulnerabilities. *Molecular Systems Biology*, *9*(1), 696. https://doi.org/10.1038/msb.2013.54

Wang, L. E., Li, C., Xiong, P., Gershenwald, J. E., Prieto, V. G., Duvic, M., Lee, J. E., Grimm, E. A., Hsu, T. C., & Wei, Q. (2016). 4-Nitroquinoline-1-oxide-induced mutagen sensitivity and risk of cutaneous melanoma: A case-control analysis. *Melanoma Research*, *26*(2), 181–187. https://doi.org/10.1097/CMR.0000000000000106

Wiltshire, T. D., Lovejoy, C. A., Wang, T., Xia, F., O'Connor, M. J., & Cortez, D. (2010). Sensitivity to poly(ADP-ribose) polymerase (PARP) inhibition identifies ubiquitin-specific peptidase 11 (USP11) as a regulator of DNA double-strand break repair. *Journal of Biological Chemistry*, *285*(19), 14565–14571. https://doi.org/10.1074/jbc.M110.104745

Wit, N., Buoninfante, O. A., Van Den Berk, P. C. M., Jansen, J. G., Hogenbirk, M. A., De Wind, N., & Jacobs, H. (2015). Roles of PCNA ubiquitination and TLS polymerases κ and η in the bypass of methyl methanesulfonate-induced DNA damage. *Nucleic Acids Research*, *43*(1), 282–294. https://doi.org/10.1093/nar/gku1301

Woelk, T., Oldrini, B., Maspero, E., Confalonieri, S., Cavallaro, E., Di Fiore, P. P., & Polo, S. (2006). Molecular mechanisms of coupled monoubiquitination. *Nature Cell Biology*, *8*(11), 1246–1254. https://doi.org/10.1038/ncb1484

Wold, M. S. (1997). Replication protein A: A heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. In *Annual Review of Biochemistry* (Vol. 66, pp. 61–92). Annu Rev Biochem. https://doi.org/10.1146/annurev.biochem.66.1.61

Xu, Y., & Her, C. (2015). Inhibition of topoisomerase (DNA) I (TOP1): DNA damage repair and anticancer therapy. In *Biomolecules* (Vol. 5, Issue 3, pp. 1652–1670). MDPI AG. https://doi.org/10.3390/biom5031652

Yan, Z., Guo, R., Paramasivam, M., Shen, W., Ling, C., Fox, D., Wang, Y., Oostra, A. B., Kuehl, J., Lee, D. Y., Takata, M., Hoatlin, M. E., Schindler, D., Joenje, H., de Winter, J. P., Li, L., Seidman, M. M., & Wang, W. (2012). A Ubiquitin-Binding Protein, FAAP20, Links RNF8-Mediated Ubiquitination to the Fanconi Anemia DNA Repair Network. *Molecular Cell*, *47*(1), 61–75. https://doi.org/10.1016/j.molcel.2012.05.026

Yao, F., Svensjö, T., Winkler, T., Lu, M., Eriksson, C., & Eriksson, E. (1998). Tetracycline repressor, tetR, rather than the tetR-mammalian cell transcription factor fusion derivatives, regulates inducible gene expression in mammalian cells. *Human Gene Therapy*, *9*(13), 1939–1950. https://doi.org/10.1089/hum.1998.9.13-1939

Yau, R. G., Doerner, K., Castellanos, E. R., Haakonsen, D. L., Werner, A., Wang, N., Yang, X. W., Martinez-Martin, N., Matsumoto, M. L., Dixit, V. M., & Rape, M. (2017). Assembly and Function of Heterotypic Ubiquitin Chains in Cell-Cycle and Protein Quality Control. *Cell*, *171*(4), 918-933.e20. https://doi.org/10.1016/j.cell.2017.09.040

Yau, R., & Rape, M. (2016). The increasing complexity of the ubiquitin code. In *Nature Cell Biology* (Vol. 18, Issue 6, pp. 579–586). Nature Publishing Group. https://doi.org/10.1038/ncb3358

Yu, M., Liu, K., Mao, Z., Luo, J., Gu, W., & Zhao, W. (2016). USP11 is a negative regulator to γh2AX ubiquitylation by RNF8/RNF168. *Journal of Biological Chemistry*, *291*(2), 959–967. https://doi.org/10.1074/jbc.M114.624478

Zhang, H., Xiong, Y., & Chen, J. (2020). DNA-protein cross-link repair: What do we know now? In *Cell and Bioscience* (Vol. 10, Issue 1, pp. 1–10). BioMed Central Ltd. https://doi.org/10.1186/s13578-019-0366-z

Zhang, R., Sengupta, S., Yang, Q., Linke, S. P., Yanaihara, N., Bradsher, J., Blais, V., McGowan, C. H., & Harris, C. C. (2005). BLM helicase facilitates Mus81 endonuclease activity in human cells. *Cancer Research*, *65*(7), 2526–2531. https://doi.org/10.1158/0008-5472.CAN-04-2421

Zhao, Y., Majid, M. C., Soll, J. M., Brickner, J. R., Dango, S., & Mosammaparast, N. (2015). Noncanonical regulation of alkylation damage resistance by the OTUD 4 deubiquitinase . *The EMBO Journal*, *34*(12), 1687–1703. https://doi.org/10.15252/embj.201490497

Zhu, X., Ménard, R., & Sulea, T. (2007). High incidence of ubiquitin-like domains in human ubiquitin-specific proteases. *Proteins: Structure, Function and Genetics*, *69*(1), 1–7. https://doi.org/10.1002/prot.21546