# Characterization of E2E Ubiquitin-Conjugating Enzymes and Ubiquitin-Specific Protease 7 (USP7) in Histone H2A Ubiquitination

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# Abstract

Ubiquitin(Ub)-conjugating E2 enzymes play essential roles in ubiquitination of proteins. The UbE2E sub-family members UbE2E1, UbE2E2, and UbE2E3 have N-terminal extensions to the conserved E2 core which contain Ubiquitin-Specific Protease 7 (USP7) binding sequences (P/A/ExxS). Here, we continued our investigations to established that USP7 can interact with E2Es *in vitro* and *in vivo*. Our new data indicated that the N-terminal extensions of E2E2 or E2E3 can directly associate with USP7 TRAF domain. We demonstrated that E2E2 or E2E3 are stabilized by USP7 in cells. We also showed that E2Es interact with Ring1B:BMI1, the core components of the Polycomb Repressive Complex 1 (PRC1) and established E2E1 as an *in vivo* E2 for monoubiquitination of histone H2A on lysine(K) 119. We demonstrated that E2Es can modulate the levels of H2A monoubiquitination in cells and that USP7 may exert an effect on K119-UbH2A levels through regulating E2Es.

# Dedications

For my dearest, and departed grandmother. Your memories, love, and grace are eternal.

I also dedicate this to Agnieszka, GhoorGhoor, and Charlie for their invaluable support.

And for science; may our collective and ever developing understanding of the universe and life lead us to make the world a better and a more peaceful place, for all life is precious.

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# List of Abbreviations

**A**........alanine

**aa**. ........amno acid

**APC/C**........anaphase-promoting complex/cyclosome

**APF-1** ........ ATP-dependent proteolytic factor-1

**ATP** ........ adenosine triphosphate

**BMI1** ........B cell-specific Moloney murine leukemia virus integration site 1

**C**... ........ cysteine

**CD**. ........catalytic domain

**CHIP** ........ carboxyl terminus of Hsc70-interacting protein

**CHX** ........cycloheximide

**D**... ........ aspartic acid

**DDR**........DNA damage response

**DUB**........deubiquitinating enzyme

**E6-AP** ........E6-associated protein

**ECL** ........electrogenerated chemiluminescence

**ENaC** ........epithelial Na+ channel

**ER**.........endoplasmic reticulum

**ERAD**........ endoplasmic reticulum– associated degradation

**FAT10**........HLA-F adjacent transcript 10

**FL**.. ........full-length

**GABARAP**........ gamma-aminobutyric acid-A receptor-associated protein

**H**... ........histidine

**HECT** ........homologous to E6-AP carboxyl terminus

**ICP0**........infected cell polypeptide 0

**IGF-1R** ........ insulin-like growth factor R1

**IPTG** ........ isopropyl-1-thio-β-D-galactopyranoside

**ISG15** ........ Interferon-stimulated gene 15

**JAMM** ........ JAB1/MPN/MOV34

**K**........ lysine

**MINDY** ........ Motif Interacting with Ub-containing Novel DUB family

**MJD**........ Machado-Josephin domain

**NEDD4** ........ neural precursor cell expressed developmentally down-regulated protein 4

**NEDD8** ........ neural-precursor-cell-expressed developmentally down-regulated 8

**NEM**........ N-Ethylmaleimide

**NMR**........ nuclear magnetic resonance

**N-t**........ N(amine)-terminal

**P/A/ExxS**........ proline/alanine/glutamic acid, (any aa), (any aa), serine

**PTEN** ........ phosphatase and tensin homologue

**PTM** ........ post-translational modification

**RBR** ........ Ring-inBetweenRing-Ring

**RCC1** ........ regulators of chromosome condensation-1

**RING**........ Really Interesting New Gene

**RLD**........ RCC1-like domain

**SCA1**........ Spinocerebellar ataxia type 1

**SDS-PAGE** ........ sodium dodecyl sulfate polyacrylamide gel electrophoresis

**SUMO** ........ small ubiquitin-like modifier

**TRAF**........ TNF receptor-associated factor

**Ub**. ........ ubiquitin

**UBC**........ ubiquitin-conjugating

**UBIP**........ ubiquitous immunopoietic polypeptide

**UBL** ........ ubiquitin-like

**UCH**............................................................................................................................ubiquitin C-terminal hydrolase

**USP** ..................................................................................................................................ubiquitin-specific protease

**WT**. ........................................................................................................................................................ wild-type

# Chapter 1: Ubiquitin Systems

## 1.1 Ubiquitin: A Ubiquitous Small Signalling Protein

Ubiquitin (Ub) was first isolated from bovine thymus tissue and characterized as an inducer of immunocyte differentiation (Goldstein *et al.* 1975). This novel protein was initially termed Ubiquitous Immunopoietic Polypeptide (UBIP) as it was found to be expressed across all tissues of multi-cellular organisms. Following its discovery, UBIP was sequenced using automated EDMAN degradation (Schlesinger *et al.* 1975). In 1978, other studies independently identified ubiquitin as ATP-dependent Proteolytic Factor-1 (APF-1), a component of a proteolytic system which stimulated protein breakdown without protease activity on its own (Ciechanover *et al.* 1978). Later, studies on the mechanisms of APF-1-associated protein degradation revealed that APF-1 was covalently attached to substrates through a peptide bond which served as a cellular signal to target the modified protein for degradation (Hershko *et al.* 1980). Comparison between the sequences and characteristics of UBIP and APF-1 eventually led to the realization that the two proteins were identical (Wilkinson *et al.* 1980). Subsequently, components of the ubiquitin-protein conjugation system were identified through ubiquitin affinity chromatography and mass spectroscopy (Hershko *et al.* 1983), and the first structure of ubiquitin became available through X-ray crystallography at a resolution of 2.8 Å (Vijay-Kumar *et al.* 1985). The structure of Ub revealed that this small protein constituted a 5-stranded β-sheet, an α-helix, and one short 3-10 helix (Figure 1-1) (Vijay-Kumar *et al.* 1987).

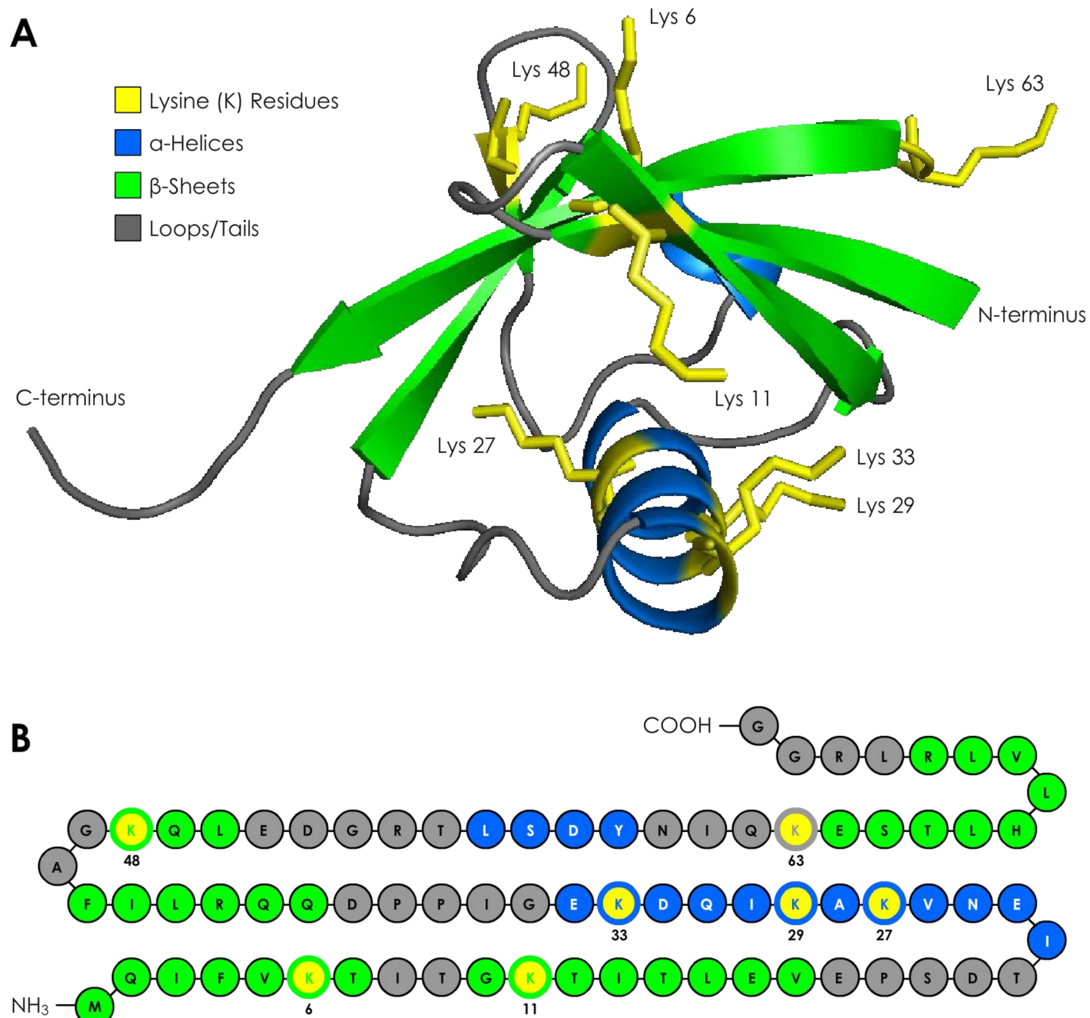



**Figure 1-1 – Ubiquitin: Sequence and Structure. (A)** Depiction of the structure of ubiquitin using accession number 1UBI from the Protein Data Bank (PDB) rendered by PyMol software (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.). **(B)** Primary structure of ubiquitin showing linear sequence of its 76 amino acids.

## 1.2 Ubiquitin and Ubiquitin-Like Post-Translational Modification

Proteins often undergo modifications by acquiring diverse functional groups to alter their structure or functions. For example, glycosylation of newly synthesized proteins in the ER/Golgi systems is required for proper protein folding, and such modifications to membrane proteins provides contact specificity for cell-adhesion proteins or receptors at cell surface (Moremen *et*

*al.* 2012). In addition, a variety of other post-translational modifications (PTMs) are involved in cellular signaling events leading to diverse and complex inhibitory or excitatory consequences. The most studied examples of such PTMs include acetylation, methylation, phosphorylation, and ubiquitination (Wang *et al.* 2014). All of these PTMS are associated with respective enzymes catalyzing their attachment or removal.

The covalent attachment of ubiquitin to lysine residues of the target proteins is referred to as ubiquitination (also termed ubiquitylation) and requires the formation of a peptide bond between the C-terminal carboxyl group of ubiquitin and the ε-amine group of lysine residues on targets (Rape 2017). Other Ub-bound molecules exist, most of which have Ub attached on thiol groups of cysteine residues such as intermediates of some ubiquitination processes. In addition to ubiquitin, a number of ubiquitin-like (UBL) modifiers such as SUMO, ISG15, and NEDD8, Atg8/12 have been identified which share similar properties as ubiquitin such as their β-grasp fold, size (8-20 kDa), a C-terminal segment which allows for flexibility of tethering, and in most cases a C-terminal glycine residue (Vierstra 2012). Structural comparison of several UBLs are shown in Figure 1-2.

As ubiquitin itself contains 7 lysine residues (Figure 1-1-A), each lysine can serve as a site for ubiquitination therefore allowing ubiquitin to form polyubiquitin chains with various linkages (Rape 2017). In addition to the lysine residues of Ub, the N-terminal amine group of ubiquitin can also be used to form a peptide bond with the C-terminal carboxyl group of the next ubiquitin molecule in a linear Ub chain (Ciechanover and Ben-Saadon 2004, Kirisako *et al.* 2006).

Due to the topological diversity and complexity of Ub chains, ubiquitination is unique among the crucial PTMs in the sense that it elicits diverse signals involved in various important cellular processes (Prabakaran *et al.* 2012).

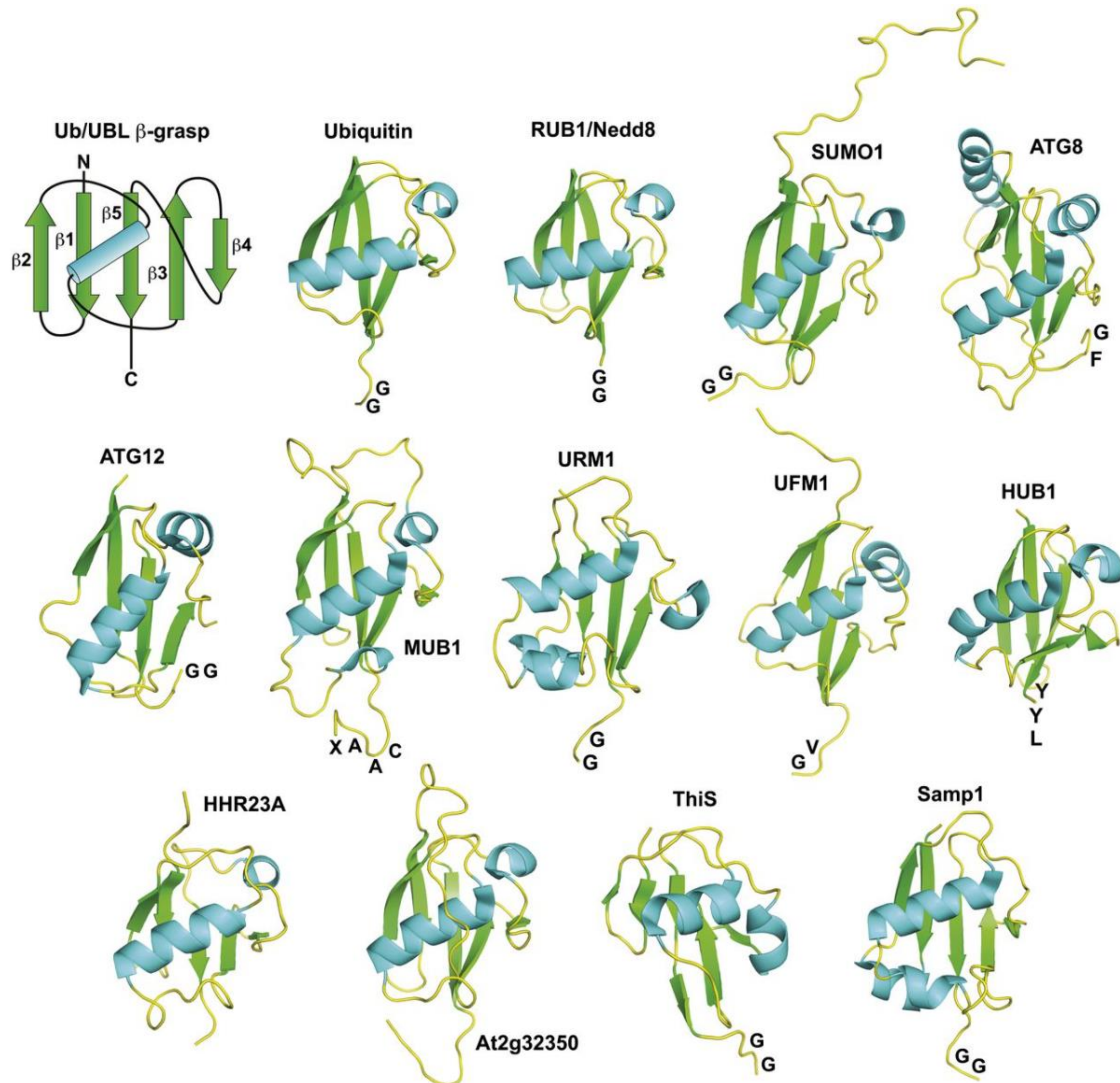



**Figure 1-2 - Ubiquitin-Like (UBL) Modifiers.** The structures of several UBL modifiers are compared showing the common UBL β-grasp fold and two C-terminal glycine residues. Adapted from Vierstra (2012).

## 1.3 Diversity of Ubiquitin Signaling

Protein polyubiquitination was initially found to be associated with targeted protein degradation by the 26S proteasome complex. Ciechanover, Hershko, and Rose were awarded the 2004 Noble Prize in Chemistry for their contribution in elucidation of the mechanism of ubiquitin-proteasome associated protein degradation (Ciechanover *et al.* 1978, Hershko *et al.* 1980, 1983, 1988). In the ubiquitin-proteasome pathway, K48- or K11-linked polyubiquitinated proteins are recognized by the 26S proteasome and become deubiquitinated, unfolded, internalized, and finally degraded by the proteasome. The 26S proteasome, found in both the nucleus and cytosol, is composed of 60 subunits that form the core 20S hollow cylindrical particle where protein degradation occurs, and the 19S component at one or both ends which aids in unfolding and translocation of substrates into the 20S (Lecker *et al.* 2006, Gallastegui and Groll 2010). In addition, the 19S contains a lid-like structure where other regulatory components and deubiquitinating enzymes may interact tightly to regulate protein entry and avoid non-specific protein degradation (Lecker et al. 2006).

Since the late 1990s, researchers have identified ubiquitin-signals that are not associated with protein degradation. These signals can be monoubiquitin or multi-monoubiquitins (Hicke 2001, Rape 2017), or K63-linked polyubiquitin chains (Fujita *et al.* 2014, Iwai *et al.* 2014). These ubiquitin-mediated signals regulate cell cycle progression, apoptosis, proliferation, DNA damage responses, signal transduction, endocytosis, localization, transcription, gene silencing, and more (Hicke 2001, Rape 2017).

## 1.4 The Ubiquitination Pathway & Machinery

Ubiquitination processes encompass a cascade of catalytic activities carried out by different enzymes. Ubiquitination is always initiated by a ubiquitin-activating (E1) enzyme which catalyzes the ATP-dependent ubiquitin activation (Figure 1-3) (Schulman and Harper 2009). The activated Ub bound to E1 is then transferable to a Ub-Conjugating (E2) enzyme to form an E2-Ub intermediate (Stewart *et al.* 2016). The E3 ligase binds to specific substrate proteins and coordinates with Ub-charged E2 to mediate substrate ubiquitination (Figure 1-3) (Morreale and Walden 2016). Besides differences in targets, the outputs of ubiquitin pathways vary in the location, type, and number of linkages between ubiquitin and target, and between multiple ubiquitins assembled in a chain, all of which are directed by the composition of the E2:E3 complex. These variations result in targeted proteins that can be monoubiquitinated (a single Ub at a particular lysine), multi-monoubiquitinated (more than one Ub at distinct lysines, or polyubiquitinated (more than one Ub at one or more sites) (Figure 1-3).

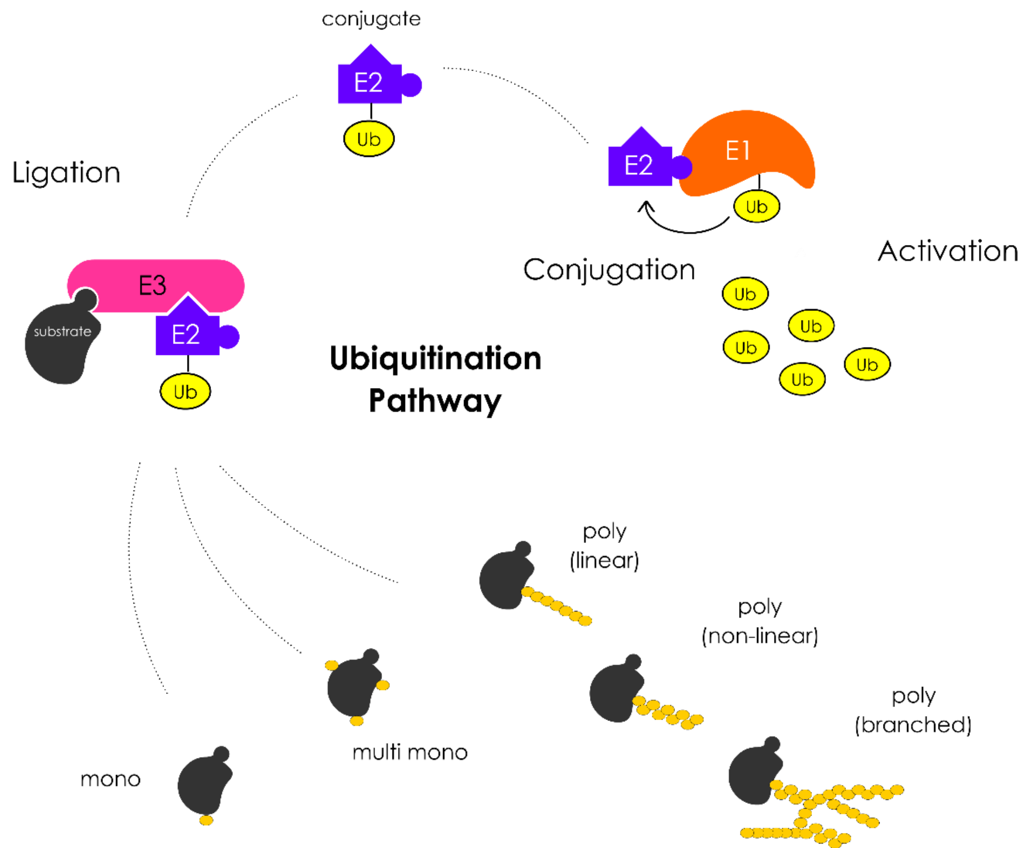



**Figure 1-3 – General Schematic of Ubiquitination Pathways.** A general ubiquitination pathway is demonstrated showing actitation of Ub by E1, transfer of Ub to an E2:Ub conjugate, and formation of a complex between a substrate, an E3 Ub-ligase, and E2:Ub. Variations of ubiquitination topology are shown for substrates (monoubiquitination, multi-monoubiquitination, and polyubiquitination with varying linkages).

## 1.5 E1 Ubiquitin-Activating Enzymes

E1s are a superfamily of activating enzymes which form the apex of the enzyme cascades involving UBLs, all of which have their own associated E1 enzyme(s). The two E1 activating enzymes, UBA1 and UBA6, have been identified for ubiquitin so far and function as monomeric proteins. UBA6 was initially indicated as an E1 for the UBL modifier FAT10 but has also been shown to activate Ub *in vitro* and *in vivo* (Pelzer *et al.* 2007); however, while UBA6 has been linked to few E2 enzymes, UBA1 is associated with the vast majority of known ubiquitin signalling

pathways and activates Ub for conjugation with almost all ubiquitin-conjugating E2s and serves as the canonical model for understanding UBL modifier activation (Schulman and Harper 2009).

## 1.6 E3 Ubiquitin-Ligases

More than a thousand E3s have been identified, with over 600 E3s alone in humans. Based on the structures and functional domains, E3s are characterized by three categories: RING E3s, HECT E3s, and the newly designated RBR E3s (Figure 1-4) (Morreale and Walden 2016).

RING E3s make up the majority of Ub-ligases. This family of proteins constitute enzymes with RING (Really Interesting New Gene) (Zheng *et al.* 2000) or U-box domains (Figure 1-4) (Hatakeyama *et al.* 2001). Both of these domains are ~40-70 residues in size and share a similar structural fold. They interact with an Ub charged E2 enzyme and promote Ub transfer directly from an E2 to a specific lysine residue of the substrate protein (Figure 1-8A). RING domains are distinct from U-box domains in that they feature zinc fingers, a domain that binds 2 zinc ions in a cross-braced coordination, a property that is important in the folding of the RING domain. RING E3s are further recognized by their mode of function with respect to complex formation (Morreale and Walden 2016). RING E3s containing both RING and U-box type domains can function as monomeric or homodimeric enzymes. Some RING E3s may also function as heterodimers or as multi-subunit complexes such as the multi-subunit Cullin RING enzymes, which are composed form RING-boxes, adaptors, and variations of substrate receptors.

While RING E3s act in some capacity as adaptors to bring Ub-E2 and a specific substrate in a proximity that favours transfers of Ub to substrates directly from E2s, HECT (homologous to E6-AP carboxyl terminus) E3s first transiently receive Ub on an internal cysteine as a thioester intermediate prior to ligation of the Ub onto the substrate (Figure 1-8B). HECT E3s bind the Ub charged E2 at their N-terminal lobe while the catalytic activity is facilitated by a cysteine at their C-terminal lobe. The N- and C-terminal lobes are tethered by a flexible loop. The major families of HECT E3s are NEDD4s and HERCs (contain HECT domains and RLDs) although several other HECT E3s are known but less characterized.

Recently the RBR E3 class has been characterized to share common features with both RING and HECT E3s (Spratt *et al.* 2014). RBRs (RING-inBetweenRING(IBR)-RING) contain the three RING1, IBR, and RING2 domains of which RING1 interacts with Ub:E2, RING2 is required for catalytic activity (houses the catalytic cysteine), and IBR augments the activity of RING1/2. In addition to RING domains, RBRs may include other functional domains which diversify them. Like HECT E3s, RBRs also ubiquitinate targets in a two-step manner (Figure 1-4) by accepting Ub from E2s first before passing it to targets.

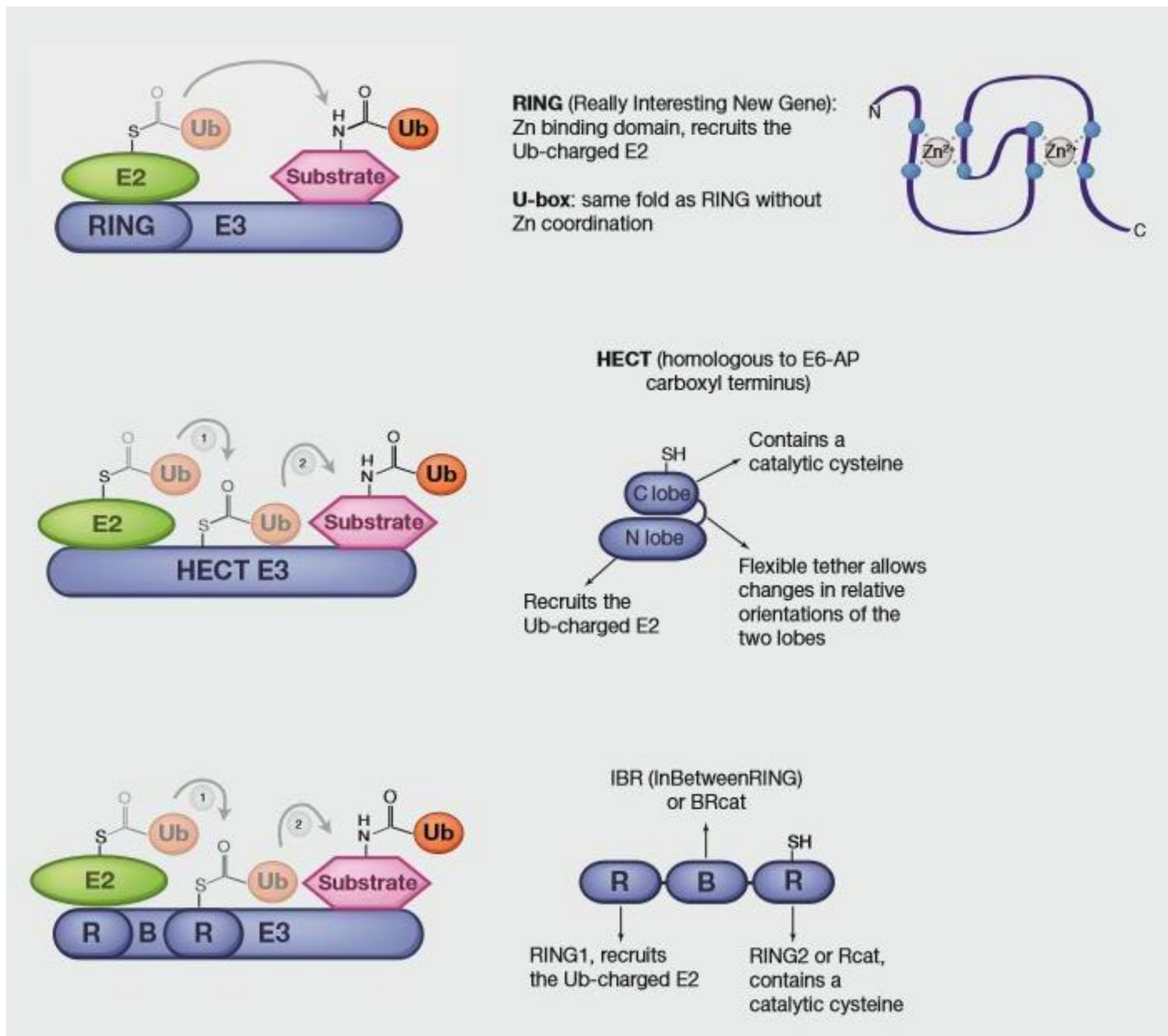



**Figure 1-4 - Types of E3 Ubiquitin-Ligases.** Classification of E3 Ub ligases are shown highlighting the mechanism of Ub transfer and their characterizing domains. **RING E3s** include RING and U-box E3s which directly transfer Ub to substrates **HECT E3s** become autoubiquitinated initially before transferring the Ub to substrate **RBR E3s** have RING domains but share Ub transfer mechanism with HECTs. Modified from Morreale and Walden (2016).

## 1.7 E2 Ubiquitin-Conjugating Enzymes

Ubiquitin-conjugated E2s have been the focus of important structural and mechanistic assays as (E2-Ub):E3 complexes play a role in directing the ubiquitination architecture. Under in vitro conditions, most E2s can directly (without an E3) transfer Ub to free lysines/cysteines of proteins

in environments that favor the chemical reaction (Stewart *et al.* 2016). However, E2s generally depend on an E3 ubiquitin-ligase to provide substrate and Ub linkage specificity. This is due to low intrinsic activity of E2s, a property that is enhanced by many fold in complex with an E3. Structural analyses of Ub-E2 report that these conjugates shift between an open state in which the C-terminally anchored Ub is free moving on a short tether, and a closed state where Ub makes surface contact with helix 2 of E2s (Stewart *et al.* 2016). Structural approaches using NMR and crystallography have identified the surface comprising of helix 1 and surrounding loops (4/7) (Figure 1-5) as the canonical site where E2s bind HECT/RING E3s which is also partly shared with the region binding E1s (Stewart *et al.* 2016). In association with RING E3s, E2s favor the closed conformation with ubiquitin (Pruneda *et al.* 2012) on helix 2 which orients the Ub molecule in such special spatial conformation that favours a nucleophilic attack for the transfer of Ub (Soss *et al.* 2013). In this manner, although E2s are mostly found in a Ub-bound state in cells, RING/HECT E3s are the limiting factors in the pathways of their associated ubiquitination. Interestingly, it is known that the E3 interaction site on E2 helix α1 is 15 Å away from the crossover α2 helix where ubiquitin makes contact with E2 in the closed conformation. Further structural analyses have identified that conserved K/R/N residues in RING E3s (RING + Ubox) hydrogen bond with the backbone-carbonyl groups of E2s and Ub, and allosterically induce the conformational effect necessary for the E2-Ub closed conformation (Pruneda *et al.* 2012). HECT/RBR-E3s on the hand do not promote the E2-Ub closed conformations and crystal structures of the E3:E2-Ub complexes show Ub  in the open state. Interestingly, E2s readily undergo transthioesterification (Figure 1-8B, initial step) in the open state and can transfer ubiquitin to cysteines of E3s (Kamadurai *et al.* 2009, Lechtenberg *et al.* 2016).

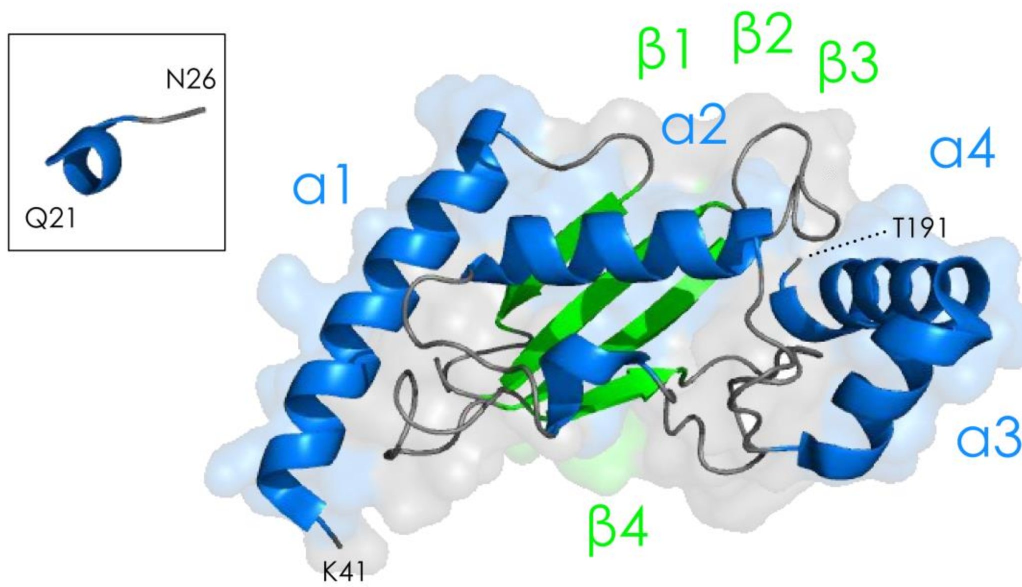



**Figure 1-5 - Structure of E2 UBC Core.** Cartoon depiction of E2E1 (UbcH6) structure using accession number 3BZH from the Protein Data Bank (PDB). Image rendered by PyMol software (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.) showing the UBC core (41-191) and a short segment of the N-t extension (21-26). The secondary structures shown are α-helices (blue) and β-sheet (green) connected by loops (gray). Surfaces are depicted as transparent background.

### 1.7.1 E2 Classification: A Tail of Tails

All E2s share a single and highly conserved UBC (ubiquitin-conjugating) domain at their core which is sufficient for catalysis. About forty E2s have been identified so far based on sequence similarity of the UBC domain in humans. Varying extensions (tails) of the UBC N- or C-termini were found in E2s and used to classify the E2s into 4 distinct classes (van Wijk and Timmers 2010). Unlike Class IV E2s, which are extended at both ends, Class I members lack tails and consist only of UBC cores. Class II and III E2s, however, are extended at C- and N-termini, respectively (Figure 1-6). In addition to varying extensions, there are insertions in the UBC domains of some E2s, however this property does not currently contribute to their classifications scheme.

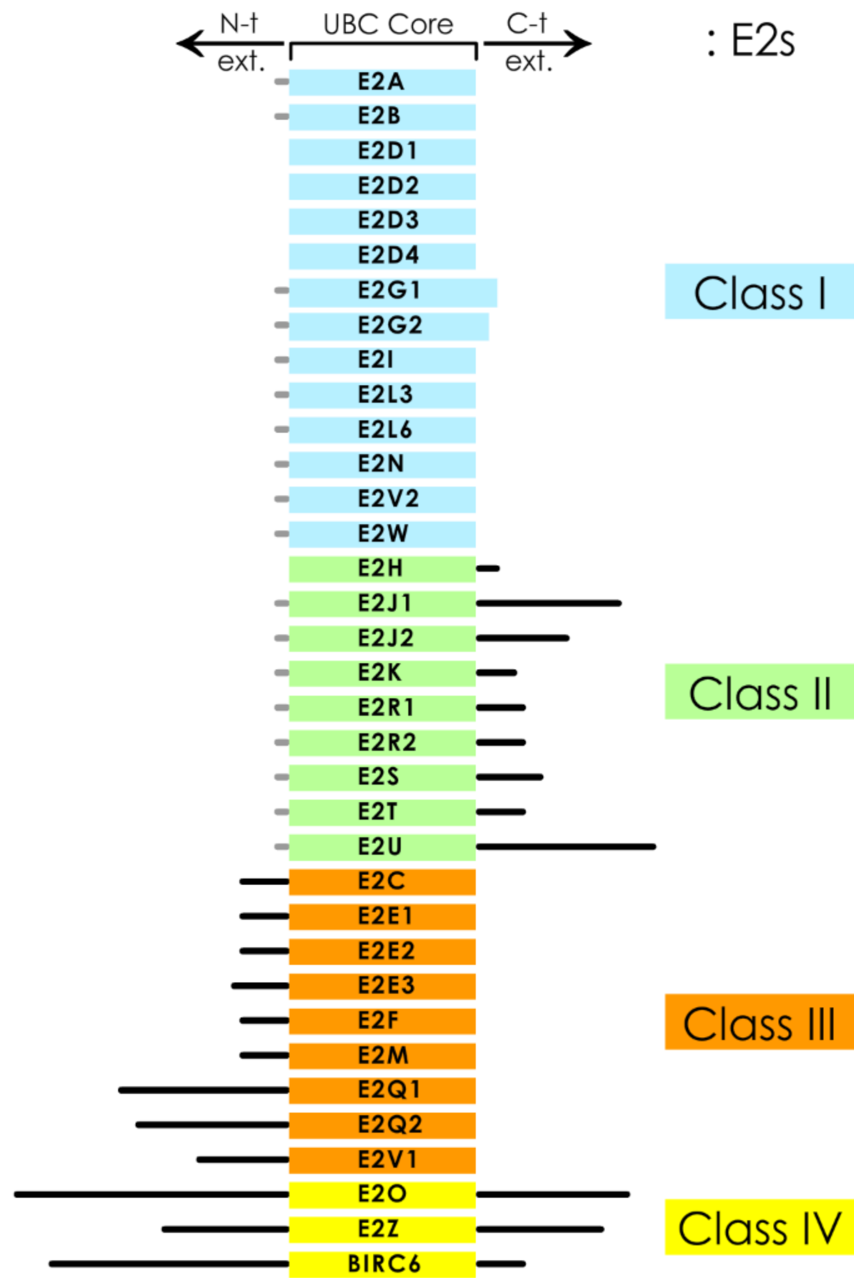



**Figure 1-6 – Ubiquitin-Conjugating Enzymes.** E2 enzymes are shown as categorized by the presence of N- or C-terminal extensions to their conserved ubiquitin-conjugating (UBC) cores.

### 1.7.2 Mechanism of E2 Conjugation by UBA1

E2 ubiquitin-conjugating enzymes require interaction with E1 Ub-activating enzymes for conjugation with ubiquitin. E2s bind dual Ub-loaded UBA1 with nanomolar affinity (Haas *et al.* 1988) and accept Ub from the catalytic cysteine [donor] of UBA1 to a catalytic cysteine residue within the UBC domain of their own [acceptor] which is shared across all families of E2 enzymes (Figure 1-7). The resulting trans-thioesterification transfer forms a covalent bond between the sulfhydryl group of the cysteine [E2] and the C-terminus of ubiquitin, and the E2-Ub complex dissociates from UBA1.

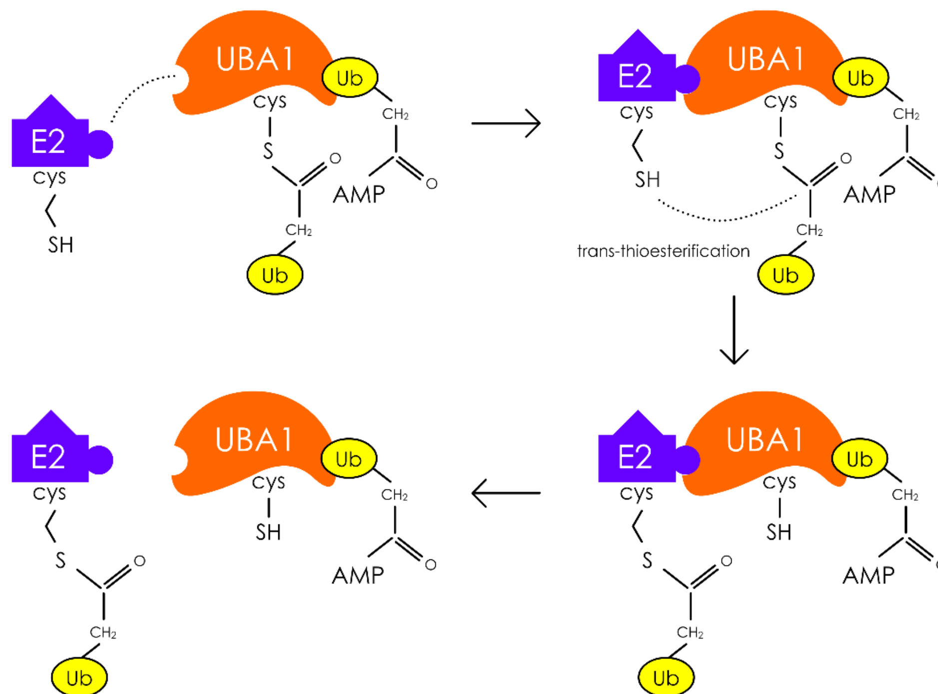



**Figure 1-7 - Mechanism of Ub-E2 Conjugation mediated by UBA1.** Dual Ub-preloaded UBA1 passes its thioester-bound Ub to the catalytic cysteine of E2 via a trans-thioesterification reaction.

### 1.7.3 E2 Functional Roles and Specificities

E2s mainly catalyze trans-thioesterification (transferring Ub from their own cysteine thioester linkage to the same linkage type on another protein) or aminolysis (transfer Ub to lysine or methionine amine groups) (Figure 1-8); however, E2s in some cases do not conform to this classic functional characterization. For example, E2J2 has been reported to preferentially transfer Ub to a hydroxyl group of ERAD's serine/threonine residues (oxyesterification) in association with mK3 E3 (a type III ER membrane protein kinase encoded by murine γ herpesvirus 68) even in the presence of free lysines on the substrate (Wang *et al.* 2007, Wang *et al.* 2009). E2W, as another instance, although usually pairs with target-lysine associated E3s, almost exclusively attaches Ub to the (α)amine group of the target's N-terminus. Contrary to E2W's method of function, E2s such as E2D3 function by only ubiquitinating lysine residues but not α-amines of the target protein.

Since HECT E3s perform the last catalytic step in ubiquitination of substrate, product determination by E2s are possible by those which partner with RING E3s. Such E2s (RING-associated) can be categorized to those which are dedicated to monoubiquitinating a target (priming E2s) and those E2s which polyubiquitinate (chain-builder E2s); however, some E2s are known to work in either mode.

E2 plays an important role in determining the mode of ubiquitin chain assembly. Some E2s preferentially favor the formation or elongation of a specific ubiquitin-chain linkage with respect to the acceptor residues within ubiquitin. For example, E2s that regulate cell-cycle progression factors (e.g. cyclins) in complex with APC/C generally form K11-linked ubiquitin chains

(Williamson *et al.* 2009). E2N and E2K, on the other hand, are K63- and K48-specific Ub-conjugating enzymes, respectively. Insights from structural studies have revealed that the sequences in the regions surrounding the active site or in the vicinity of UBC domains (or at times insertions) provide E2s with contacts that interact with specific residues or motifs on substrate or Ub itself which direct the Ub transfer to a specific lysine in the interacting region (Stewart *et al.* 2016). Depending on the coordinated E3s, E2s may exhibit preference or strictly be committed to a specific type of E3 (Stewart *et al.* 2016). E2s such as E2L3 strictly behave as trans-thiolation agents therefore they always pair with HECT or RBR E3s.

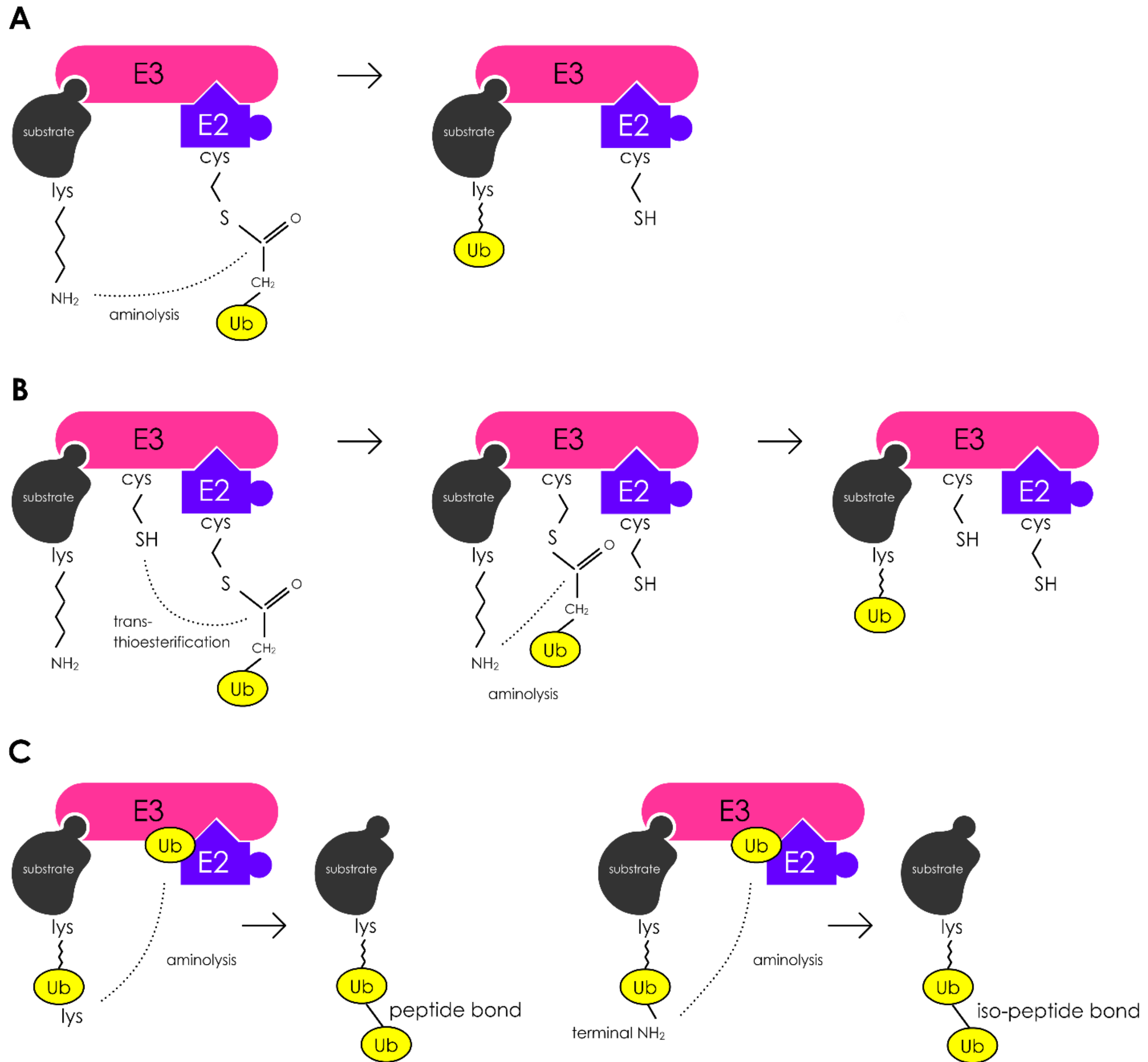



**Figure 1-8 - Mechanisms of E2-Mediated Ubiquitination. (A)** Ub-E2:E3:substrate complex for RING-type E3. A second incoming Ub attaching at the N-terminus of the first moiety results in an iso-peptide bond where as any internal lysines (63/48/33/29/27/11/6 of the first Ub will result in formation of a peptide bond. **(B)** HECT- or RBR-type E3 transiently accepts Ub on a cysteine. A second reaction passes the Ub to the substrate.

### 1.7.4 E2E Sub-Family of Ubiquitin-Conjugating Enzymes

The three members of the E2E sub-family (Class III) share similar functional and structural characteristics and have been linked to several pathways and diseases.

Some E2E members have been indicated to participate in ubiquitin pathways involving NEDD4 E3 ligases which link them to membrane/cross-membrane cellular functions, cell immunity, proliferative signalling, and other pathways. In *Xenopus laevis* oocytes, E2E3 has been shown to interact with NEDD4-2 and regulate the activity of epithelial $Na^+$ channel (ENaC) proteins which control the homeostasis of $Na^+$ by transportation across the plasma membrane and contribute to the regulation of blood pressure (Debonneville and Staub 2004). E2E3 has also been shown to regulate the localization and activity of anti-oxidant transcription factors involved in the expression of proteins that maintain redox homeostasis (Plafker and Plafker 2015). There is growing evidence that associates E2E2 as one of the susceptibility genes for inherited [type II] *diabetes mellitus* most prevalent in the east Asian populations (Yamauchi *et al.* 2010). Other studies have found variants of the E2E2 gene (*UbE2E2*) to be related to gestational diabetes (Kim *et al.* 2013). Furthermore, both E2E2 and E2E3 have been identified as interactors with endoplasmic reticulum (ER)-bound E3 ligases that play roles in autophagy and mitophagy (Ambivero *et al.* 2014), and indicated as components of the ubiquitin pathway contributing to Parkinson's disease (Ramsey and Giasson 2010).

NEDD4 has been suggested to be involved in co-regulating the ubiquitination of PKCθ/PLCγ1, a process that contributes to the maintenance of T-cell anergy (unresponsiveness due to lack of additional stimuli). Furthermore NEDD4 has been identified as a requirement for proper development of neuromuscular junction in mice, and for the downregulation of insulin-like growth factor R1 (IGF-1R) (Cao *et al.* 2008) which are associated with oncogenesis (Werner and Bruchim 2009). NEDD4 can also be recruited by Ebola (Harty *et al.* 2000), Epstein-Barr (Ikeda *et al.* 2000), and Rabies viruses through WW motifs and plays a major role in their release by

vesicularization (budding) (Malakhova and Zhang 2008). E2E1 is one of the few specific E2s that have been shown to interact with and transfer Ub to NEDD4 (Anan *et al.* 1998) and the disruption of NEDD4:E2E1 interaction by the ubiquitin-like molecule ISG15 has been shown to enhance anti-viral response (Malakhova and Zhang 2008). Therefore similarly, E2E1 can potentially regulate other NEDD4 pathways reviewed above. E2E1 has also been demonstrated (*in vitro)* to participate in NEDD4-1-mediated polyubiquitination (Sugeno *et al.* 2014) while mice studies show that PTEN/PI3K pathways control neural development by regulating the expression of NEDD4-1 via mTOR1C (Hsia *et al.* 2014), suggesting more pathways in which E2E1 may be involved.

Other than NEDD4-related pathways, E2E1 has been shown to ubiquitinate DNA-binding protein ataxin-1, which in mutant form contributes to the neurodegenerative disorder Spinocerebellar ataxia type 1 (SCA1) (Hong *et al.* 2008). Ubiquitination of ataxin-1 by E2E1 has been further linked to its degradation through the proteasomal pathway. Recently, E2E1 was discovered as a critical component of the PRC1 complex which regulates gene silencing by ubiquitinating histone H2A (featured in Chapter 3) (Wheaton *et al.* 2017).

While many studies focus on determining the network of proteins that involve E2s, some highlight the intra-molecular biochemistry which affect their interactions or rate of reactions. For instance, the N-terminal extensions of the E2E sub-family have been indicated in recent studies to conduct regulatory functions for their UBC domains. In E2E1, in addition to serving as a docking site for a ubiquitin-specific protease (Sarkari *et al.* 2013), the N-terminal extension has been shown to limit Ub chain assembly through auto-inhibition (Schumacher *et al.* 2013). Other studies

have shown that CHIP E3 ligase binds an SPA motif of a number of E2 enzymes including all members of the E2E sub-family (Soss *et al.* 2011); It was further shown that while E2Ds polyubiquitinate CHIP, all E2Es only add a single Ub to CHIP however to varying levels.

Considering the published literature concerning E2Es, the roles of E2E1 have been far more studied and understood than the other two members E2E2 and E2E3. Therefore, further research is needed to understand the roles and functions of these closely related enzymes.

# 1.8 Deubiquitinating Enzymes (DUBs)

## 1.8.1 DUB Classification

Similar to other PTMs, the catalytic processes that modify targets by adding ubiquitin molecules can be reversed. In ubiquitin systems, deubiquitinating enzymes (DUBs) are responsible for removing Ub from the ubiquitinated proteins. DUBs are essential to cells as they are regulators of major cellular pathways. DUBs are classified (Figure 1-9) into the five thiol-(cysteine) families according to their protease domains,: Machado-Josephin Domain (MJDs), Ubiquitin-Specific Proteases (USPs) [largest group], Ubiquitin C-Terminal Hydrolase (UCHs), Ovarian Tumour (OTUs), and the recently identified Motif Interacting with Ub-containing Novel DUB family (MINDYs) (Abdul Rehman *et al.* 2016) (Figure 1-10). The other family of DUBs are zinc-metalloproteases which contain JAB1/MPN/MOV34 (JAMM) domains (Lee *et al.* 2011, Pfoh *et al.* 2015).

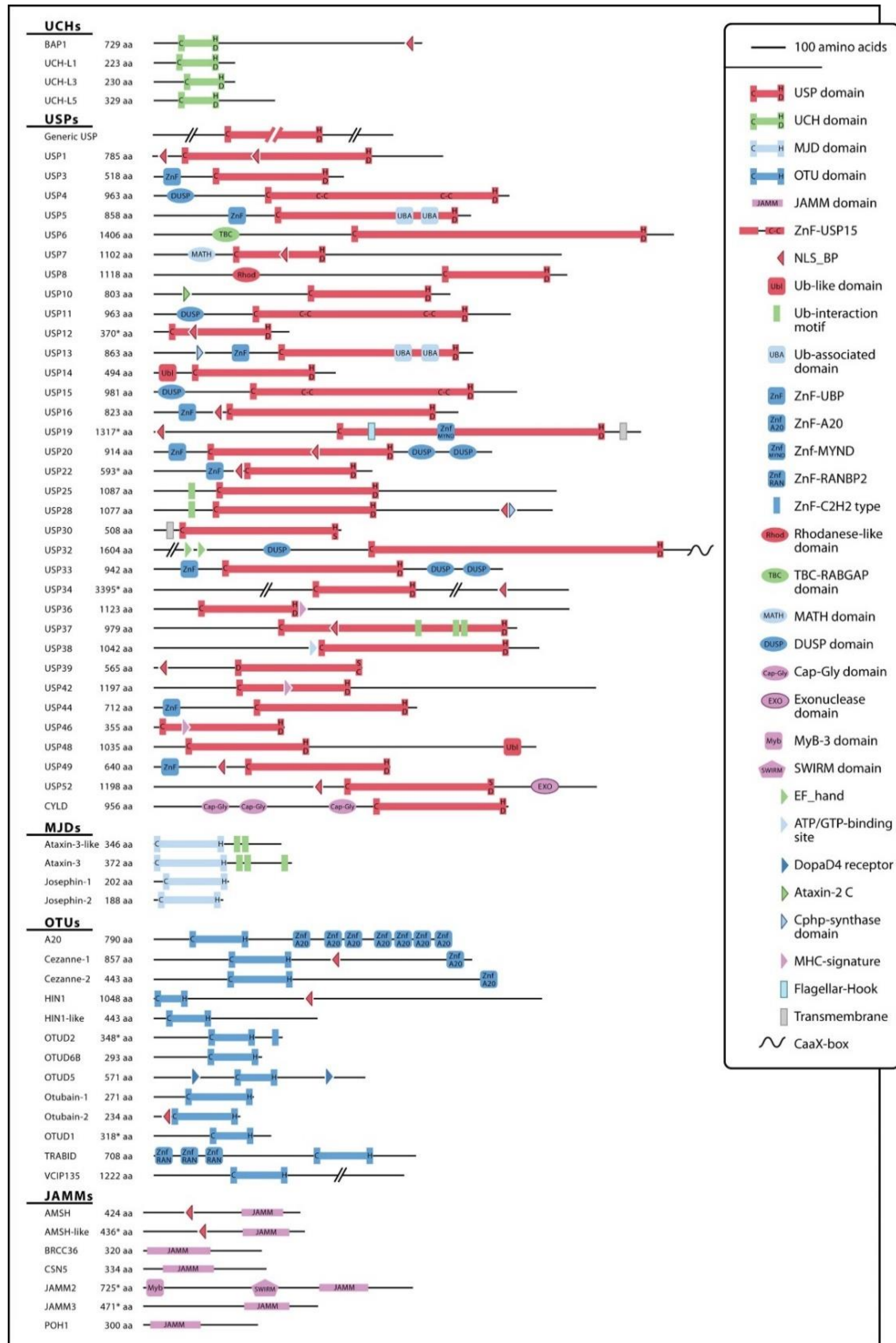



**Figure 1-9 - DUB Classifications and Functional Domains.** Schematics of deubiquitinating (DUB) enzymes in established families (UCH, USP, MJD, OUT, JAMM) are shown. The legend on the right lists characterized domains and features common to DUBs or other proteins. USPs constitute the largest family of DUBs. Refer to **Figure 1-10** for the new MINDY family of DUBs. Adapted from Nijman *et al.* (2005).

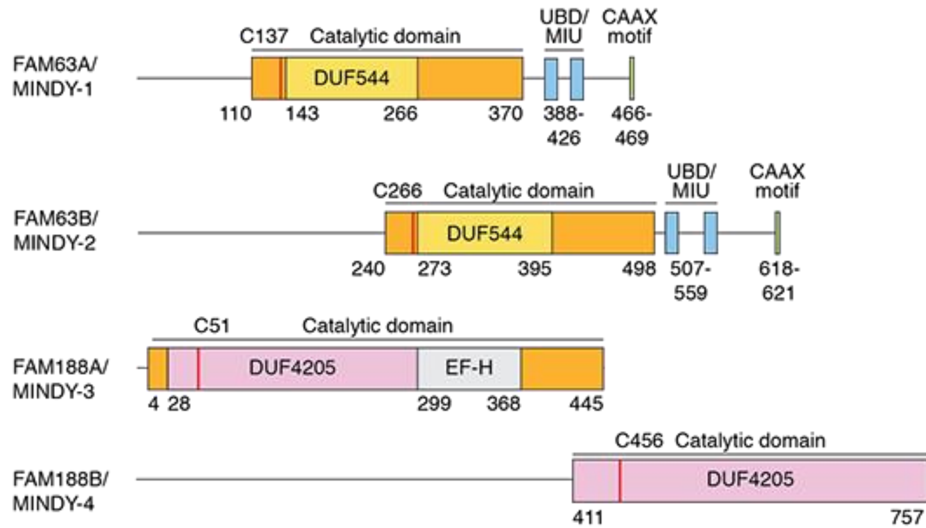



**Figure 1-10 – The Recently Identified MINDY Family of DUBs.** The enzymes shown are characterized as a new family of DUBs with share a characteristics catalytic domain which can include various additional domains or motifs. Adapted from (Abdul Rehman *et al.* 2016).

## 1.8.2 DUBs Regulate Ubiquitin Pathways

Acting as ubiquitin proteases, DUBs generally recognize and cleave Ub from their substrates. DUBs regulate cellular processes by assuming different roles, such as attenuating the activity of E3s through reversal of Ub conjugation, producing mature Ub by processing Ub-precursors, editing Ub chains, or maintaining the pool of free ubiquitin for those DUBs associated with the 26S proteasome (Nijman *et al.* 2005).

In the cascade of ubiquitination pathways, DUBs can attenuate the rate at which Ub-E2 conjugates can provide Ub to ubiquitinating complexes such as the case for Ataxin-3 (an MJD type DUB) and E2L3. In addition, DUBs such as USP7 can alter the stability of E2E1 as substrate (Sarkari *et al.* 2013).

DUBs can also counteract the activity of E3 ligases. For example, USP7/10 can stabilize p53 by regulating the levels of the E3 ligase Hdm2 which under normal physiological conditions keeps

p53 polyubiquitinated and at low levels. Inhibition or downregulation of USP7 through signalling can therefore indirectly increase p53 levels to the threshold required for its essential cellular activities after important events such as DNA damage responses (Sheng *et al.* 2006, Sarkari *et al.* 2010). DUBs can also interact with and deubiquitinate autoubiquitinated E3s. USP15 and USP7, for instance, can also affect the p53 stabilization pathway by deubiquitinating self-ubiquitinated Hdm2 therefor stabilizing the destabiliser of p53.

Several DUBs are associated with the 26S proteasomal complex. POH1/RPN1, USP14, and UCH37 are all JAMMs which assist in removal and recycling of ubiquitin from marked proteins which is required before proteins are unfolded and degraded in the proteasome (Hussain *et al.* 2009). The activity of USP14, however, has been shown to selectively stabilize some substrates such as cyclin B (Lee *et al.* 2016). Furthermore, Ub removal by this enzyme is not always limited to cleaving Ub in single units as USP14 can remove Ub in short (2-3 linkers) chain segments or entire blocks.

Some DUBs may not show specificity to substrate itself, rather a preference to the type of linkages in their polyubiquitin chains; USP14, UCH37, and RPN11 are examples of DUBS associated with the 26S proteasome in humans which behave in this manner. Other DUBs such as USP7 act on specific targets which are recognized through additional domains such as TRAF (Tumour necrosis factor Receptor Associated Factor) (Saridakis *et al.* 2005, Sheng *et al.* 2006, Sarkari *et al.* 2010).

### 1.8.3 Ubiquitin-Specific Protease 7 (USP7): One DUB, Many Roles

USP7 was initially characterized as herpes-virus associated ubiquitin-specific protease (HAUSP) through its discovery via its interaction with the ICP0 protein from the herpes simplex virus (Meredith *et al.* 1994). USP7 has since been established as an important regulatory enzyme as the list of its interacting partners are expanding which include a number of cancer-related viral and cellular proteins:

USP7 is known to interact with EBNA1 (Holowaty *et al.* 2003) and vIRF1 (Chavoshi *et al.* 2016) from Epstein-Barr and Kaposi sarcoma herpesviruses, respectively, which through competition inhibit USP7's ability to regulate its cellular targets. One of the most important USP7 functions is regulating the turnover of the tumour suppressor protein p53 either directly, or indirectly via regulation of the human double minute 2 (HDM2) ubiquitin-ligase that suppresses p53 levels in mammalian cells under physiologically normal conditions.

USP7 acts on diverse substrate proteins in the cell. For example, USP7 targets Retinoblastoma (Rb) (Bhattacharya and Ghosh 2014) which is a protein that inhibits the S-phase of the cell cycle by inactivating the transcription of S-phase related cyclins. In other studies USP7 has been shown to interact with and downregulate transcription factors such as FOXO4 in response to oxidative stress (van der Horst *et al.* 2006). USP7 also plays a role in the localization of the oncogene PTEN (phosphatase and tensin homologue) which by means of ubiquitination/deubiquitination performs dual functions in the nucleus versus the cytosol (Trotman *et al.* 2007, Song *et al.* 2008).

Recently USP7 has been implicated to maintain required SUMOylated proteins at sites of DNA replication. Inhibition of the interaction of USP7 with these proteins at replication sites has been

shown to cause accumulation of ubiquitin on these targets which causes their removal from the replisomes which disrupts their required interaction with nascent DNA (Lecona *et al.* 2016). Other studies have indicated that high levels of USP7 in the nucleus of non-small cell lung carcinomas are associated with a high level of Ki-67 antigen, a major marker of cell proliferation in these tumours (Zhang *et al.* 2016). USP7 has been established as an essential cellular protein in mouse models as germline knockouts show lethality in developing embryos. Moreover, deletions or mutations have shown to result in developmental malfunction in the nervous system (Kon *et al.* 2011). Research on medulloblastoma models indicate the role of USP7 in proliferation and metastasis of tumour cells. These studies show that while overexpression of USP7 increases migration of medulloblastomas, knockdown of USP7 inhibits the ability of these cells to grow and metastasize (Zhan *et al.* 2017).

The involvement and roles of USP7 in important pathways and malignancies have established this protease as a therapeutically important target in cancer, DDR (DNA damage response), epigenetics, and viral/immune responses.

#### 1.8.3.1 USP7 Structure and Mechanism of Function

USP7 is a single polypeptide of 1102 amino acid residues (~128 kDa) constituting its TRAF (TNF receptor-associated factor) domain located in the proximity of the N-terminus, catalytic domain, and 5 smaller UBL domains stretching the C-terminal end connected by short linker sequences (Figure 1-11) (Pfoh *et al.* 2015a, Pfoh *et al.* 2015b).

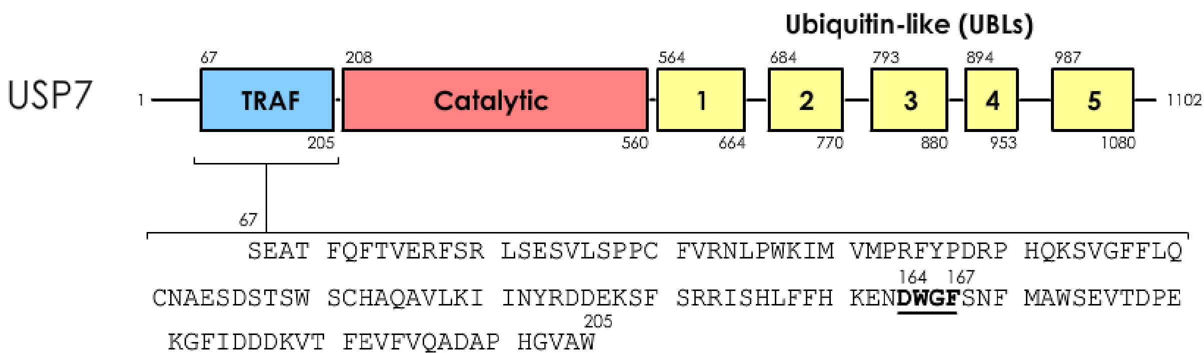



**Figure 1-11 – The Ubiquitin-Specific Protease 7 Protein Domains.** USP7 domains are shown to scale with flanking numbers indicating amino acid residue regions. The amino acid sequences for the TRAF (N-terminal) domain is demonstrated with key binding residues (discussed in Chapter 2) bolded and underlined.

The USP7 UBL domains, unlike conventional UBLs, shared little sequential similarity to Ub or each other. Generally, the function of UBLs may vary within a protein or from protein to protein. In USP7, UBL12 are important in substrate binding whereas UBL45 in addition to the C-terminus aid in autoactivation.

Although the catalytic domain of USP7 can perform independently, the full-length enzyme is more than a 100-fold active (Fernández-Montalván *et al.* 2007). The proteolytic activity of USP7 is centered around coordination of three key residues in the catalytic domain (CD). During this process, cysteine 223 (C223) acts as a nucleophile and attacks the peptide bond between the C-terminus of ubiquitin and the lysine of a substrate. The two other key residues, aspartic acid 481 (D481) and histidine 464 (H464) which are 10 Å apart from C223 during inactivity, are required for proper deprotonation of C223 prior to this reaction (Hu *et al.* 2002). Collective insights from partial structures have proposed a full-length model of USP7 (Pfoh *et al.* 2015). Crystal structures identify an allosteric self-activating mechanism of USP7 in which UBL 45 bind a groove on CD in proximity to Ub binding site and trigger conformational changes on the CD, which results in

activation of UPS7 by means of facilitating contact between the very C-terminal tail of USP7 residues 1098 and 1100 and the residues from the catalytic site C223, D481, and H646 (Faesen *et al.* 2011, Pfoh *et al.* 2015, Kim and Sixma 2017). In the absence of a whole USP7 structure, available models may underestimate spatial hindrance with respect to the required stretching/looping of USP7 and the degree of flexibility between the UBL domains are not fully understood. However, UBL3 has appeared in two distinct orientations in crystal structures (with respect to UBL1 and UBL2) and residue H792 at the junction of UBL23 has been identified to act as a hinge (Pfoh *et al.* 2015). Due to changes in the hydrogen bond network centred around this key hinge residue and other residues on UBL3 and UBL1, UBL3 may pivot at a $35^{o}$ angle bringing UBL345 to closer proximity of the CD (Figure 1-12). Furthermore a second potential hinge has been proposed in the junction of UBL45 which could further account for the necessary contacts between the CD and the C-terminal residues of UBL5 (Pfoh *et al.* 2015).

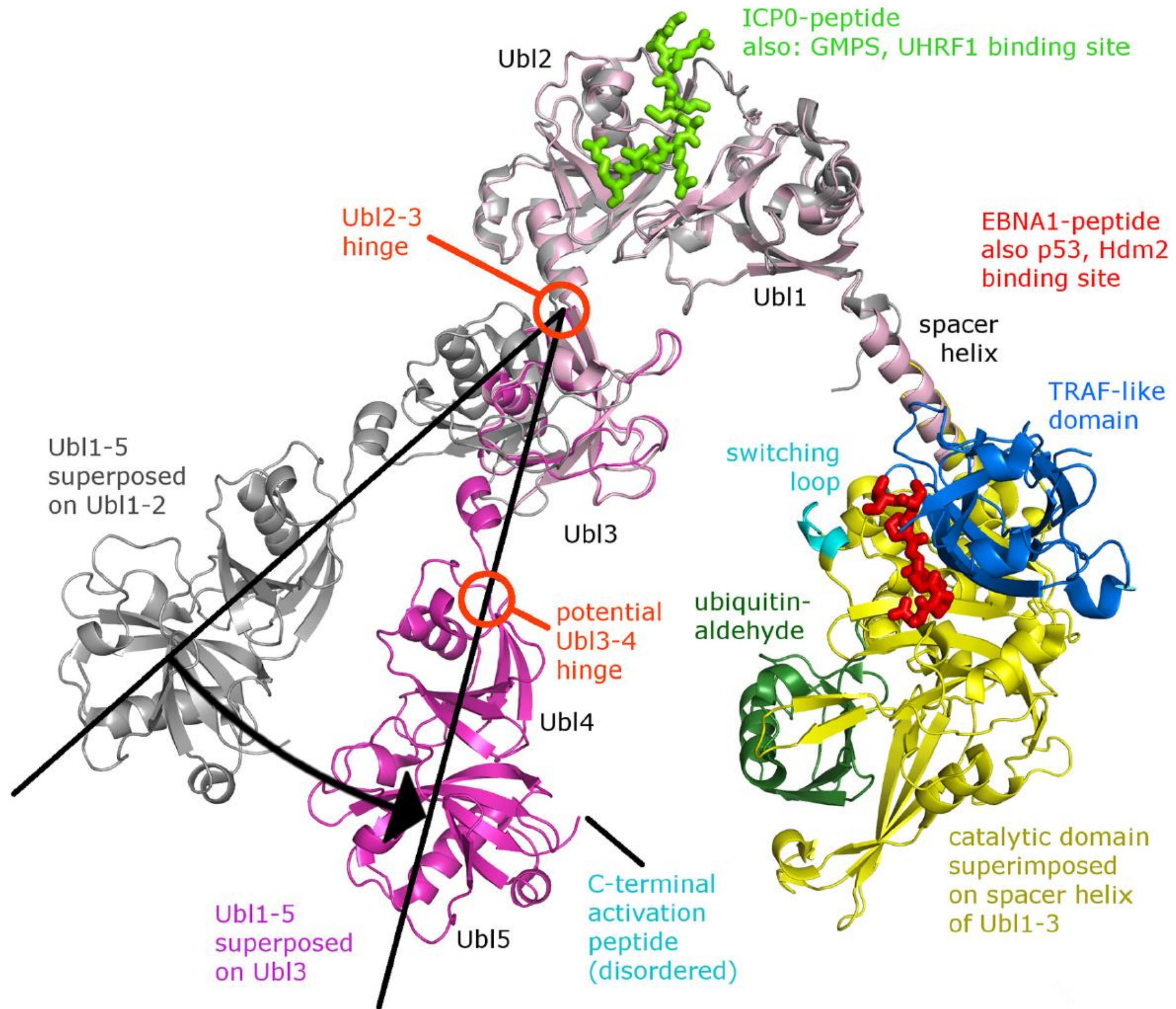



**Figure 1-12 - A Full-Length Model of USP7.** A proposed full-length model of UPS7 showing C-terminal UBLs (4/5) looping to contact the catalytic domain (CD) for activation. The model and rendered image, adapted from (Pfoh *et al.* 2015), were constructed by super-positioning of Protein Data Bank (PDB) structures 2F1Z, 1NBF, 1YY6, 2YLM, 4WPH, and 4WPI.

#### 1.8.3.2 USP7's TRAF Domain has a Consensus Binding Site

The TRAF domain, which is not present in other USP family members, is required for nuclear localization of USP7 (Zapata *et al.* 2001). The TRAF domain in USP7 binds a number of its targets in a manner more unique to USP7 compared to other proteins with TRAF domains. A non-canonical consensus P/A/ExxS sequence motif was identified as a USP7 TRAF domain-binding

motif. This sequence motif was found in the USP7 substrate proteins including cellular target proteins p53, HDM2/HDMX, E2E1, and MCM-BP as well as viral target proteins vIRF1, and EBNA1 (Holowaty *et al.* 2003, Sheng *et al.* 2006, Sarkari *et al.* 2010, Jagannathan *et al.* 2014, Chavoshi *et al.* 2016). Importantly, the appearance of the E residue within this sequence motif (P/A/ExxS) has been predominantly reported to be associated with viral proteins with higher affinities than cellular proteins (Figure 1-13). This preferential binding of USP7 could result in a competitive inhibition of USP7 binding to its cellular target proteins by the viral proteins.

```
                      P/A/ExxS
E2E1 N-t  1 MSD-----DDSRASTSSSSSSSSN-----QQTEKETNTP-----KKKESKVSMSKNSKLLST 47
E2E2 N-t  1 **TEAQRV**-SPSTSGG**DGDQRESVQQEP*REQVQP-----K**EG*I*S--------- 47
E2E3 N-t  1 **SDRQRS**ESPSTSSG**DADQRDPAAPEP*EQEERKPSATQQ**NT*L*---------- 52
p53                     PGGS
Hdm2                    PSSS
HdmX                    AHSS
CHFR                    PSTS
MCM-BP                  PSTS
EBNA1                   EGPS
vIRF1                   EGPS
ORF45                   EGPS
```

**Figure 1-13 – The P/A/ExxS USP7 Binding Motif on E2E2 and E2E3 N-t Extensions.** E2 peptide sequences were obtained from NCBI protein database (accessions P51965 [E2E1], Q96LR5 [E2E2], Q969T4 [E2E3]) and aligned using ExPasy Multiple Protein Alignment Tool (**https://www.expasy.org**). Asterisk symbols (*) and dashes (-) indicate identical amino acid residues and gaps within the alignments, respectively.

## 1.9 Ubiquitin & The Histone Code

The human nucleosome is the smallest unit of DNA compactions and is comprised of an octamer formed from 2 copies of core histone proteins H2A, H2B, H3 and H4 and the DNA that wraps around them (Luger *et al.* 1997, Khorasanizadeh 2004). H1 proteins are not nucleosomal

histones, however, as nucleosomal linkers, they participate in higher-order packaging of DNA into chromatin. Post-transcriptional modifications of histones modulate the interaction of histones with both DNA and transcriptional machinery by remodeling the chromatin structure through dynamic changes (Geiman and Robertson 2002, Bannister and Kouzarides 2011). The transcriptional memory that progresses the development of cells and differentiation of their lineages, for example, arise from structural changes to chromatin (remodelling). PTMs act as molecular switches to change transcriptional activity between on and off states. Understanding of histone modification codes have been characterized based on PTM type and acceptor histone peptide residues; together these properties show an association theme with respect to the switch states (code). However, the histone code becomes more complicated as multiple PTMs occur on the same nucleosome (combination switches). Therefore, different combinatorial patterns of PTM at different residues of histones constitute the histone codes, which serve as the instructions for various nuclear functions including gene silencing, transcriptional regulation and chromatin remodelling.

### 1.9.1 Mono-Ubiquitination of H2A by Polycomb Group Proteins

Histone proteins are subjected to ubiquitination. Studies estimate that H2A, the first protein that was discovered to be modified by Ub, comprises up to 15% of total ubiquitinated proteins in humans and other vertebra.

Lysine (K)119-monoubiquitination of histone H2A by the E3 ligase Polycomb Repressive Complex 1 (PRC1) has emerged as an important PTM and is associated with long term maintenance of

transcriptional repression. It is estimated that about 5-15% of total H2A is found to be K119-ubiquitanted. Mammalian polycomb group proteins (PcGs) were initially discovered to be involved in H2A ubiquitination based on their *Drosophila melanogaster* counterparts (Lewis 1978) and are categorized into different sub-classes depending on the makeup of their constituents.

Two major polycomb group protein complexes were identified as PRC 1 and PRC2 which modify histones H2A and H3 respectively. Functional PRC1 is involved in X-chromosome inactivation during human female development (Fang *et al.* 2004) and regulation of Hox genes which are important in body plan and anterior-posterior development in *D. melanogaster* (Cao *et al.* 2005). PRC2 catalyzes trimethylation of H3 on lysine 27 (3meK27-H3) which is associated with initiation of gene repression and serves as a signal for recruiting PRC1. In addition, PRC2 has been shown to monomethylates H2B at K120 which competitively inhibits ubiquitination at that site (Kogure *et al.* 2013).

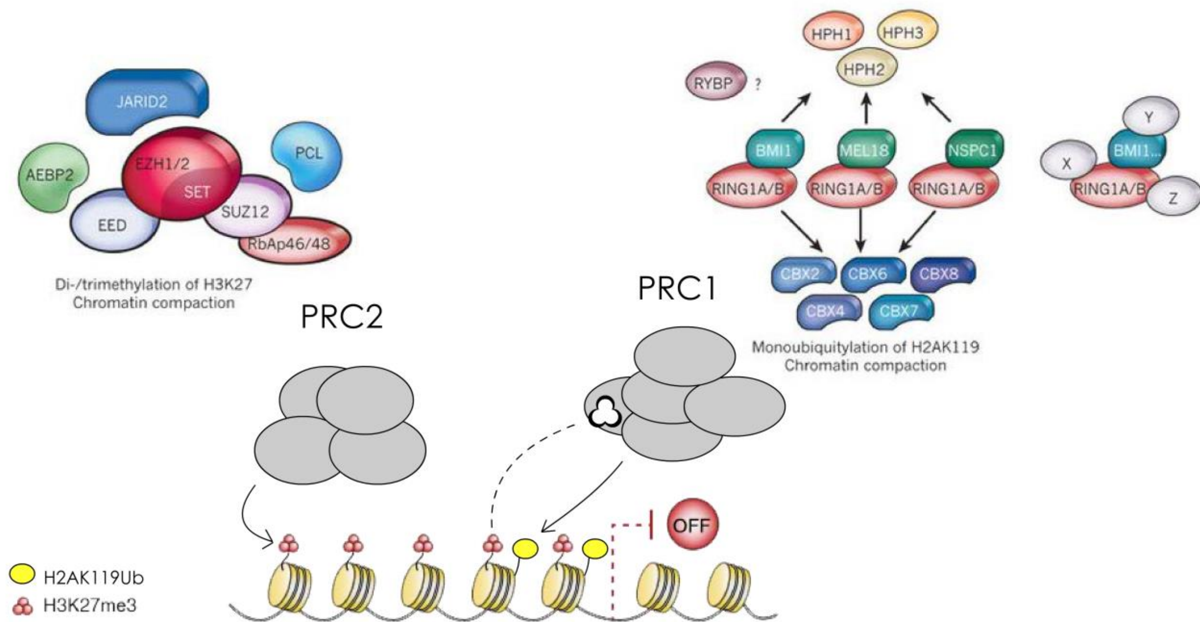



**Figure 1-14 – PRC1 and PRC2 Complexes and Maintenance of Gene Repression.** Variations of PRC1 and PRC2 components are shown. Trimethylation of H3K27 by PRC2 recruits PRC1 for ubiquitination of H2AK119. Modified from Margueron and Reinberg (2011) and Kim and Roberts (2016).

Both PRC1 and PRC2 are diversified into sub-classes (variants) by their respective and varying individual subunits, each with distinct function (Figure 1-14). PRC2 consists of Eed, E(z) (enhancer of zeste: Ezh1/2) which has the methyl-transferase activity, Su(z)12 (suppressor of zeste12), and RbAp46/48 subunits as core components which can recruit JARID2, AEBP2, and PCL as accessory proteins.

PRC1 on the other hand, consists of variations of Pc (Polycomb: Cbx2/4/6/8/7), Ph (polyhomeotic: Edr1/2), Psc (posterior sex comb: BMI1, Mel18), and Sce (sex comb extra: Ring1A, Ring1B). From these classes (PRC1), the Pc family bears chromodomains for binding methylated histones, Ph and Psc families consist of zinc finger motifs also found in Sce in specialized RING domains which play a role in ubiquitination of targets. BMI1 is required for recognition of nucleosomal H2A and has previously

been linked to cancers, morphological abnormalities, and mechanisms involved in stem cell maintenance.

## 1.10 Ub-Ligases and Ub-Conjugases as Targets of USP7

In the growing list of USP7 interacting partners, a number of targets have interestingly been identified as ubiquitin pathway enzymes (E2/E3) themselves such as HDM2, Ring1B, and E2E1. More interestingly, USP7 can directly regulate p53 (HDM2 target) (Li *et al.* 2002, Saridakis *et al.* 2005), and Ring1B and E2E1 function together in PRC1 complex (Wheaton *et al.* 2017). In such pathways, USP7 plays a more complicated regulatory role as it can be involved in multiple steps.

### 1.10.1 USP7 is a Regulator of Ring1B

Ubiquitinated RING1B is perhaps one of the best examples for diversity of ubiquitin signalling. RING1B that is self polyubiquitinated generates a mixture of K6/27/48 linkages which are required for its ubiquitin ligase activity (Ben-Saadon *et al.* 2006). However polyubiquitination of RING1B by E6-AP (another E3 ligase), which generates K48 ubiquitin chains exclusively, is associated with the destruction of RING1B through the proteasomal pathway (Zaaroor-Regev *et al.* 2010). USP7 has been identified as a DUB associated with RING1B and interestingly without discrimination of the type of RING1B polyubiquitin chains (self- versus E6-AP-assembled) (de Bie *et al.* 2010). These studies have identified that USP7 physically binds RING1B and other PcG proteins and that USP7 deubiquitinates RINGB both *in vitro* and *in vivo* which stabilizes RING1B levels (de Bie *et al.* 2010).

### 1.10.2 USP7 is a Regulator of E2E1

In E2E1, a ubiquitin-conjugating enzyme, the N-terminal extension contains an ASTS motif which was demonstrated by our group to recruit USP7 (Sarkari *et al.* 2013). We further showed that USP7 catalyzed attenuation of E2E1-mediated poly-ubiquitination and showed the dependence of this activity on the N-terminal extension in experiments comparing the core versus full-length E1E1. It was also shown that the wildtype $^{164}$DWGF$^{167}$ sequence on the TRAF domain of USP7 (Figure 1-11) is required for interacting with E2E1 as loss of activity was observed when this sequence was mutated. Moreover, our group demonstrated that USP7 regulates cellular levels of E2E1 (Sarkari *et al.* 2013).

### 1.10.3 USP7 Is a Potential Regulator of E2E2 and E2E3

#### 1.10.3.1 E2E2 and E2E3 Contain TRAF Binding Motif

In our investigation of other USP7-interacting proteins, we turned to the remaining two members of the E2E sub-family of Class III ubiquitin-conjugating enzymes, E2E2 and E2E3, as they also contained N-terminal domains of similar length and properties as E2E1. Additionally, PSTS sequences were identified in both proteins. The alignment of the E2E N-terminal domains and the comparison of P/A/ExxS motifs of several USP7 binding partners are shown in Figure 1-13.

#### 1.10.3.2 Full-Length E2E2 and E2E3 Proteins Bind USP7 TRAF

Pulldown data by Majda Mohammed (unpublished) provided preliminary evidence that USP7 can physically associate with E2E2 and E2E3. In these experiments full-length E2E2 and E2E3 were shown to bind the TRAF domain whereas the E2E2 or E2E3 Core domains did not show any binding.

#### 1.10.3.3 USP7 Binds E2E2 and E2E3 *in vivo*

To investigate any physical interaction between USP7 and E2E2 or E2E3, Ashurov (2014) performed reciprocal co-immunoprecipitations *in vivo*. In these experiments, lysates from HEK 293T cells which over-expressed Myc-USP7 and FLAG-E2E2 and FLAG-E2E3, were subjected to coimmunoprecipitation using Myc and FLAG antibodies separately. In each trial, recovered precipitates showed both Myc and FLAG signals when probed with their respective antibodies suggesting that USP7 interacts with both E2E2 and E2E3 *in vivo*. Negative-control trials including IgG precipitations or overexpression of single/empty vectors did not result in co-immuonoprecipitation of the two tagged proteins.

#### 1.10.3.4 Molecular Analysis of the USP7:E2E2 or E2E3 Interactions

X-ray crystallography was used to visualize the interaction between USP7 and the two ubiquitin-conjugating enzymes (E2s) at the molecular level (Ashurov 2014). For these crystallization trials, $^{9}$DDSPSTSGGS$^{18}$ (E2E2) and $^{10}$DESPSTSSGS$^{19}$ (E2E3) peptides were synthesized and separately co-crystalized with USP7 TRAF. The structures of TRAF:peptide complexes revealed that the peptide residues within the complexes were situated on the shallow groove at the TRAF surface where they encompass the β7 strand of TRAF and make contact with two other β-strands throughout their lengths. Furthermore, Mohammed (unpublished) measured the dissociation constant ($K_d$) of the binding between USP7 and  E2E2 or E2E3 in the 6.0-6.3 μM range.

# 1.11 Rationale

Previous work by Ashurov and Mohammed (reviewed in previous section) established E2E2 and E2E3 as interacting partners of USP7 both *in vitro* and *in vivo*. These consisted of E2E2 and E2E3 (FL and core) pulldowns with USP7 TRAF (WT), crystallization of E2E2 and E2E3 N-terminal tails with TRAF, determination of E2E2 or E2E3:USP7 dissociation constant, and reciprocal co-immunoprecipitation of E2E2 or E2E3 and USP7 in cells. These findings, served as basis for the continuation of the line of investigation related to the project presented in Chapter 2 which aimed to provide more evidence to further establish E2E2 and E2E3 as USP7 targets and to provide evidence of functional effects from USP7 on E2E2 and E2E3. In my investigations, we optimized and enhanced the pull-down data and performed new pull-down experiments using mutant TRAF and N-terminal E2E2 or E2E3 tails only to show the dependence of these interactions on WT TRAF and the E2E2 or E2E3 N-terminal tails.

Our group also identified an association between E2E1 and Ring1B:BMI1 through mass spectroscopy, linking this E2E enzyme to the PRC1 complex which monoubiquitinates histone H2A on K119 (Wheaton *et al.* 2017). Complementary to work by other authors, in a second project (Chapter 3), we performed a series of experiments to investigate any roles that E2E1, and also E2E2 and E2E3 due to structural properties and sequences, may play in H2A ubiquitination in association with PRC1 complex. We therefore performed *in vitro* co-immunoprecipitation assays to address whether the three E2E enzymes can directly bind Ring1B:BMI1. We further performed *in vitro* H2A ubiquitination assays to test the enzymatic ability of E2E1, E2E2, and E2E3

in complex with PRC1. Additional experiments using mutant E2E or Ring1B were designed to confirm any H2A observation would be as a result of catalytic activity from wildtype E2E and Ring1B. Furthermore, we silenced or overexpressed the expression of E2Es *in vivo* as to manipulate the hypothesized machinery for H2A ubiquitination and compared the levels of H2A K119 monoubiquitination with control samples in which E2E levels were unaltered. In these two projects (chapters) we investigated the relationship between USP7 and E2E enzymes, and between E2E enzymes and PRC1. In order to converge the findings of these two projects and link USP7 to K119 monoubiquitin of H2A, we performed an experiment to demonstrate whether silencing of USP7 can exert an effect on Ub-K119 H2A

# Chapter 2: Materials & Methods

## 2.1 Bacteria | Culture, Gene Expression, and Protein Purification

*E. coli* BL21 (DE3) cultures were used for gene expression and protein purification purposes in bacteria.

### 2.1.1 DNA Plasmid Constructs and Amplifications

Table 2-1 summarizes the constructs which were transformed in to bacteria for various experimentation.

Plasmid amplifications were performed using Miniprep Kit or Plasmid Maxi Kit (Qiagen) as per the manufacturer's instructions manual.

**Table 2-1 – DNA Plasmid Constructs for Bacterial Transformations.** Peptides were expressed in the pET28a and/or pET15b vectors which expressed a Glutathione-S-transferase (GST) or a 6xHistidine (His)-tag respectively.

| pET28a | | | pET15b | | |
|---|---|---|---|---|---|
| **Tag** | **Protein** | **Sequence** | **Tag** | **Protein** | **Sequence** |
| GST | E2E2 (N-terminal) | 1-47 | His | E2E1 | full-length |
| GST | E2E3 (N-terminal) | 1-52 | His | E2E2 | full-length |
| GST | USP7 (TRAF) | 54-205 | His | E2E2 (Core) | ΔN-terminal |
| GST | USP7 ($TRAF^{AAGF}$) | 62-205 | His | E2E3 | full-length |
| GST | -------- | -------- | His | E2E3 (Core) | ΔN-terminal |
| | | | His | USP7 (TRAF) | 1-205 |
| | | | His | USP7 (TRAF) | 54-205 |
| | | | His | Ub | full-length |

### 2.1.2 Preparation of Chemically-Competent Bacteria

A 1 mL sample from an overnight culture of bacteria (37 $^{o}$C, shaking at 200 RPM) in LB (Luria Broth) media (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) (BioShop) was transferred to 100 mL of LB media and grown to $OD_{590\ nm}$ of 0.375 (250 RPM). The culture was divided to two 50 mL volumes in pre-chilled vessels and incubated on ice for 10 minutes. The samples were centrifuged at 4 $^{o}$C for 7 min (1,600 ×g). Pelleted cells were cleared of supernatant, re-suspended in pre-chilled $CaCl_2$ (30 mM), and re-pelleted at 1,100 ×g for 5 min (step repeated after 30 min incubation on ice). Cells were resuspended in 2 mL of buffer and rapidly frozen in 100 µL aliquots (ethanol-dry ice bath) and stored at -80 $^{o}$C.

### 2.1.3 Gene Expression (Transformations)

For each transformation, 100 µL of chemically-competent bacteria were transformed with 2.0 µg of plasmid DNA (Table 2-1). DNA was mixed with bacteria that were briefly thawed then incubated at 4 $^{o}$C for 10 min. The mixture was heat-shocked for 1 min at 42 $^{o}$C and immediately returned to 4 $^{o}$C for 5 min. The transformed culture was then supplemented with 250 µL of LB (Luria Broth) media (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) (BioShop) and incubated at 37 $^{o}$C for 1 hour while shaking at 250 RPM. The mixture was then added to 5 mL of LB media (100 µg/mL ampicillin or 50 µg/mL kanamycin) and incubated overnight (37 $^{o}$C, 500 RPM). Freezer stocks were prepared using transformed bacterial cultures and glycerol (75%:25% v/v) and stored in -80 $^{o}$C.

### 2.1.4 Inducible Protein Expression

Starter cultures were prepared by inoculating 100 mL of LB media (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) (BioShop) (100 µg/mL ampicillin or 50 µg/mL kanamycin) with a small mass scraped from frozen transformed bacterial stocks (refer to 2.1.3). Starter cultures were incubated at 37 $^{o}$C overnight, transferred to 1 L of TB (Terrific Broth) media (12 g/L Tryptone, 24 g/L yeas extract, 9.4 g/L $K_2HPO_4$, 2.2 g/L $KH_2PO_4$) (BioShop) (+ antibiotics), grown to $OD_{600\ nm}$ 1.5, and induced with 1 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) (16 $^{o}$C, overnight). Cultures were harvested by centrifuging (7,500 ×g, 4 $^{o}$C, 30 min) and collected as dry pellets for immediate purification or stored in -80 $^{o}$C.

### 2.1.5 Harvest and Lysis

Cells (pre-thawed on ice where applicable) were fully suspended in 10 mL of Bacterial Lysis Buffer (500 mM NaCl, 20 mM Tris, 10% glycerol, 1 mM benzamidine, 0.5 mM PMSF, 10 mM βME, 0.01 % triton x-100, pH7.5) (+5 mM imidazole for histidine-nickel purifications) per 1 g of pellet on ice. The mixtures were sonicated on ice in 40 mL volume using ultrasonication (Branson Sonifier®) (½" output tip, 40% amplitude, 0.5 s pulses, 2.0 s gaps, 4 min total pulse time). Cell lysates were centrifuged (39,000 ×g, 4 $^{o}$C, 30 min) and separated from pelleted debris.

### 2.1.6 Histidine-Nickel Protein Purifications (His-Tag)

Cleared cell lysates were incubated with 10 mL of Nickel-Nitrilotriacetic Acid Resin (50% suspension stock) (Qiagen) in 100 mL affinity-chromatography columns. Columns were incubated on a rocker at 4 $^{o}$C for 1 hour. The columns were drained and washed with 400 mL of Nickel Wash Buffer (20 mM Tris, 500 mM NaCl, 0.5 mM PMSF, 10 mM βME, 1 mM benzamidine, 20 mM

imidazole, pH7.5). To elute purified proteins, Nickel Elution Buffer (20 mM Tris, 500 mM NaCl, 0.5 mM PMSF, 10 mM βME, 1 mM benzamidine, 500 mM imidazole, pH7.5) was used. Purified proteins were dialyzed with 2 L of buffer (50 mM Tris, 0.5 mM PMSF, 5mM βME, 5% glycerol, pH7.5) at 4 $^{o}$C (1 L for 4 hours, 1 L overnight). Refer to **Error! Reference source not found.** for samples of protein purifications.

### 2.1.7 GST-Glutathione Protein Purifications (GST-Tag)

GST constructs were purified using the same general procedure as described in section 2.1.6 except for the following corresponding resins and buffers: Glutathione-Sepharose Resin (GE Healthcare), GST Wash Buffer (20 mM Tris, 10mM βME, 0.5 mM PMSF, 1 mM benzamidine, 500 mM NaCl, 10% glycerol, pH7.5), GST Elution Buffer (50 mM Tris, 10mM βME, 500 mM NaCl, 10% glycerol, 30 mM Glutathione, pH7.5). Refer to **Error! Reference source not found.** for samples of protein purifications.

## 2.2 Mammalian Cells | Culture, Gene Expression, and Protein Purifications

U2OS [osteosarcoma], HEK 293T [human embryonic kidney], and HeLa [Henrietta Lacks] cell-lines were used for *in vivo* studies. FLAG (tag) purifications and FLAG pull-downs were performed with U2OS cells only.

### 2.2.1 DNA Plasmid and siRNA Constructs

Table 2-2 summarizes the DNA plasmid constructs used for expression of proteins in the mammalian cell-lines for *in vivo* experiments, FLAG purifications and FLAG-pulldowns.

**Table 2-2 – DNA Plasmid Constructs for Mammalian Transfections.** Peptides were expressed in the pCDNA3.1 or pCMV3F vectors.

| pCDNA3.1 | | | pCMV3F | | |
|---|---|---|---|---|---|
| **Tag** | **Protein** | **Sequence** | **Tag** | **Protein** | **Sequence** |
| -------- | BMI1 | full-length | FLAG | Ring1B | full-length |
| | | | FLAG | Ring1B I53A | full-length |
| | | | FLAG | E2E1 | full-length |
| | | | FLAG | E2E1 C131A | full-length |
| | | | FLAG | E2E3 | full-length |
| | | | FLAG | E2D2 | full-length |
| | | | FLAG | E2D2 C85A | full-length |

The siRNA oligonucleotides used in gene-silencing experiments were ordered from GenePharma Co. Ltd. (Shanghai) and are listed in Table 2-3.

**Table 2-3 – siRNA Oligonucleotide Sequences. The TT sequences were designated to produce overhangs for the 21-23 bp long (inclusive) oligonucleotides.**

| siRNA Oligonucleotide | 5' –Sequence– 3' |
|---|---|
| Negative Control | ACGUGACACGUUCGGAGAATT |
| USP7 | UUUGCCGCGGAAUAAUUUGGGTT |
| E2E1 (Oligo #1) | GACCAAGAGAUACGCUACA |
| E2E1 (Oligo #2) | GUGUAUUCUUUCUCGAUAUTT |
| E2E2 (Oligo #1) | CGCUGCUAAAUUGUCAACUTT |
| E2E2 (Oligo #2) | GGGUGUUCUUUCUUGACAUTT |
| E2E2 (Oligo #3) | CUUCCGAACAAGAAUCUAUTT |
| E2E3 (Oligo #1) | GAAGGAGCUAGCUGAAAUATT |
| E2E3 (Oligo #2) | CCCGCUUUGACUAUUUCAATT |
| E2E3 (Oligo #3) | GACCAAGAGAUACGCAACATT |

### 2.2.2 Cell Culture

Cells were incubated in a Thermo Scientific® Forma™ Steri-Cycle™ $CO_2$ Incubator (37 $^oC$, 5% $CO_2$). U2OS cells were grown in McCoy's media (Wisent #317-010-CL) (with L-glutamine, without Hepes) and HEK 293T cells were grown in DMEM media (Wisent #319-005-CL) (4.5g/L glucose + L-glutamine, sodium pyruvate); both media were supplemented with 10% FBS (fetal bovine serum) and gentamicin (10 µg/mL). Cells were washed with PBS (Wisent #311-010-CL) (without calcium and magnesium). Cells were treated with trypsin (Wisent) for detachment during passaging or harvest.

### 2.2.3 Gene Over-Expression (DNA Transfections)

Cells were washed and incubated with fresh media 1 hour prior to transfections. Cultures were transfected with plasmid DNA (Table 2-2) using PolyJet™ reagent (Signagen). Transfections were prepared as per manufacturer's standard protocol and incubated for 12 hours before aspiration and recovery with fresh media. Cultures were harvested 24-36 hours post-transfections.

### 2.2.4 Gene Silencing (siRNA Transfections)

Cells were washed and incubated with fresh media 1 hour prior to transfections. Cultures were transfected with siRNA oligonucleotide (20 nM, 1 round) (Table 2-3) using LipoJet™ reagent (Signagen). Transfections were performed as per manufacturer's standard protocol and incubated for 18 hours before aspiration and recovery with fresh media. Cultures were harvested 60 hours post-transfections.

### 2.2.5 Harvest

Cells were detached from plates using cell dissociation reagent (Wisent #325-542-EL) (0.05 % trypsin, 0.53 mM EDTA) and agitation of plates. Unanchored cells were collected with PBS and cells were pelleted by centrifugation (800 RPM, 4 min, room temperature).

### 2.2.6 Lysis

Cell pellets were re-suspended in either RIPA Buffer (50 mM Tris, 150 mM NaCl, 0.5% nonidet-P-40, pH 7.5) or for enzymatic assays in PBS (Wisent #311-010-CL) (without calcium and magnesium, 1mM DTT + protease inhibitor [Roche cOmplete™ tablets]). The suspensions were sonicated on ice using ultrasonication (Branson Sonifier®). Cell lysates were centrifuged (16,200 ×g, 4 $^{o}$C, 30 min) and separated from pelleted debris.

### 2.2.7 FLAG Affinity Protein Purifications (FLAG-Tag)

FLAG-tagged constructs were purified using M2-FLAG Affinity Resin (Sigma Aldrich). Purification columns were loaded with resins (100 µL) and cleared cell lysates (refer to 2.2.6) and incubated at 4 $^{o}$C while rotating. Columns were washed with pre-chilled PBS (Wisent #311-010-CL) (without calcium and magnesium, 1mM DTT + Roche cOmplete™ protease inhibitor tablets). Resin-bound proteins were eluted with competitor 3×FLAG peptide (100 ng/µL) (Sigma Aldrich) in TBS.

## 2.3 Electrophoresis and Western Blots

### 2.3.1 SDS-PAGE

For in vivo experiments, concentrations of cell lysates were measured by Bradford Reagent (standard curve). For loading, 5-10 µg of lysates were separated on 12% polyacrylamide gels.

For in vitro experiments, refer to figure captions or corresponding methods for protein masses or volumes loaded on gels.

### 2.3.2 Western Blots

For Western blotting, polyacrylamide gels were transferred to PVDF membranes (GE Healthcare) in Western Transfer Buffer (20% MeOH, 25 mM Tris, 190 mM glycine). Membranes were incubated in PBST (5% skim milk powder w/v) for blocking (1 hour) followed by primary and secondary antibody (Table 2-4) incubations (1 hour). PVDF membranes were washed with PBST between primary and secondary antibody incubations and prior to chemiluminescence treatment with ECL (GE Healthcare #RPN2232). Chemiluminescence signals were either developed on to film or captured directly from the membrane by Carestream Image Station 4000MM device (Kodak).

**Table 2-4 - List of Primary and Secondary Antibodies.**

| Target | Company | Product Number |
|---:|:---:|:---|
| 3mK4-H3 | Active Motif | 39159 |
| BMI1 (D42B3) | Cell Signaling | 5856S |
| E2E1 | Boston Biochem | A630 |
| E2E2 | Bethyl | A303-485A |
| E2E3 | Aviva Systems Biology | ARPP43070-P050 |
| E2E3 | Abcam | AB151255 |
| FLAG®-Tag | Sigma-Aldrich | F1804 |
| GAPDH [0411] | Santa Cruz | SC-47724 |
| GST | Novagen | 71097-3 |
| H2A | Cell Signalling | 2578 |
| His-Tag | Qiagen | 34660 |
| K119Ub-H2A | Cell Signaling | 8240 |
| p16 [H-156] | Santa Cruz | SC-759 |
| Ring1a | Cell Signaling | 2820S |
| Ring1B (D22F2) | Cell Signaling | 5694S |
| Ubiquitin | BioLegend | MMS-258R |
| USP7 | Bethyl | A300-033A |
| IgG Mouse | Jackson | 115-035-166 |
| IgG Rabbit | Jackson | 111-035-003 |

## 2.4 Pulldown Assays

### 2.4.1 GST-Pulldowns

GST-pulldowns were performed by co-incubating 500 µg of purified GST-tagged proteins [bait] with purified proteins of interest [prey] in columns preloaded with 100 µL of glutathione-sepharose resins (50% suspension stock) (GE Healthcare). Column volumes were adjusted to 1 mL with Pulldown Buffer (0.1 mM benzamidine, 0.05 mM PMSF, 5 % glycerol, 50 mM Tris pH 7.5, 100 mM NaCl, 5 mM DTT) and were set to rotate at 4$^{o}$ C for 1 hour. Columns were washed with Pulldown Buffer and eluted GST Elution Buffer (50 mM Tris, 10mM βME, 500 mM NaCl, 10% glycerol, 30 mM glutathione). Samples from the eluted proteins, column input, and last wash were subjected to SDS-PAGE and Western blotting (refer to section 2.3).

### 2.4.2 FLAG Pulldowns

FLAG®-tagged proteins (FLAG-RING1B) were expressed in and purified from U2OS cells as described in section 2.2.7 with the exception that for the purpose of these pulldowns, isolated proteins were not eluted from purification columns [bait]. To these resin:FLAG-RING1B preloaded columns, 500 µg of [prey] purified protein (E2Es or negative control) was added which were purified from bacteria as described in section 2.1. Column volumes were adjusted to 1 mL with buffer (100 mM NaCl, 50 mM Tris, 0.5 mM PMSF, 5mM βME, 5% glycerol, pH 7.5). Columns were incubated at 4 $^{o}$C while rotating for 1 hour for protein binding. Columns were washed with PBS (Wisent #311-010-CL) (without calcium and magnesium) and eluted with 3×FLAG® peptide (Sigma Aldrich). Elution samples were subjected to SDS-PAGE and Western Blotting as described in section 2.3.

## 2.5 Protein Turnover Assays

For the turnover assays, U2OS cells were transfected for 72 hours with siRNA (refer to section 2.2.4 for gene silencing procedures). At the end of the transfection incubation period, cells were washed and split for 5 time-points then treated with cycloheximide (50 µg/mL) and harvested at their corresponding time-points.

## 2.6 Ubiquitination Assays

Ubiquitination assays were performed *in vitro* by incubating E1 (0.4 µM), E2 (0.4 µM), E3 (Nedd-4 HECT domain (0.5 µM), and reconstituted nucleosomes (2 µg) in 50 mM Tris, 5 mM $MgCl_2$, 2 mM DTT, pH 7.5 + protease inhibitor (Roche) using a thermal cycler (90 min, 30 $^oC$). E1 (UBA1) enzymes were provided by Rahima Khatun from our laboratories (purified from SF9 insect cells). Reconstituted nucleosomes were provided by Lilia Kaustov (Princess Margaret Cancer, Toronto). Full-length E2s were expressed in and purified from bacteria as described in section 2.1. The E3 enzymes Ring1B:BMI1 where co-expressed and co-purified from U2OS cells using protocols described in section 2.2.

## 2.7 De-Ubiquitination Assays

De-Ubiquitination assays were performed *in vitro* by incubating E1 (0.4 µM), E2 (full-length or core only) (0.4 µM), E3 Nedd4 HECT domain (0.5 µM), reconstituted nucleosomes (2 µg), and USP7 (full-length or TRAF only) (0.0-2.0 µg) in 50 mM Tris, 5 mM $MgCl_2$. 2 mM DTT + protease inhibitor (Roche) using a thermal cycler (90 min, 30 $^oC$). E1 (UBA1) enzymes were provided by

Rahima Khatun from our laboratories (purified from SF9 insect cells). USP7 full-length enzymes were provided by Ira Lacdao from our laboratories (purified from SF9 insect cells). Reconstituted nucleosomes were provided by Lilia Kaustov (Princess Margaret Cancer, Toronto). E2s, USP7 TRAF, and E3 (Nedd4 HECT) were expressed in and purified from bacteria as described in section 2.1.

# Chapter 3: E2E2 and E2E3 are Novel USP7-Interacting Enzymes

## 3.1 Work Contributions

The experimentations for the data presented in this chapter were performed solely by the author of this thesis in their entirety.

The GST pull-down data for the sub-section "USP7 TRAF Interacts with E2E2 and E2E3 N-terminal Extensions *in vitro*" were initially established for full-length E2E2 and E2E3 and their core domains by Majda Mohammed (unpublished); the author of this thesis repeated these experiments for optimization and enhancement of data as shown in this chapter (Figure 3-1A and Figure 3-2A) and extended the work to include the $TRAF^{AAGF}$ mutant and N-terminal extensions of E2E2 and E2E3 as separate constructs and experiments.

## 3.2 Introduction

The deubiquitinating enzyme USP7 binds a number of its cellular and viral targets via recognition of a P/A/ExxS motif by its TRAF domain. The ubiquitin-conjugating enzyme E2E1, which expresses an ASTS motif in the N-terminal extension to its ubiquitin-conjugating core, has been identified as a USP7 target consistent with this profile (Sarkari *et al.* 2013). E2E1 is one of the three members of the E2E sub-family of Ub-conjugases and shares a similar structure with E2E2 and E2E3, the other two enzymes in this group which express PSTS motifs in their N-t extensions. Earlier work by Ashurov (2014) provided preliminary evidence to show an association between USP7 and E2E2 or E2E3 using *in vivo* reciprocal co-immunoprecipitations, inhibition assays, and crystal structures of USP7 bound to synthetic peptides of E2E2 or E2E3 PSTS motifs. In this project (Chapter 2), we further explored the observed USP7:E2E2 or E2E3 interaction through other techniques and expanded our investigations by addressing whether the activity and function of the two E2 enzymes are regulated by USP7 as a consequence of the observed interactions, similar to their E2E1 counterpart (Sarkari *et al.* 2013).

# 3.3 Results

## 3.3.1 USP7 TRAF Interacts with E2E2 and E2E3 N-terminal Extensions *in vitro*

We performed a series of GST-pulldowns with purified E2E2 or E2E3 and USP7 TRAF to further investigate the physical association between the two E2Es and USP7. We used GST-tagged TRAF as bait or a GST-only construct as negative control and incubated these recombinant proteins with E2E2 or E2E3. Samples from input and elution fractions of the pulldown columns were analyzed by SDS-PAGE and visualized by staining the proteins on acrylamide gel. Our results demonstrated that USP7 interacts with both E2E2 and E2E3 as indicated by the co-elution of either E2 enzyme with GST-TRAF (Figure 3-1A and Figure 3-2A). GST-alone did not interact with either of the E2 proteins as shown by their absence in the elution fraction visualized on the gel (Figure 3-1A and Figure 3-2A). To further confirm that the binding of E2E2 or E2E3 to USP7 is specific, we mutated the TRAF $^{164}$DWGF$^{167}$ site (Figure 1-11) that is required for binding P/A/ExxS motifs. In this mutant construct (GST-TRAF$^{AAGF}$), both the D (aspartic acid) and the W (tryptophan) residues were replaced with A (alanine). Contrary to GST-TRAF samples, in pulldowns with GST-TRAF$^{AAGF}$ we no longer detected E2E2 or E2E3 (Figure 3-1A and Figure 3-2A) indicating that wildtype TRAF$^{164-167}$ was required for this interaction consistent with other P/A/ExxS-containing proteins that bind USP7 TRAF.

In addition to the E2E2 and E2E3 full-length proteins, we used their [UBC] cores or the N-t extensions as separate constructs (GST-TRAF with E2E core, or TRAF with GST-E2E N-t). We performed these experiments in order to determine whether the observed USP7 interactions were specific only to the N-t extensions due to the presence of the identified PSTS motifs. While

we did not observe any binding between GST-TRAF and E2E2 or E2E3 cores (Figure 3-1B and Figure 3-2B), the GST-N-t extensions for both E2E2 and E2E3 were retrieved in the elution fractions of their pulldowns with TRAF; this confirmed that the interaction between UPS7 and E2E2 or E2E3 was mediated via the N-terminal tails of the E2s as hypothesized (Figure 3-1C and Figure 3-2C).

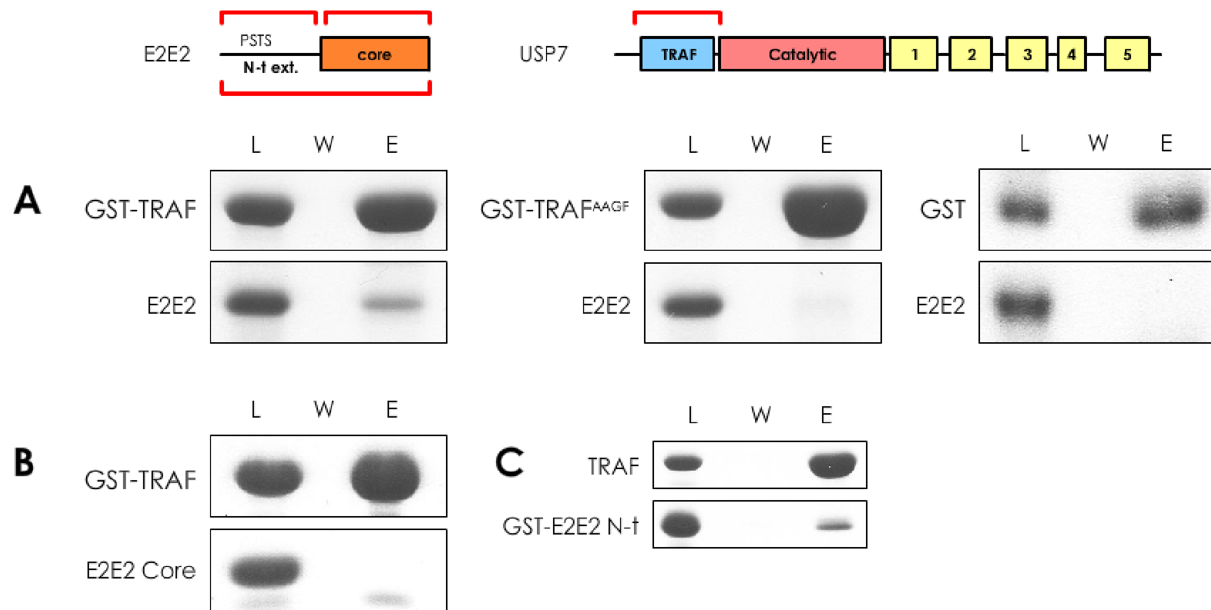



**Figure 3-1 – GST-Pulldowns Between USP7 TRAF and E2E2.** Constructs were incubated in equal molar masses in columns. 10 µL volumes from the input (load, L), wash (W), and elution (E) samples were subject to SDS-PAGE and visualized by Coomasie Blue staining. **(A)** GST-USP7 TRAF or mutant (AAGF) constructs with His-E2E2 full-length. GST-only was used as negative control. **(B)** GST-USP7 TRAF with His-E2E2 Core or **(C)** His-USP7 TRAF with GST-E2E2 N-terminal (N-t) extension.

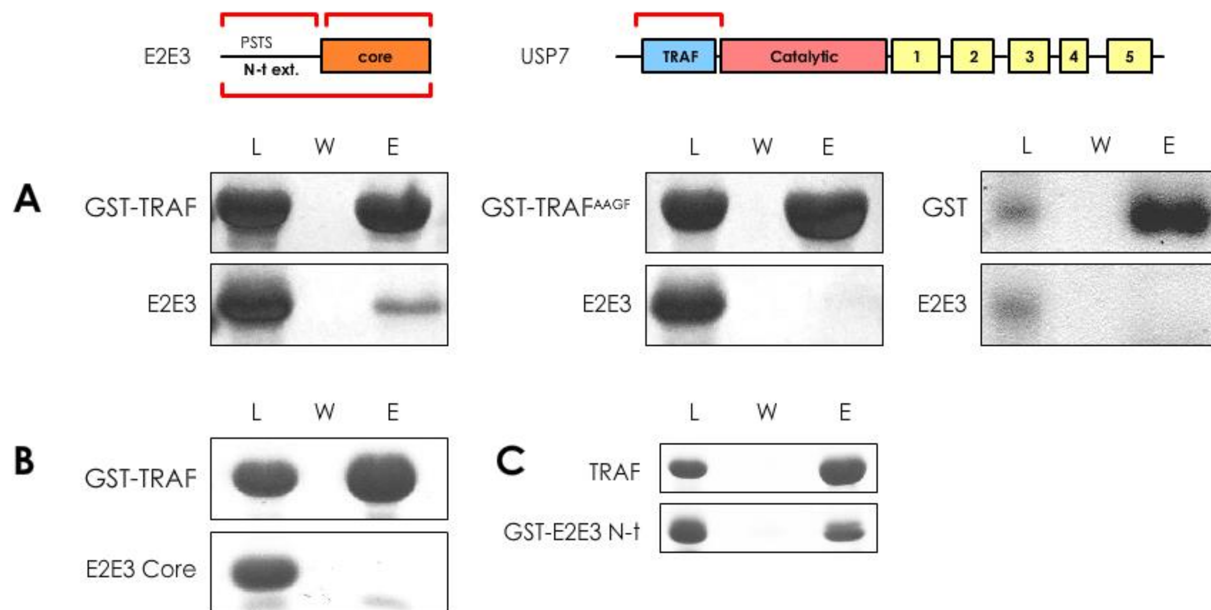
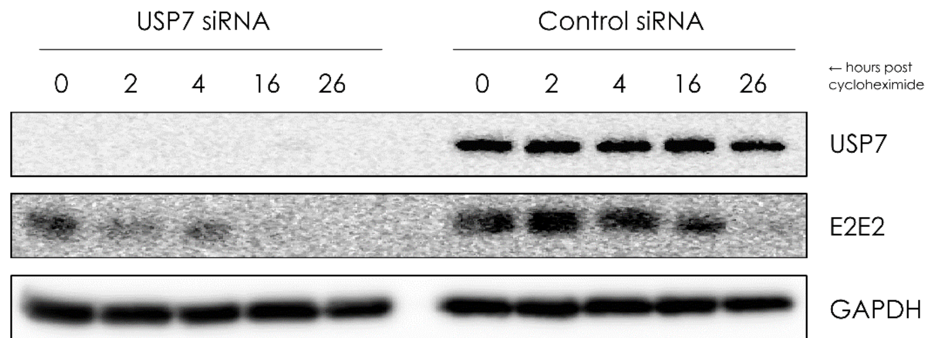



**Figure 3-2 – GST-Pulldowns Between USP7 TRAF and E2E3.** Constructs were incubated in equal molar masses in columns. 10 µL volumes from the input (load, L), wash (W), and elution (E) samples were subject to SDS-PAGE and visualized by Coomasie Blue staining. **(A)** GST-USP7 TRAF or mutant (AAGF) constructs with His-E2E3 full-length. GST-only was used as negative control. **(B)** GST-USP7 TRAF with His-E2E3 Core or **(C)** His-USP7 TRAF with GST-E2E3 N-terminal (N-t) extension.

### 3.3.2 USP7 Maintains Cellular Levels of E2E2 and E2E3

To investigate whether USP7 contributes to the steady-state levels of E2E2 and E2E3 *in vivo*, we silenced gene expression of USP7 in U2OS cell lines using siRNA oligonucleotides and sampled E2E2 or E2E3 levels at various times in USP7-silenced (siUSP7) or USP7-wildtype (siCtrl) [control] cells. In order to enhance the visualization of changes in E2E2 or E2E3 levels to a more detectable threshold, at time zero, all cells were treated with cycloheximide (CHX), a protein synthesis inhibitor that ceases nascent protein production.

As shown in Figure 3-3, when USP7 expression was silenced, E2E2 levels started to decrease in the first 2 hours and became barely detectable after 4 hours. However, in the control samples in which the endogenous expression of USP7 was unaltered, E2E2 levels were stable and relatively high as long as 16-hours before diminishing. This data suggested that USP7 maintains cellular levels of E2E2 and contributes to its steady-state and that in the absence of USP7, E2E2 is degraded faster.



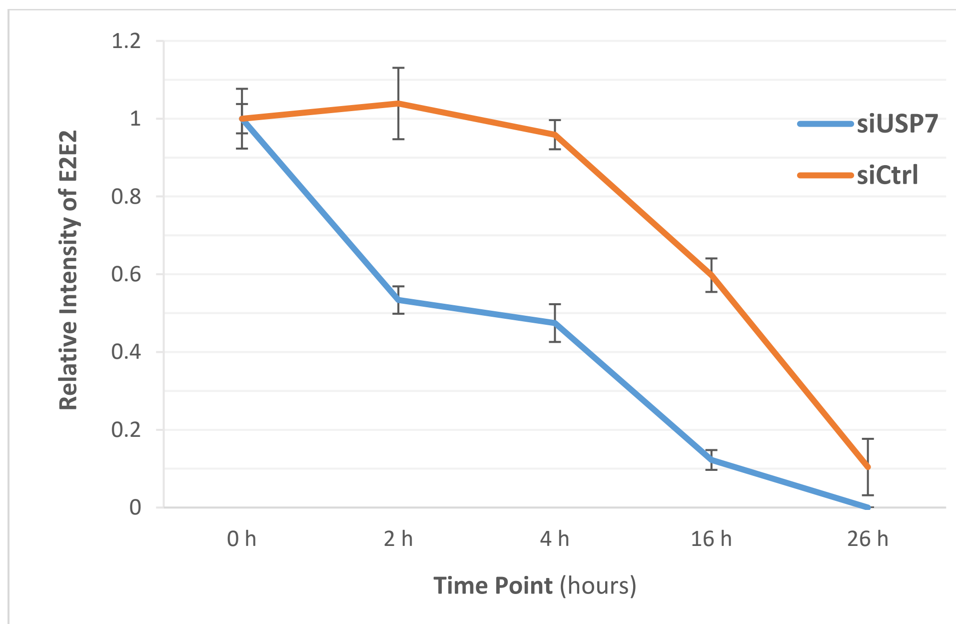
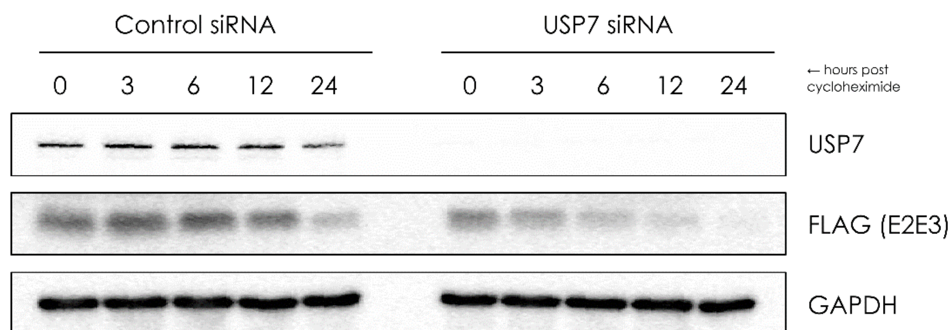




**Figure 3-3 – E2E2 Turnover Assays. (Left)** U2OS cells were transfected with control and USP7 siRNA. Following knockdown period, cells were treated with cycloheximide at time zero. Time points reflect samples harvested at corresponding times. **(Bottom)** Quantification of time points (N=3) using ImageJ software (Wayne Rasband, National Institute of Health, USA) measuring average grey pixel density (0-255) as relative ratio to GAPDH (loading control) levels. For each time point, averages were calculated as a fraction of time zero (relative fraction) with corresponding standard deviation of the means. Measurements were equalized between Western blots by setting GAPDH USP7siRNA at 0h as reference for comparison.

We attempted a similar experiment for E2E3, however we were unsuccessful in detecting endogenous E2E3 using antibodies from two different companies as tested in various cell-lines or conditions (**Error! Reference source not found.** - Supplemental). We therefore re-designed the experiment by transiently overexpressing FLAG-E2E3 and monitoring the fate of tagged E2E3 with FLAG antibodies instead of the endogenous protein. Similar to our findings for E2E2's turnover, in the absence of USP7 we observed FLAG-E2E3 levels to be unstable and decrease earlier than controls. As shown in Figure 3-4, in siUSP7 samples FLAG-E2E3 levels decrease within the first 3 hours post-CHX treatment and diminish by the 24h time point. However, in siCtrl samples, FLAG-E2E3 levels are relatively stable until the 12h time point and only show a major decrease at the 24h mark. These findings confirmed that USP7 regulates E2E3 levels *in vivo* in addition to E2E2.



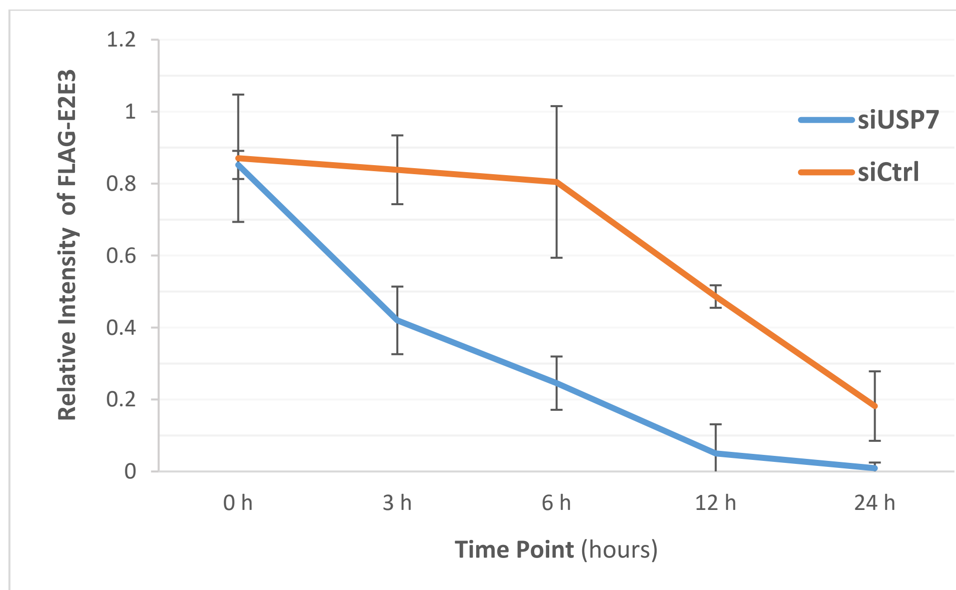




**Figure 3-4 – E2E3 Turnover Assay.** U2OS cells were transfected with FLAG-E2E3 plasmid DNA. Following over-expressions of FLAG-E2E3, cells were transfected with USP7 or non-specific (control) siRNA oligos. Cells were then treated with cycloheximide and harvested at corresponding time points. Quantifications were performed as described in **Figure 3-3** (N=3).

### 3.3.3 USP7 Attenuates E2E2 and E2E3-mediated ubiquitination

To determine whether USP7 can have any effect on E2E2 or E2E3-mediated polyubiquitination in complex with E3 enzymes, we setup *in vitro* de-ubiquitination assays. In these experiments, we used either E2E2 or E2E3 as E2 components which were either in full-length or core form. As the E3 component, we used NEDD4, a Ub-ligase previously shown to assemble poly-Ub chains and undergo auto-ubiquitination in association with E2Es. To these reactions, we added either full-length or TRAF-only USP7 in varying dosages (0-2 µg) and performed Western blots probing for ubiquitin as to visualize changes in ubiquitin chain assembly or breakdown.

Our preliminary data showed that prior to adding USP7 (at 0 µg), poly-ubiquitinated species were detected with the E2 enzymes either in full-length or core forms as shown by the smearing of the ubiquitin antibody signal at the high molecular weight ranges (Figure 3-5 and Figure 3-6, 0 µg lanes). With increasing dosages of full-length USP7 (0.5, 1, 2 µg), while little effect was observed for core E2E2 or E2E3 samples, the full-length E2 enzyme samples demonstrated a decrease in poly-Ub levels with as little as 0.5 µg of added USP7 (Figure 3-5 and Figure 3-6 respectively, compare lanes 1-4 with 5-8) suggesting that the N-t extensions played a role in the exertion of this effect.

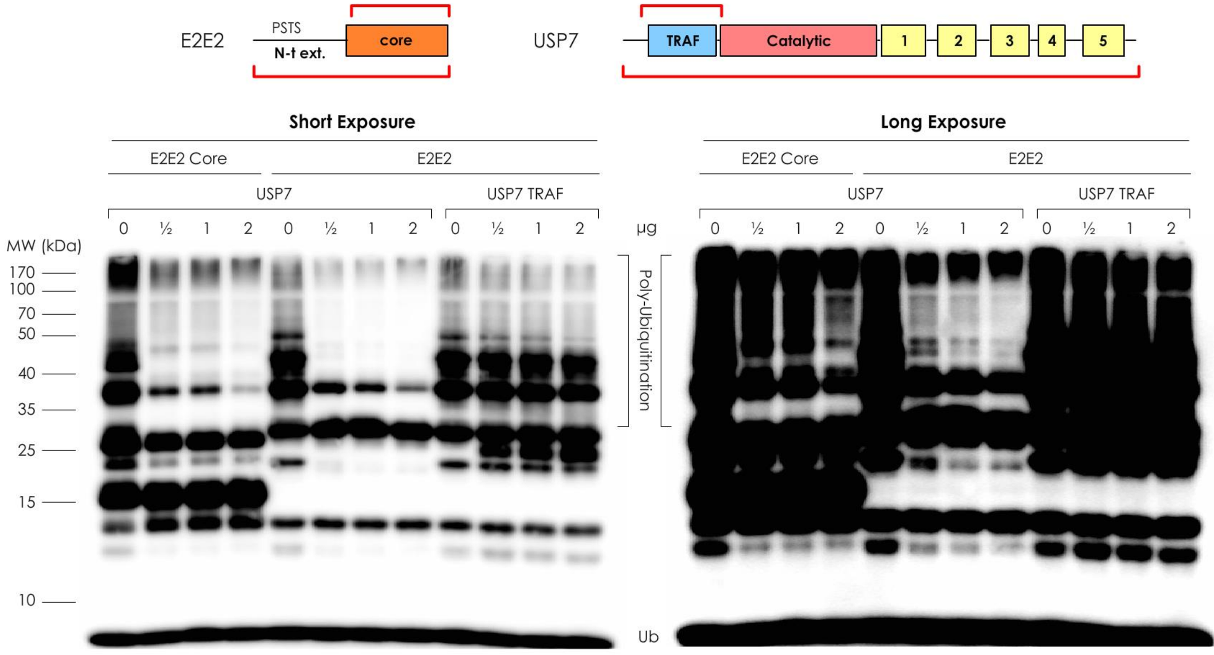



**Figure 3-5 – USP7 DeUbiquitination Assays for E2E2.** Reaction were incubated with E1(UbA1), E3 (Nedd4 HECT domain), Ub and E2E2 either in full-length or Core forms. Full-length USP7 or TRAF-only were added to reactions in increasing concentrations. Blots were probed with Ub antibody (N=2).

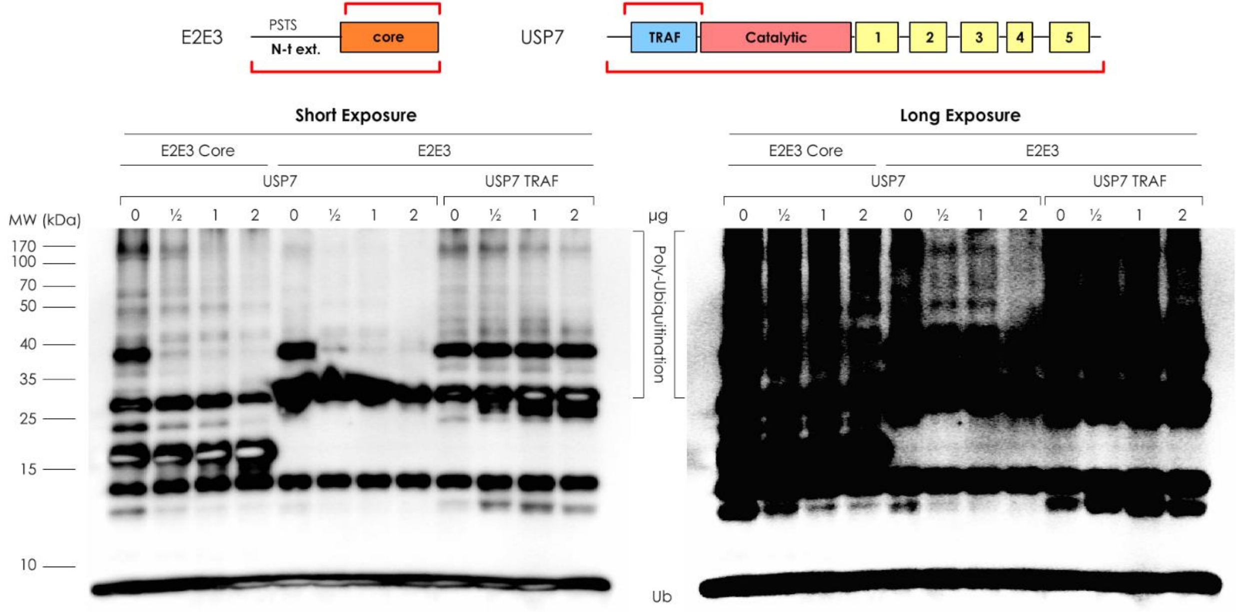



**Figure 3-6 – USP7 DeUbiquitination Assays for E2E3.** Reaction were incubated with E1(UbA1), E3 (NEDD4), Ub and E2E2 either in full-length or Core forms. Full-length USP7 or TRAF-only were added to reactions in increasing concentrations. Blots were probed with Ub antibody (N=1).

To further determine the role of USP7 in the observed reduction of poly-Ub chains, we repeated the full-length E2E2 or E2E3 assays but substituted full-length USP7 with its TRAF domain only. While there were reductions in poly-Ub levels with full-length USP7, in the absence of the catalytic [and other] component of USP7, we did not observe any noticeable change in poly-Ub levels with any additional amount of TRAF-alone for both E2E2 and E2E3 experiments (Figure 3-5 and Figure 3-6, compare lanes 5-8 with 9-12).

## 3.4 Discussion

Growing evidence indicates the P/A/ExxS sequence as a conventional binding site for USP7 via its TRAF domain. E2E1 (Sarkari *et al.* 2013), E2E2 and E2E3, all members of the Class III ubiquitin-conjugating enzyme sub-families, express this motif at their N-t extensions and were selected candidates as USP7 targets. GST pull-down assays demonstrated that USP7 (TRAF) physically associates with E2E2 and E2E3. Comparing pulldown results between the UBC Core and N-t segments of E2E2 or E2E3 confirmed that the interaction took place between TRAF and the N-t domain where PSTS motifs are localized. The USP7:E2E2 or E2E3 interaction was further confirmed *in vivo* by experiments in which Myc-TRAF and FLAG-E2E2 or E2E3 co-immunoprecipitated each other reciprocally (Ashurov 2014). Our molecular analysis of the binding region confirmed that E2E2 or E2E3 make contact with the $^{164}$DWGF$^{167}$ sequence on USP7 (Ashurov 2014) which is similar and consistent with other P/A/ExxS-expressing proteins that interact with TRAF such as p53, Mdm2 (Sheng *et al.* 2006), and E2E1 (Sarkari *et al.* 2013). Collectively, our interaction assays established E2E2 and E2E3 as novel binding partners for USP7, however the nature of E2E2 and E2E3 as ubiquitin-conjugating enzymes raises multiple questions in addressing the effects of these interactions on cellular pathways.

One possibility suggests that the E2E N-t extensions, as they have the capability for binding USP7, could act as adapters by recruiting USP7 to a substrate:(E3:E2) complex, where anchored in close proximity, USP7 could act as a catalytic antagonist by removing ubiquitin molecules from either E2 (Figure 3-7A) or E3 (Figure 3-7B) in the assembly (downregulating ubiquitination kinetics); although in cases such as Ring1B, USP7 may engage directly with the E3. In the absence of a

substrate, our "deubiquitination" assays (Figure 3-5 and Figure 3-6) aimed at demonstrating this possibility by investigating changes to poly-Ub chain assemblies as result of the interaction between USP7 and E2E2 or E2E3. Although preliminary and in need of establishment of further statistical significance, these assays hint at explaining the observed reduction of poly-Ub chains to be as a result of the interaction between the E2 N-t tails and USP7; this was shown by the dramatic difference between the reductions in samples of full-length versus core-only E2E2 or E2E3. We further attempted to demonstrate that the catalytic domain of USP7 was responsible for the decreases observed in the amount of poly-Ub chains and to establish this as USP7 catalyzed deubiquitination. Our results showed that the TRAF domain alone did not contribute to the reduction effect and that the catalytic domain of USP7 was present in all samples in which any reduction was observed. However, these experiments are not sufficient to make a direct connection between catalytic activity by USP7 resulting in these effects (deubiquitination). This is in part due to the possibility of non-catalytic related effects such as the spatial hindrance as a result of recruiting a ~135 kDa multi-domain protein (USP7) to the E2:E3 complex. In such scenario, the observed effect would be no catalytic reduction of poly-Ub chains (deubiquitination) at all, rather merely an inhibition of their accumulation (ubiquitination). Considering all aspects, the preliminary data at the very least or best suggests that USP7 attenuates levels of E2E2 or E2E3:NEDD4-mediated poly ubiquitination.

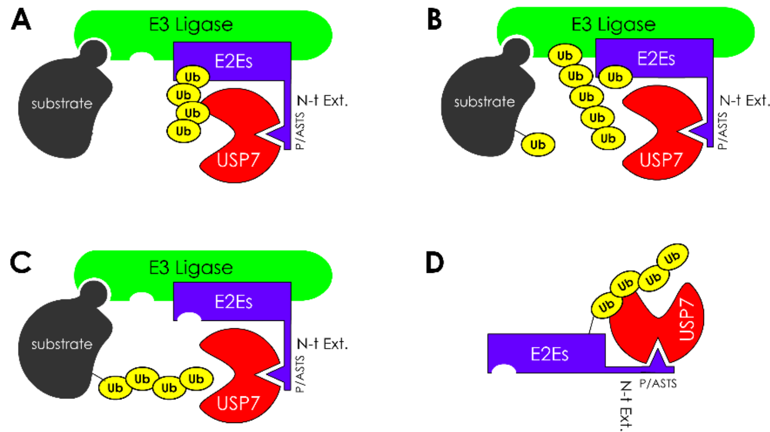



**Figure 3-7 – Proposed USP7 Regulatory Mechanisms for E2Es with PSTS Motifs. (A)** USP7 deubiquitinating an E2E poly-ubiquitinated by E3s **(B)** USP7 interacting with E2Es as an adapter to deubiquitinate E3s **(C)** USP7 interacting with an E2E:E3 complex as an adapter to deubiquitinate end-target **(D)** USP7 deubiquitinating free E2Es.

Although E2E2 and E2E3 have been found to have high expression profiles in the pancreas, liver, and adipose tissue (Ito *et al.* 1999), no known targets have been established for these enzymes in their specific tissues. However, histone H2A (introduced as a potential output gene in the next chapter), and GABARAP (autophagy-related protein) have been very recently identified and may be used as output in future assays. More output genes must be established for studying these enzymes.

Another outcome could result from USP7 interacting with E2Es that are themselves destined for proteasomal degradation (rescue by USP7), therefore regulating E2Es independent of the E3:E2 complex (Figure 3-7D) and indirectly regulating the targets of USP7-binding E2s. Our turnover assays gave insights to this pathway by showing that USP7 affects the stability of E2E2 and E2E3 *in vivo*. This was demonstrated by the abolishment of the E2s' cellular levels within a shorter period when USP7 gene expression was silenced compared to its endogenous levels (Figure 3-3

and Figure 3-4). While the results show that USP7 has an effect on the turnover of E2E2 and E2E3, the effect is mostly visible when the fate of these proteins are monitored over time and after CHX treatment as they are not as apparent at time zero. This suggests that such pathway may not have an immediately measurable impact on downstream targets. Furthermore, this pathway could face a complex outcome as multiple USP7-interacting E2s may share the same target such as Histone H2A for E2E1, E2E2, and possibly E2E3 (explored in Chapter 3).

The genes coding for ubiquitin-interacting proteins constitute about 10% of the human genome, therefore, ubiquitin signalling pathways are vulnerable to mutations (Kessler 2013). Aberrations in ubiquitin pathways have also been linked to inflammation, bacterial and viral infections, and cancer. It is, therefore, important to understand the roles of ubiquitin and its associated machinery at the molecular level in cellular pathways and processes. Ubiquitin systems offer a wide range of therapeutically important targets; however, only a handful of drugs have made it to the market. Perhaps the major challenge in targeting ubiquitin systems is the commonality of enzymes at the level of E1s, E2s, and DUBs associated with the proteasome. However, as the degree of target specificity increases in E3s and non-proteasomal DUBs, such as USP7, these classes of enzymes have become more promising drug targets. Drug development studies continue to benefit from more insights on the growing network of interacting partners and the structural and biochemical analyses for these interactions. In recent years, thousands of compounds have been screened for USP7 inhibition by a few pharmaceutical companies and a few reversible, non-/covalent USP7 inhibitors have been identified with varying levels of specificity to UPS7 and potency ranging from $IC_{50}$ < 200 nM (FORMA Therapeutics) to 1-200 μM

concentrations reviewed in depth in Wu *et al.* (2017). Very recently more compounds have been reported to inhibit USP7 with high affinity ($IC_{50}$ < 2 μM) (Turnbull *et al.* 2017).

# Chapter 4: E2Es Associate with PRC1 and Contribute to Histone H2A Ubiquitination

## 4.1 Work Contributions

The experimentations for the data presented in this chapter were performed solely by the author of this thesis in their entirety. Some of these data (Figure 4-1-E2E1, Figure 4-5) are published in the Journal of Biological Chemistry together with experiments, data, and analyses from other authors and contributors (Wheaton *et al.* 2017) (see Appendix C: Publication).

## 4.2 Introduction

E2Es are a 3 membered sub-family of ubiquitin-conjugases that have N-terminal extensions to the conserved E2 ubiquitin-conjugating core common in all E2s. Our investigations in identifying binding partners for USP7 established E2E1 as one of the targets of the deubiquitinating enzyme (Sarkari *et al.* 2013). USP7 has been shown to regulate RING1B (de Bie *et al.* 2010, Zaaroor-Regev *et al.* 2010) of the PRC1 complex which monoubiquitinates H2A on K119 (Fang *et al.* 2004). Our mass spectroscopy analysis of E2E1-interacting proteins also linked E2E1 to RING1B (Wheaton *et al.* 2017). For the project presented in this chapter, we hypothesized that E2E1, and potentially E2E2 and E2E3 due to similar characteristics, may associate with PRC1 as E2 components for the ubiquitination of H2A. We designed experiments to detect direct association between E2Es and PRC1 and investigated K119-H2A levels by manipulating these E2s *in vivo*. Related to this project, data from other authors from our group expand these findings in the context of p16 as a target gene and USP7 (Wheaton *et al.* 2017) (USP7 partially visited in this chapter).

# 4.3 Results

## 4.3.1 E2E1, E2E2 and E2E3 Interact with Ring1B:BMI1

Ring1B and BMI1 have been shown to act as the critical and core components of PRC1 required for H2A ubiquitination (Buchwald *et al.* 2006). Therefore, to investigate a physical interaction between E2Es and PRC1, we used Ring1B and BMI1 to perform *in vitro* FLAG-pulldowns. These experiments were conducted by immobilizing FLAG-Ring1B:BMI1 on FLAG affinity agarose and incubating the complex with all E2Es in separate trials. As negative controls for the specificity of binding, we used GST in place of E2Es in another trial. The Western blots in Figure 4-1 demonstrate the results of these pulldowns. As shown, when columns were eluted using excess competitor FLAG-peptides, we found E2E1, E2E2, and E2E3 co-eluted with FLAG-Ring1B:BMI1. GST however was not found in any of the elution samples indicating that the interaction of all three E2Es with the immobilized Ring1B:BMI1 were specific.

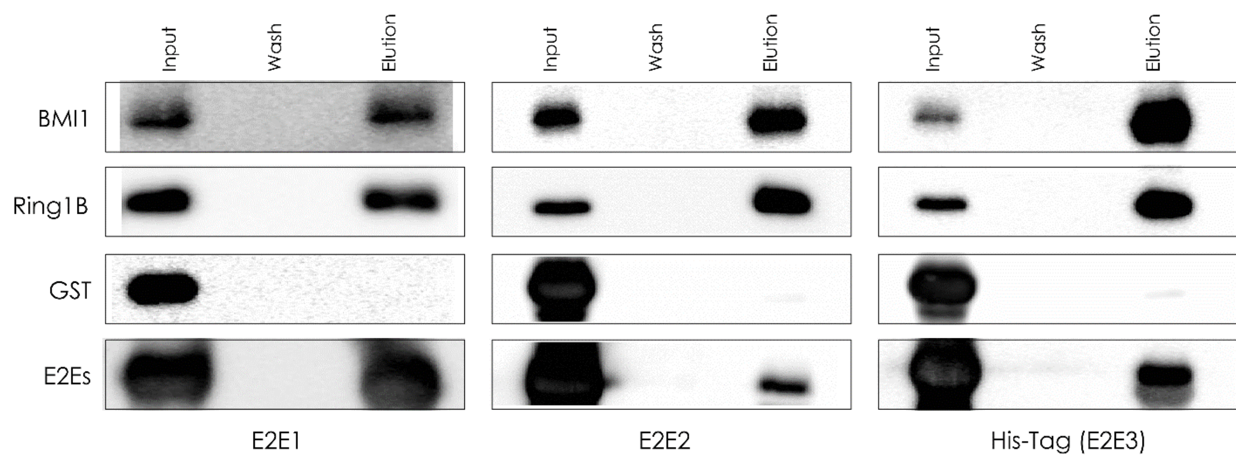



**Figure 4-1 – Ring1B:BMI1 *in vitro* FLAG-Pulldowns with E2E1, E2E2, and E2E3.** FLAG-Ring1B and BMI1 were co-expressed in U2OS cell-lines and isolated as a complex in purification columns. Recombinant E2Es and GST were purified from *E. coli* and co-incubated with immobilized FLAG-Ring1B:BMI1. Input, wash, and elution fractions were subject to SDS-PAGE and Western blotting.

## 4.3.2 E2Es Contribute to Ubiquitinated H2A Levels

In order to investigate whether the observed interactions between Ring1B:BMI1 and E2Es have any implications on H2A ubiquitination, we performed a series of experiments including *in vivo* silencing and overexpression of E2E genes and compared the levels of K119-H2A ubiquitinations to endogenous E2E expressions (controls) using Western blots with an antibody that detects H2A which is ubiquitinated specifically on K119. In addition, we performed *in vitro* ubiquitination assays for K119Ub-H2A using Ring1B:BMI1 and the three E2E enzymes.

### 4.3.2.1 Silenced Expression of E2Es Decreases K119Ub-H2A Levels *in vivo*

In our E2E silencing experiments, we used RNA interference and although the siRNA oligonucleotides (oligos) were designed specifically for their corresponding E2, we used more than one oligo to account for any potential cross-reactivity to other E2Es resulting in non-specific silencing in two cell lines (U2OS and HEK 293T). We used E2E1 and E2E2 antibodies for detection of their corresponding protein levels from the cell lysates, however E2E3 blots could not be produced due to unavailability of a potent antibody.

In U2OS cells (Figure 4-2), we found an overall decrease of K119Ub-H2A levels when using siRNA targeting any individual E2Es compared to the control. However, silencing (knockdown) of one E2E with a particular oligo also caused reduction in levels of another E2E indicating that the corresponding decrease in K119Ub-H2A may be due to non-specific targeting of other members of the E2E subfamily.

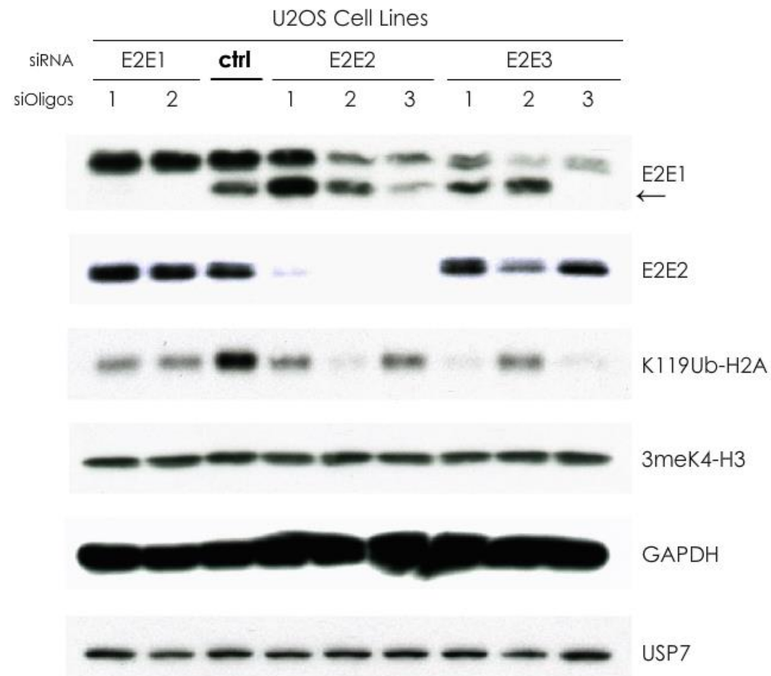



**Figure 4-2 – Silencing of E2Es and K119Ub-H2A Levels in U2OS Cells.** Cells were transfected with multiple siOligos designed against the corresponding E2E mRNA. Cell extracts were subject to SDS-PAGE and Western blotting using E2E1, E2E2, K119Ub-H2A, GAPDH, USP7, and 3meK4-H3 antibodies.

In U2OS cells, E2E1 has two isoforms that were detected using our E2E1 antibody. Knockdowns using two E2E1 oligos effectively silenced the shorter E2E1 isoform (bottom band), which resulted in a major decrease in K119Ub-H2A. E2E1 oligos had no cross-activities on the levels of E2E2.

Using 3 different oligos against the E2E2 mRNA resulted in silencing of E2E2 protein but in some cases, we also observed silencing of E2E1. Silencing with E2E2 oligos in all cases caused a decrease in K119Ub-H2A levels. For E2E3 knockdowns, although we observed lowered K119Ub-H2A levels using any of the E2E3 oligos, we also observed changes in the expression of E2E1 isoforms and in one case a reduction of E2E2. We could not confirm E2E3 silencing due the lack of E2E3 antibodies. Our data indicated that silencing of E2E proteins result in decreased K119Ub-H2A levels suggesting that these enzymes play a role in histone H2A ubiquitination. However due to cross-reactivity of the siRNA oligos among the E2E subfamily, these experiments alone could not pinpoint the observed effects to a particular E2E.

Figure 4-3 demonstrates the results of another E2E silencing trial performed in HEK 293T cells. In this experiment, compared to U2OS cells (Figure 4-2), we observed similar decreases in K119Ub-H2A levels when we introduced siRNA oligos to target the expression of any of the E2E proteins.

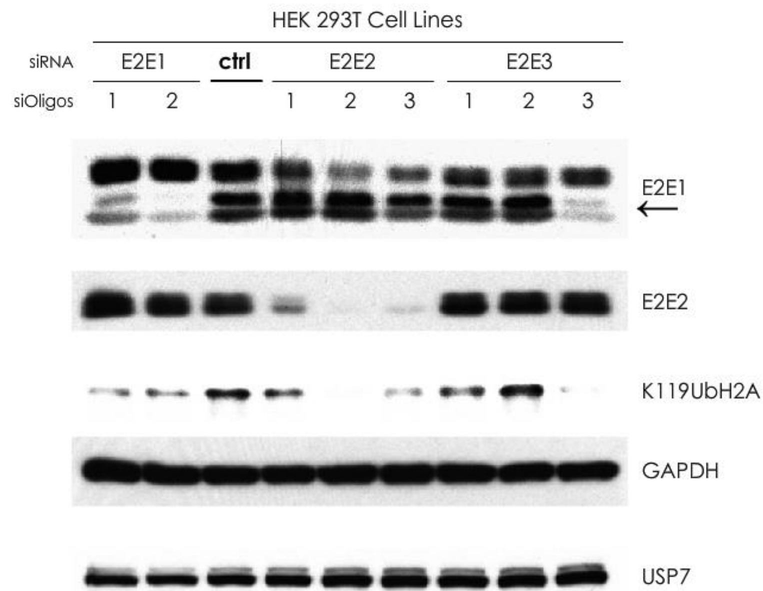



**Figure 4-3 – Silencing of E2Es and K119Ub-H2A Levels in HEK 293T Cells.** Cells were transfected with siOligos (as shown) designed against the corresponding E2E mRNA. Cell extracts were subject to SDS-PAGE and Western blotting using E2E1, E2E2, K119Ub-H2A, GAPDH, and USP7 antibodies.

#### 4.3.2.2 E2E1 is a PRC1-Associated E2 for Ubiquitination of H2A *in vitro*

To further examine K119 ubiquitination of H2A with respect to the members of the E2E family, we performed assays in which we compared the activity of the three E2Es in the presence of Ring1B:BMI1 (Figure 4-4). While E2E1 and E2E2 (and potentially E2E3) were shown to contribute to K119 ubiquitination of H2A by our earlier *[in vivo]* gene silencing experiments, we found only E2E1 to demonstrate a major effect for this reaction *in vitro.* As shown in Figure 4-4, while E2E1 efficiently catalyzed H2A ubiquitination, E2E2 showed no activity and E2E3 showed a relatively weak effect in these reactions.

In order to confirm that the observed K119 ubiquitination of H2A is a result of association of E2E1 with Ring1B:BMI1, we performed additional ubiquitination reactions in the absence of either Ring1B:BMI1 or E2E1; we observed K119-ubiquitinated H2A when both enzymes were present together but not with either one alone (Wheaton *et al.* 2017).

We further confirmed the requirement of Ring1B:BMI1 for K119 ubiquitination of H2A by mutating Ring1B to a catalytically inactive form, in which the residue Ile 53 (I53) located within the RING domain, was mutated to Ala (A), and performed additional Ub assays. As shown in Figure 4-5, in reactions with wildtype Ring1B (WT), we detected the same species using ubiquitin, H2A, or K119Ub-H2A antibodies. However, no product (K119Ub-H2A) was observed for the Ring1B I53A mutant, confirming that the K119-H2A ubiquitination event requires catalytically active Ring1B:BMI1.

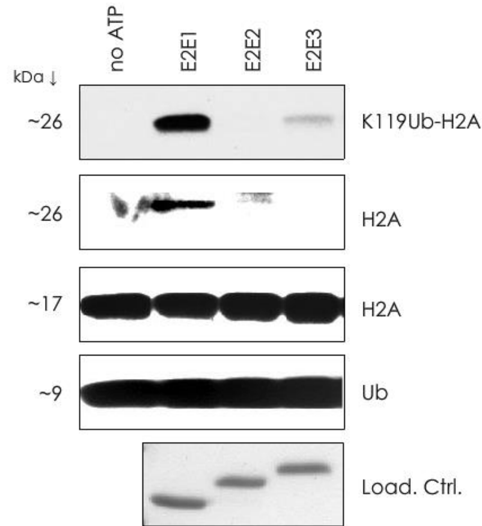



**Figure 4-4 – *In Vitro* Ubiquitination Assay of H2A with E2Es.** Ub assays were performed using Ring1B:BMI1 (E3) co-purified from U2OS cells, UbA1 (E1), Ub, reconstituted H2A, and a corresponding E2E. Reaction were stopped by denaturation and subject to Western blotting using K119Ub-H2A antibody.

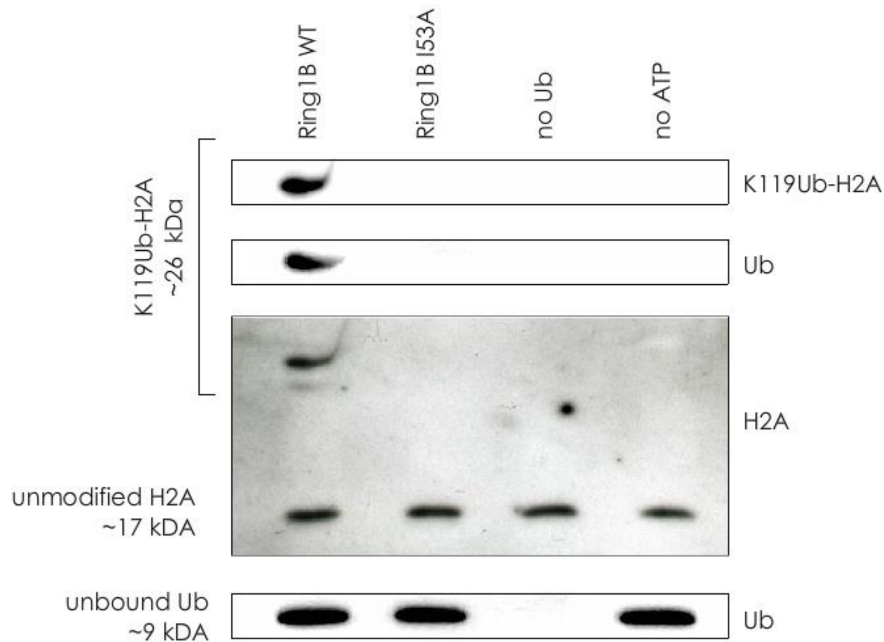



**Figure 4-5 – *In Vitro* Ubiquitination Assay of H2A with mutant Ring1B.**WT or mutant Ring1B were copurified from U2OS cells as E3s. Recombinant E2E1 was purified from *E. coli.* Assay reactions were stopped by denaturation and subject to Western blotting using K119Ub-H2A, H2A, and Ub antibodies.

We next performed E2E overexpression experiments to confirm the role of E2E1 in H2A ubiquitination. In these *in vivo* trials, we overexpressed FLAG-tagged E2E1 (WT) or a UBC-domain catalytic mutant form (C131A) in U2OS cell-lines, and compared K119Ub-H2A to endogenous levels (controls) using Western blots. As shown in Figure 4-6, we observed a corresponding increase in K119Ub-H2A when E2E1 was overexpressed but only in WT form, further confirming the requirement of catalytically active E2E1 for H2A ubiquitination. Furthermore, overexpression of E2E1 (WT) in HEK 293T cells also resulted in a major increase in K119Ub-H2A levels (Figure 4-7).

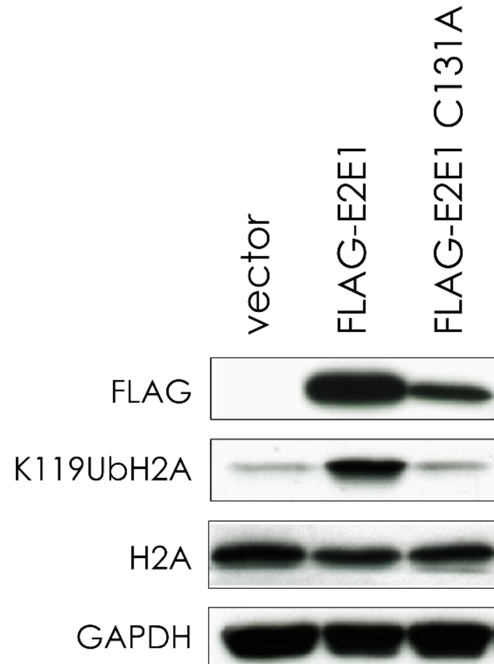



**Figure 4-6 – Overexpression of WT/mutant FLAG-E2E1 and K119Ub-H2A Levels.** Empty vector, or WT/mutant E2E1 were transfected in U2OS cell. Cell lysates were subject to SDS-PAGE and Western blotting 60 hours post transfection and probed with K119Ub-H2A antibodies.

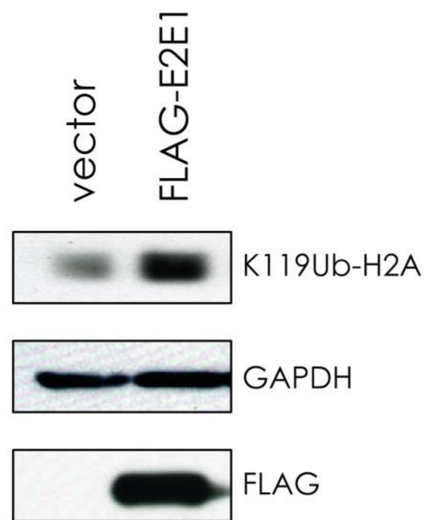



**Figure 4-7 – HEK 293T Overexpression of FLAG-E2E1 and K119Ub-H2A Levels.** Empty vector or FLAG-E2E1 were overexpressed by transfection.

#### 4.3.2.3 USP7, an E2E1 Regulator, Modulates K119Ub-H2A Levels *in vivo*

Our group previously established a regulatory effect between USP7 and E2E1 (Sarkari *et al.* 2013); our results showed that USP7 stabilizes E2E1 *in vivo* and that a reduction in USP7 levels corresponds to lower levels of E2E1; here, we demonstrated a directly proportional effect from E2E1 levels on K119Ub-H2A; therefore, we sought to investigate the relationship between H2A and E2E1 in the broader context of E2E1's regulatory network by changing USP7 levels and checking for a downstream effect on K119Ub-H2A through E2E1. As expected, with silenced USP7 levels we observed a corresponding decrease in E2E1 and K119Ub-H2A compared to the control siRNA samples (Wheaton *et al.* 2017) (partially shown in Figure 4-8).

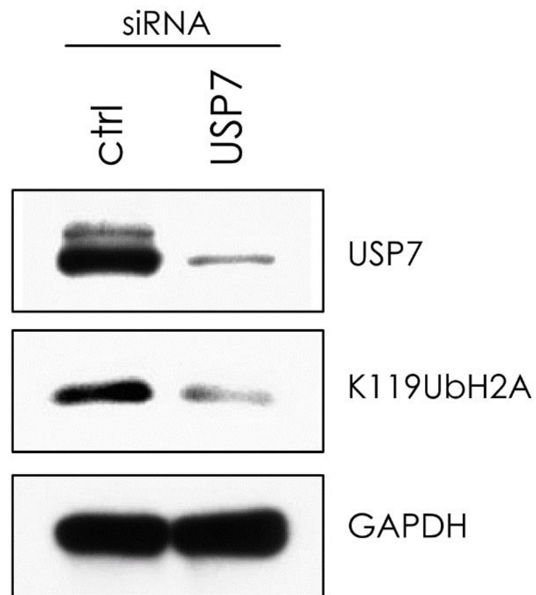



**Figure 4-8 – HEK 293T Silencing of USP7 Gene Expression and K119Ub-H2A Levels.** Cells were transfected with control (non-specific) or USP7 siOligos. Western blot performed with antibodies specific to K119Ub-H2A.

## 4.4 Discussion

Mass spectrometry data indicated an association between E2E1 and Ring1B:BMI1 (Wheaton *et al.* 2017), linking this E2E ubiquitin-conjugating enzyme to the core members of the PRC1 complex. The E2E family of ubiquitin-conjugating enzymes consist of E2E1, E2E2 and E2E3 which share much sequential and structural similarities such as the catalytic UBC core, their N-terminal extensions, and a P/A/ExxS USP7 binding motif (Figure 1-6 and Figure 1-13). Here, our *in vitro* pull-down data demonstrated that all E2E members can physically interact with Ring1B:BMI1 while the strongest association (proportion bound) occurred between E2E1 and core PRC1 followed by E2E3 and E2E2 in order (Figure 4-1). *In vitro* ubiquitination assay results indicated that Ring1B:BMI1 can efficiently ubiquitinate H2A at K119 in complex with E2E1, weakly by E2E3, and not by E2E2 (Figure 4-4). *In vivo* data demonstrated a reduction in K119Ub-H2A levels when siRNA against any of the E2E members were introduced in cells (Figure 4-2 and Figure 4-3) although E2E3 levels could not be shown due to antibody restrictions. Ubiquitination assays involving WT or catalytically inactive Ring1B:BMI1 or E2E1 confirmed that both components are required in functional form for the monoubiquitinating H2A at K119 (Figure 4-5 and Figure 4-6). Our results collectively and consistently establish E2E1 as a PRC1 associated E2 which targets H2A for K119 monoubiquitination.

We further expanded our studies and showed that USP7, a regulator of E2E1, exerts an effect on K119Ub-H2A through its association with E2E1 (Wheaton *et al.* 2017) (Figure 4-8). In the previous chapter and publication, our studies linked USP7 to E2E1, E2E2, and E2E3 stabilization. Other studies have shown that USP7 can also regulate Ring1B, both *in vivo* and *in vitro* (de Bie *et al.*

2010, Zaaroor-Regev *et al.* 2010), which further complicates our understanding of USP7's mode of regulation for the downstream H2A pathway as it is able to regulate both the ubiquitin-conjugating (E2) and the ubiquitin-ligase (E3) component for this ubiquitination.

Recent studies have also indicated that the interaction between USP7 and Ring1B modulates an oscillatory molecular switch that is the state of Ring1B autoubiquitination and ubiquitination by E6-AP, a balance of which affects the H2A pathway (Nguyen *et al.* 2011). Furthermore, E2s that interact with Ring1B also play a role in the fate of Ring1B [and subsequently uH2A] as they may direct ubiquitination between activation or degradation of Ring1B.

In this chapter, we demonstrated a pattern that also links E2E2 and E2E3 to K119Ub-H2A; however, unlike E2E1, these two homologues did not or did not efficiently ubiquitinate H2A *in vitro* while their manipulation showed an effect *in vivo*. It is unclear whether the interaction of E2Es with Ring1B could be subject to cross-talking or upregulation as a result of changes to the expression profile of another E2E sub-family member or even the E2 superclass as other E2 families such as E2D1 (Bentley *et al.* 2011), E2D2 (Wheaton *et al.* 2017), and E2D3 (McGinty *et al.* 2014) have also been shown to interact with Ring1B:BMI1. The discrepancy between the *in vitro* and *in vivo* data may also indicate that other factors may be involved in addition to PRC1 with E2E2 or E2E3 for the ubiquitination of H2A in cells.

Our preliminary experimentation with a third cell line (HeLa) showed that introducing siRNA against any of the E2Es results in total silencing of all E2E1 and E2E2 (and possibly E2E3) (data

not shown). Therefore, tissue specificity of E2Es may play a major role in how E2Es function in general or with respect to their involvement in the K119Ub-H2A pathway.

# Chapter 5: Summary and Future Directions

## 5.1 E2Es as USP7 Targets

Previous work by Majda Mohammad (unpublished) and Ashurov (2014) indicated that E2E2 and E2E3 interact with USP7. In this work, we extended our *in vitro* pull-down data by demonstrating that E2E2 and E2E3 N-terminal extensions alone physically interact with TRAF domain of USP7, and that $TRAF^{AAGF}$ mutant is incapable of binding E2E2 and E2E3 (Figure 3-1 and Figure 3-2). In protein turnover assays (*in vivo*) in which USP7 gene expression were silenced, we demonstrated that USP7 contributes to the steady-state levels of E2E2 and E2E3 proteins (Figure 3-3 and Figure 3-4). Furthermore, my preliminary *in vitro* data indicated that USP7 attenuates E2E2- or E2E3-mediated polyubiquitination (Figure 3-5 and Figure 3-6).

In the light of the ambiguities concerning our USP7 deubiquitination assay, two future approaches are suggested to establish the observed effect as actual catalytic activity by USP7. Many ubiquitin system studies make use of NEM (N-Ethylmaleimide), a maleic acid derivative with a highly reactive alkene bond, which readily attacks thiol groups therefore covalently binds cysteines (Hill *et al.* 2009). Although this approach would deactivate the catalytic site of USP7 (C223) and provide basis for comparison of its catalytic activity, this sort of experimentation must be subject to careful laboratory practices such as extensive dialysis of deactivated USP7 as residual NEM could potentially target catalytic cysteines in E1, E2 and E3 in this type of assay and inhibit their function in addition to USP7. A secondary approach using ubiquitin-aldehyde (Ub-H) may serve as a more suitable alternative as it is a more specific inhibitor of USP- and UCH-type DUB activities by blocking the hydrolysis of poly-Ub chains (Hershko and Rose 1987).

Furthermore, a number of assays have been developed for detecting inhibition of USP7 activity which may be employed for our future assays. Some of these approaches utilize fusion of ubiquitin with fluorescence molecules (e.g. Ub-AMC, Ub-Rh110, Ub-AML, and others) which emitted light after DUB activity released the fluorophore from Ub allowing for excitation of the molecule. While these methods later improved interference and non-specificity issues of this technique, new approaches take advantage of coupling fluorescence polarization with ubiquitinated USP7-specific target peptides such as TAMRA-PTEN[5-21] (5-carboxytetramethylrhodamine) (Wu *et al.* 2017).

Our USP7 catalytic activity assays could benefit from comparing USP7 activity in the presence of a known substrate for the E2E2 or E2E3:E3 complex which would further aid in understanding of the mechanisms or modes of USP7 function by addressing whether E2:E3s can act as complex adapters and engage USP7 directly with their substrate (Figure 3-7C). In addition, identification of ubiquitinated species is of great importance in these assays. It is recommended that future Western blots assays be enhanced by using antibodies to probe for all present proteins (E1,E2,E3, substrate, USP7) in addition to ubiquitin in order to provide further insight on the changes to the ubiquitination level profile.

## 5.2 The Role of E2Es in H2A Ubiquitination

Previous work (mass spectroscopy) indicated that E2E1 is associated with Ring1B:BMI1 which are the core components of the PRC1 complex (Wheaton *et al.* 2017). In this work, using *in vivo* coimmunoprecipitation techniques, we demonstrated that E2E1, E2E2, and E2E3, which constitute all members of the E2E subfamily of ubiquitin-conjugating enzyme, physically interact

with Ring1B:BMI1 (Figure 4-1). With *in vitro* ubiquitination assays, we demonstrated that E2E1 together with Ring1B:BMI1 can monoubiquitinate H2A on K119 (Figure 4-4).We further showed and that catalytically active Ring1B *(in vitro* assay) (Figure 4-5) and E2E1 (*in vivo*) (Figure 4-6) are required for this ubiquitination. Furthermore, we demonstrated that silencing of E2E1, E2E2, and potentially E2E3 genes *in vivo* can modulate H2A K119 monoubiquitination (Figure 4-2 and Figure 4-3), and that overexpression of E1E1 in two cell-lines increases H2A K119 monoubiquitination (Figure 4-6 and Figure 4-7). We also demonstrated that silencing of USP7 gene expression (an E2E1 regulator) decreases the levels of H2A K119 monoubiquitination.

An ideal way for understanding the interaction between E2Es and PRC1 is to investigate the molecular interactions between these proteins. A crystal structure of E2D3 in complex with PRC1 and the nucleosome was recently published and highlighted the importance of the E2 in the stability of this complex (McGinty *et al.* 2014). As N-terminal extension of E2Es may play various roles in the PRC1 complex, crystal structures of any of these enzymes may provide insights to the topology of how other factors such as USP7 may be recruited to PRC1 or whether the N-terminal tail may hinder or favour H2A ubiquitination by making contact with PRC1. While we investigated the role of E2E1 more extensively, more experiments are suggested for E2E2 and E2E3 such as the overexpression of WT and catalytically inactive forms. Although we performed this experiment for E2E1, for all three E2Es it is suggested to investigate the differences between overexpressing full-length versus core or N-terminal extensions as it may further provide insight in how the N-terminal tails may regulate the behaviour of these proteins.

While we showed the direct association between E2E1 and PRC1 both *in vitro* (Figure 4-1) and *in vivo* (Wheaton *et al.* 2017), E2E2 and E2E3 were only shown to interact *in vitro* (Figure 4-1).

Interestingly, while E2E2 and E2E3 seem to not ubiquitinate H2A *in vitro* (or not to the same extent as E2E1) (Figure 4-4), the silencing of these genes appears to have comparable effects on H2A K119 ubiquitination *in vivo* (Figure 4-2 and Figure 4-3). It is therefore important to further investigate the interaction of E2E2 and E2E3 with PRC1 by performing reciprocal coimmunoprecipitations to show any direct association *in vivo* or subjecting the pulldown complex to mass spectroscopy for identifying whether other proteins may be necessary for this complex to function.

In our experiments in the previous chapters and publications, we showed an effect on E2E levels by manipulating USP7. We also showed an effect from manipulating E2Es on H2A ubiquitination. Although we aim to establish that manipulations of USP7 exerts an effect on H2A via E2Es considering the evidence in which USP7 silencing led to both a decrease in levels of E2E1 and K119-monoubiquitinated H2A in the same experiment (Wheaton *et al.* 2017), given the evidence that all E2Es manipulate H2A monoubiquitination and considering that USP7 regulates PRC1 components, further experimentation is needed to establish the relationship (USP7→ particular E2E → K119-Ub H2A) exclusively. For this purpose, as preliminary experiments, it is suggested to obtain E2E knockout cell-lines in which the effect of any E2E may be studied in isolation of their closely related sub-family members.

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# Appendix A:
# Supplemental Figures

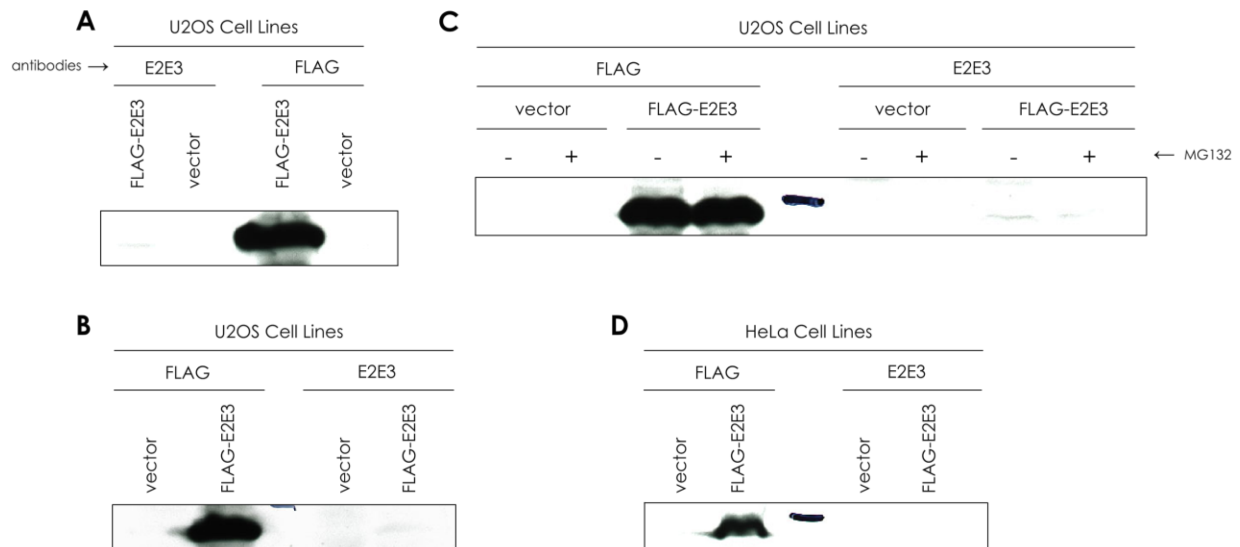


**Figure A-1 – E2E3 Antibody Troubleshooting.** All panels show Western blots for transfection of corresponding cells with empty or FLAG-E2E3 vector DNA probed with either anti-FLAG or E2E3 antibody. **(A)** 50 µg DNA **(B)** 50 µg of DNA and treatment with proteasome inhibitor MG132 **(C)** 175 µg of DNA **(D)** 50 µg of DNA.

# Appendix B:
# Protein Purifications

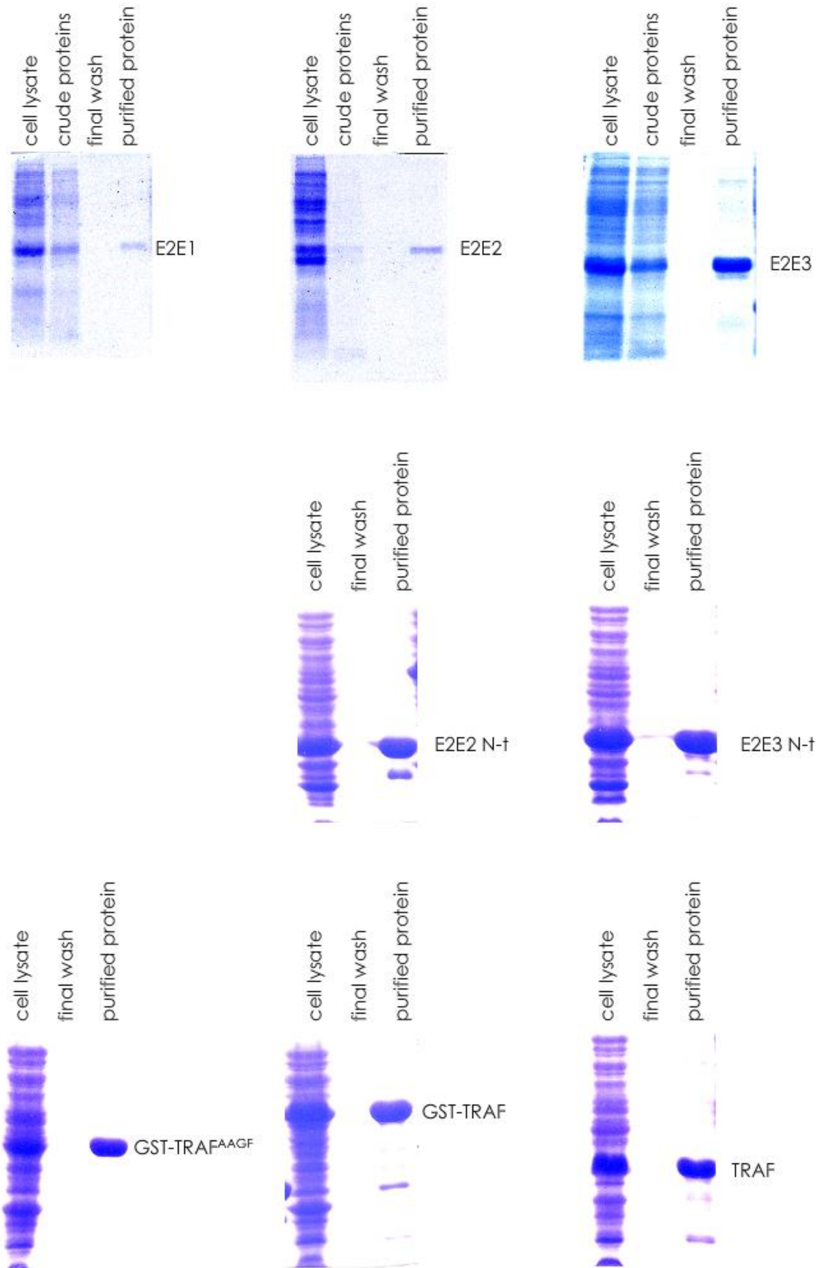


**Figure B-1 – Protein Purification Samples.** Images are scans of acrylamide gels from SDS-PAGE. Crude cell extracts or protein mixtures are shown in first lane(s) which appear as smears. Empty lanes are samples from the last wash. Purified proteins are shown as single bands (labelled).

# Appendix C: Publication

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## UbE2E1/UBCH6 Is a Critical *in Vivo* E2 for the PRC1-catalyzed Ubiquitination of H2A at Lys-119*

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**Edited by George N. DeMartino**

**UbE2E1/UbcH6 is an E2 ubiquitin-conjugating enzyme that is regulated by USP7. We identified UbE2E1 as a novel component of Polycomb repressive complex 1 (PRC1), the E3 ligase complex responsible for histone H2A ubiquitination and gene silencing. We demonstrate that UbE2E1 is critical for the monoubiquitination of H2A at residue Lys-119 (uH2AK119) through its association with the PRC1 complex. UbE2E1 interacts with PRC1 subunits including Ring1A and Ring1B. Overexpression of UbE2E1 results in increased levels of uH2AK119, whereas overexpression of catalytically inactive UbE2E1_C131A or UbE2E1 knockdown results in decreased levels of uH2AK119. The down-regulation of H2A ubiquitination by loss of function of UbE2E1 is correlated with alleviated p16$^{INK4a}$ promoter repression and induced growth inhibition in HCT116 cells. These results are specific to UbE2E1 as knockdown of UbE2D E2s does not show any effect on uH2AK119. We extended the UbE2E1 regulation of uH2AK119 to USP7 and showed that USP7 is also a key regulator for monoubiquitination at H2A Lys-119 as both knockdown and deletion of USP7 results in decreased levels of uH2AK119. This study reveals that UbE2E1 is an *in vivo* E2 for the PRC1 ligase complex and thus plays an important role in the regulation of H2A Lys-119 monoubiquitination.**

Monoubiquitination of H2A at Lys-119 (uH2AK119) is found on 5∼15% of total H2A in mammalian cells. H2A monoubiquitination plays an essential role in the maintenance of genome integrity and pluripotency of stem cells, developmental patterning, and X-chromosome inactivation (1–6). The monoubiquitination of H2A at residue Lys-119 is primarily mediated by Polycomb repressive complex 1 (PRC1),[2] which contains three RING domain proteins Ring1A (RING1), Ring1B (RING2 or RNF2), and one of the PCGF proteins. The core PRC1 proteins Ring1B and BMI1 (also known as PCGF4) form an active heterodimeric E3 ubiquitin ligase for monoubiquitination of H2A at Lys-119. Genetic studies revealed that Ring1B is the main catalytic subunit of the PRC1 complex as Ring1B knock-out leads to global loss of uH2A and embryonic lethality (4, 7, 8). However, BMI1 and Ring1A also play important roles in stimulating the E3 ligase activity and maintaining the integrity of the PRC1 complex. Deletion of BMI1 and Ring1A lead to a significant decrease of uH2AK119 levels and developmental abnormalities (3). Despite extensive studies characterizing the E3 ligases responsible for ubiquitination of H2A, little is known about the specific E2-conjugating enzyme(s) for H2A ubiquitination. Although several E2s including the UbE2D family (UbE2D1/UbcH5a, UbE2D2/UbcH5b, and UbE2D3/UbcH5c) and UbE2E1/UbcH6 have been reported to assist Ring1B·BMI1 in catalyzing monoubiquitination of H2A, these are all based on biochemical studies and no E2 has yet been identified for the PRC1 complex in a cellular context (9, 10). UbE2Ds and UbE2E1 share ∼80% sequence identity within their catalytic ubiquitin-conjugating (UBC) domains. Distinct from UbE2D subfamily members, UbE2E1 contains a 50-residue unstructured N-terminal extension rich in serine and lysine residues, which has been shown to interact with USP7 and is essential for USP7-mediated UbE2E1 stabilization (11).

Several deubiquitinating enzymes (DUBs) including UBP-M/USP16, USP3, USP21, USP22, and 2A-DUB have been reported to act directly on uH2AK119 and influence its function. Specifically, UBP-M/USP16 is required for dynamic H2A deubiquitination during cell cycle progression (12, 13). USP21, USP22, and 2A-DUB function as positive transcriptional regulators via deubiquitination of H2A (14–16). USP3 is involved in the DNA damage repair pathway and deubiquitinates H2A among other substrates in DNA damage foci (17). In addition, DUBs can indirectly regulate the levels of uH2AK119 by affecting the function and stability of enzymes involved in H2A ubiquitination. For example, USP7 and USP11 were purified with the PRC1 complex and shown to modulate its transcriptional regulation of the *INK4a/ARF* locus (18). Also USP7 has been identified as an interacting partner of Ring1B in separate studies indicating its involvement in PRC1 function (18–20). More recently, USP7 was identified to regulate PRC1 function via its association with SCML2 (21).

H2A monoubiquitination at Lys-119 has been associated with gene silencing of the *INK4A/ARF* locus in the maintenance of stem cell proliferative activity and the sustainment of unlim-

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[2] The abbreviations used are: PRC1, Polycomb repressive complex 1; UBC, ubiquitin-conjugating; DUB, deubiquitinating enzyme; IP, immunoprecipitation; PCGF, polycomb group ring finger.

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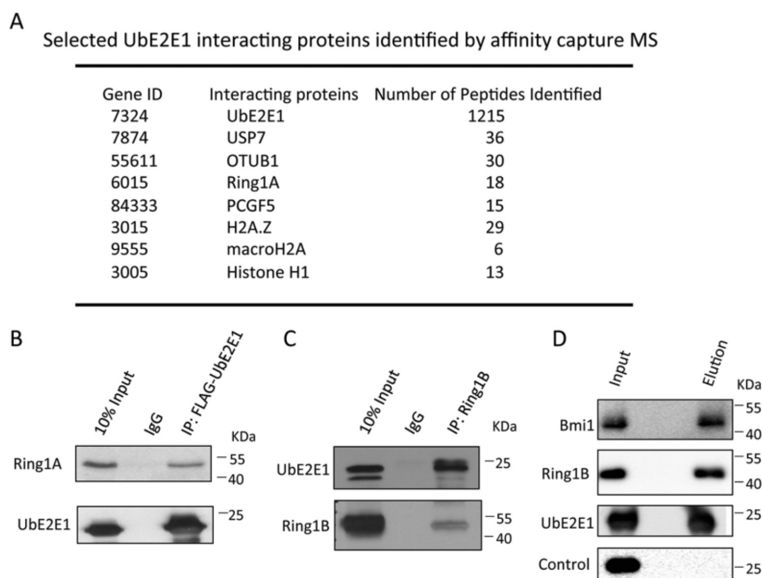


FIGURE 1. **UbE2E1 interacts with PRC1.** *A*, UbE2E1 interaction partners identified using affinity capture proteomic studies. *B*, UbE2E1 interacts with Ring1A. FLAG-tagged UbE2E1 was expressed in U2OS cells and immunoprecipitated with an antibody against the FLAG tag, followed by Western blotting using antibodies against Ring1A and UbE2E1. *C*, UbE2E1 interacts with Ring1B. U2OS cell lysates were immunoprecipitated with a Ring1B antibody followed by Western blotting with antibodies against endogenous Ring1B and UbE2E1. *D*, FLAG pulldown of UbE2E1 with FLAG-tagged Ring1B·BMI1. UbE2E1 was incubated with FLAG-Ring1B·BMI1-captured complex and eluted with FLAG-peptide followed by Western blotting with antibodies against Ring1B, BMI1, UbE2E1, and GST as negative control.

ited cell proliferation in cancer cells (22–24). Deletion or transcription repression of the *INK4A/ARF* locus are common in many malignant cancers. The *INK4A/ARF* locus encodes two tumor suppressor proteins $p16^{INK4a}$ and $p14^{ARF}$. The $p16^{INK4a}$ protein inhibits the cyclin-dependent kinase (CDK4 and CDK6) complexes by blocking cyclin D association, thereby negatively regulating cell cycle progression and cell proliferation. The $p14^{ARF}$ protein enhances the function of tumor suppressor p53 and promotes apoptosis and senescence in response to oncogenic stresses. Activation of $p16^{INK4a}$ and $p14^{ARF}$ transcription leads to cell cycle arrest and cellular senescence, whereas deletion or repression of this gene locus results in uncontrolled cell growth and transformation (reviewed in Ref. 25). Importantly, the levels of uH2AK119 appear to be correlated with transcriptional silencing of this locus. Loss of uH2AK119 at the promoter leads to de-repression of the *INK4A/ARF* locus, whereas enriched uH2AK119 results in repression of this locus (26). The level of uH2AK119 is tightly regulated and critical in determining the cellular activity of $p16^{INK4a}$ and $p14^{ARF}$.

In this study, we explored novel UbE2E1 interacting proteins and found that UbE2E1 interacts with components of the PRC1 complex. We further demonstrated that UbE2E1 is critical for PRC1-mediated monoubiquitination of H2A Lys-119. Through the regulation of the levels of uH2AK119, UbE2E1 influences transcriptional silencing of the *INK4A/ARF* locus. Our study revealed that UbE2E1 regulates H2A ubiquitination by functioning as an endogenous E2 for PRC1-mediated H2A ubiquitination. Thus, UbE2E1 plays a critical role in homeostasis of H2A ubiquitination.

## Results

*UbE2E1 Binds Components of the PRC1 Complex*—E2 enzymes are critical components of the protein ubiquitination machinery as they influence E3 ligase activity and modulate different types of ubiquitin modification (27). We performed affinity capture LC-MS/MS proteomic studies, using overexpressed FLAG-tagged UbE2E1 as bait, to identify UbE2E1 interacting proteins in U2OS cells. PRC1 proteins Ring1A and PCGF5 were identified as novel UbE2E1 interacting proteins (Fig. 1*A*). In addition, USP7 was also captured as a binding partner of UbE2E1, and was shown to be critical in controlling UbE2E1 stability (11).

To address whether UbE2E1 associates with components of the PRC1 complex, we performed co-immunoprecipitation analyses to test the interaction between UbE2E1 and Ring1A or Ring1B. Consistent with the MS data, endogenous Ring1A was readily pulled down with FLAG-UbE2E1 (Fig. 1*B*). Although Ring1B, the main E3 ligase of the PRC1 complex was not detected in the initial UbE2E1 proteomic study, the interaction between Ring1B and UbE2E1 was confirmed by immunoprecipitation using an antibody against endogenous Ring1B and immunoblotting for endogenous UbE2E1 (Fig. 1*C*). Together, these results suggest that UbE2E1 interacts with components of the PRC1 complex *in vivo*.





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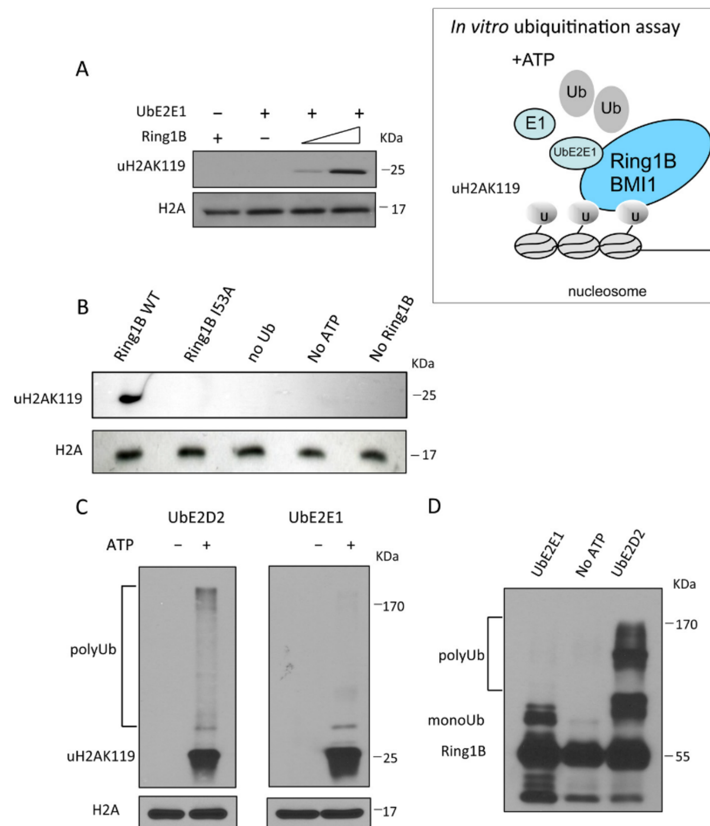


FIGURE 2. **UbE2E1 modulates PRC1 catalyzed mono-ubiquitination of H2A.** *A, in vitro* ubiquitination of H2A using reconstituted nucleosome as substrate in the presence or absence of UbE2E1 and Ring1B·BMI1 complex. In each reaction, equal amounts of Ub, E1, and nucleosome were added. Reactions were analyzed by Western blotting using antibodies against total H2A or uH2AK119. *B, in vitro* ubiquitination of H2A using reconstituted nucleosome as substrate in the presence of wild-type or mutant Ring1B. Reactions were analyzed by Western blotting using antibodies against total H2A or uH2AK119. *C, in vitro* ubiquitination of H2A using reconstituted nucleosome as substrate in the presence of UbE2E1 or UbE2D2. Reactions were analyzed by Western blotting using antibodies against total H2A or uH2AK119. *D, in vitro* autoubiquitination of Ring1B in the presence of UbE2E1 or UbE2D2. Reactions were analyzed by Western blotting using a Ring1B antibody.

An *in vitro* pulldown assay was used to demonstrate that UbE2E1 interacted directly with the FLAG-Ring1B·BMI1 dimer. FLAG-Ring1B·BMI1 was overexpressed in U2OS cells and immobilized on FLAG-agarose. UbE2E1 or GST as negative control was then incubated with the Ring1B·BMI1 FLAG-agarose. UbE2E1 but not GST was co-eluted with the Ring1B·BMI1 heterodimer indicating that UbE2E1 interacted with the Ring1B·BMI complex (Fig. 1*D*).

*UbE2E1 Is Critical for H2A Monoubiquitination*—To test whether UbE2E1 is an E2 for Ring1B·BMI1-catalyzed monoubiquitination of H2A, an *in vitro* ubiquitination assay was performed using UbE2E1, FLAG-Ring1B·BMI1, and reconstituted nucleosome as a substrate. The active Ring1B·BMI1 complex was purified from cell lysate using FLAG-agarose and eluted using a 3× FLAG peptide. Monoubiquitination of H2A was detected in the presence of both UbE2E1 and Ring1B·BMI1 but not either alone, indicating that UbE2E1 can act as the E2 for PRC1-mediated H2A monoubiquitination (Fig. 2*A*). To test whether the ubiquitination of H2A Lys-119 in the presence of UbE2E1 was specific for the PRC1 complex, an inactive Ring1B mutant (I53A) was prepared. This I53A Ring1B mutant was unable to catalyze the monoubiquitination of H2A Lys-119 (Fig. 2*B*). These results are consistent with other studies showing that UbE2E1 is involved in the monoubiquitination of H2A catalyzed by recombinant Ring1B·BMI1 *in vitro* (9). Previous studies have shown that UbE2D family proteins assist Ring1B·BMI1 to monoubiquitinate H2A at Lys-119 (9, 10, 28, 29). To compare the function of UbE2E1 and UbE2D proteins, we examined Ring1B·BMI1-mediated H2A ubiquitination and Ring1B autoubiquitination. Both UbE2E1 and UbE2D2 are active in Ring1B·BMI1-mediated H2A ubiquitination at Lys-119. As shown in Fig. 2*C*, UbE2E1-Ring1B·BMI1 catalyzes only H2A Lys-119 monoubiquitination, whereas UbE2D2-Ring1B·BMI1 carries out both monoubiquitination at H2A Lys-119 and polyubiquitination of H2A. These results suggest that UbE2E1 and UbE2D proteins have distinct functions in H2A ubiquitination. Interestingly, Ring1B was autoubiquitinated in these *in vitro* H2A ubiquitination reactions (Fig. 2*D*). In the





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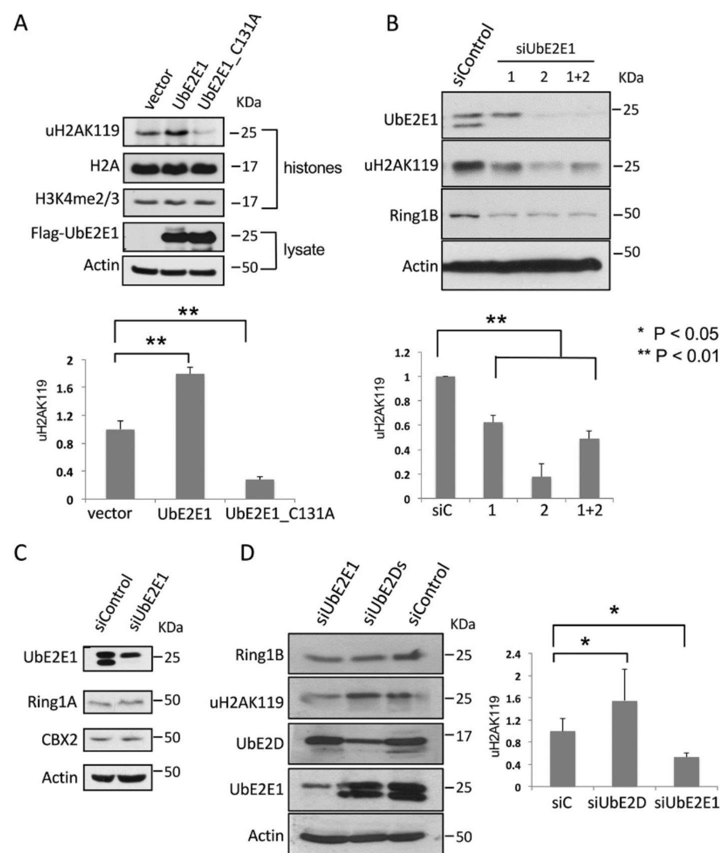


FIGURE 3. **UbE2E1 is critical for H2A monoubiquitination.** *A*, overexpression of UbE2E1 or UbE2E1_C131A. HCT116 cells were transfected with empty vector, FLAG-tagged UbE2E1, or UbE2E1_C131A. Total histones or cell lysates were immunoblotted using the indicated antibodies. *B*, knockdown of UbE2E1. U2OS cells were transfected with nonspecific control siRNA (siControl) or siRNAs targeting UbE2E1 (siUbE2E1 #1, siUbE2E1 #2, or combination of siUbE2E1 #1 and siUbE2E1 #2). Cell lysates were immunoblotted using the indicated antibodies. *C*, knockdown of UbE2E1. U2OS cells were transfected with nonspecific control siRNA (siControl) or an siRNA targeting UbE2E1. Cell lysates were immunoblotted using the indicated antibodies. *D*, knockdown of UbE2E1 and UbE2Ds in U2OS cells. U2OS cells were transfected with nonspecific control siRNA or siRNAs targeting UbE2E1 or UbE2Ds. Cell lysates were immunoblotted using the indicated antibodies. Fold-change of uH2AK119 was determined by normalizing uH2AK119 to actin. A paired *t* test was performed to evaluate statistical significance of the changes in uH2AK119 levels. *Error bars* indicate standard deviation and are from at least 2 lysates.

UbE2E1-specific assays, monoubiquitinated Ring1B was detected, whereas polyubiquitination of Ring1B was detected in the reactions using UbE2D2.

To gain better insight into the function of UbE2E1 in the PRC1 complex, we overexpressed UbE2E1 and the enzymatically inactive mutant UbE2E1_C131A. Ectopic UbE2E1 resulted in increased cellular levels of uH2AK119, but no change in total H2A or di- and trimethylated H3K4 (Fig. 3*A*). Ectopic UbE2E1_C131A, the enzymatically inactive mutant, resulted in decreased cellular levels of uH2AK119 with no effect on total H2A or di- and trimethylated H3K4 (Fig. 3*A*). Together, these results support the role of UbE2E1 in the PRC1-catalyzed monoubiquitination of H2A Lys-119. To further confirm the role of UbE2E1 in the PRC1 complex, two different siRNAs were used to knockdown UbE2E1. Both siRNAs or a combination of the two dramatically reduced endogenous UbE2E1 and resulted in decreased levels of uH2AK119. These results further support that UbE2E1 is an active E2 for H2A monoubiquitination at Lys-119 *in vivo*. Interestingly, knockdown of UbE2E1 also decreased the protein levels of Ring1B but not CBX2 or Ring1A (Fig. 3, *B* and *C*), indicating that UbE2E1 may also have a role in the stability of the main PRC1 E3 ligase, Ring1B.

To better understand the specificity of E2s for the monoubiquitination of H2A Lys-119 *in vivo*, we also examined knockdown of UbE2Ds. Surprisingly, the knockdown of UbE2Ds did not have any effects on the levels of either uH2AK119 or Ring1B (Fig. 3*D*). These results demonstrate that the UbE2Ds do not play an essential role in monoubiquitination of H2A Lys-119 *in vivo*.

*USP7 Regulates the Levels of uH2AK119*—USP7 was previously shown to directly interact with and stabilize UbE2E1 (11).

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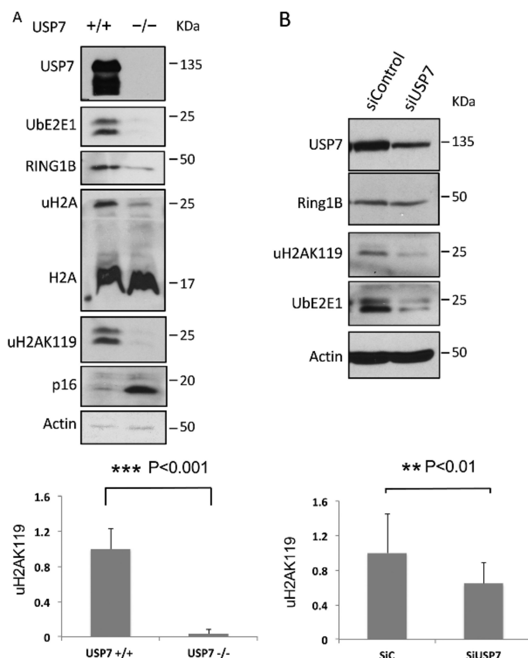


FIGURE 4. **USP7 regulates PRC1 and H2A monoubiquitination.** *A*, the lysates from HCT116 USP7$^{+/-}$ and USP7$^{-/-}$ cells were subject to immunoblotting using antibodies as indicated. *B*, USP7 knockdown was performed in HeLa cells by transfection with a USP7-specific siRNA. The lysates from USP7 knockdown or control HeLa cells were subject to immunoblotting using antibodies as indicated. Fold-change of uH2AK119 was determined by normalizing uH2AK119 to actin. A paired *t* test was performed to evaluate statistical significance of the changes in uH2AK119 levels. *Error bars* indicate S.D. and are from at least 2 lysates.

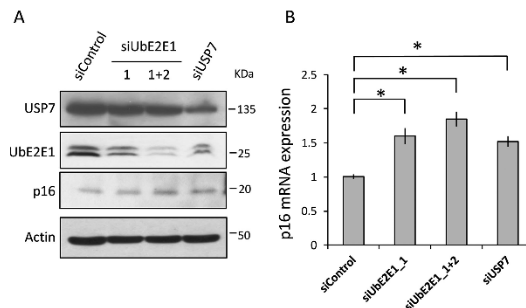


FIGURE 5. **UbE2E1 and USP7 regulate the levels of p16$^{INK4a}$ mRNA and protein levels.** *A*, knockdown of UbE2E1 and USP7 in HeLa cells. HeLa cells were transfected with nonspecific control siRNA (siControl) or siRNAs targeting UbE2E1 (siUbE2E1 #1, or combination of siUbE2E1 #1 and siUbE2E1 #2) or USP7. Cell lysates were immunoblotted using the indicated antibodies. *B*, p16$^{INK4a}$ mRNA expression levels were affected by UbE2E1 or USP7 knockdown. p16$^{INK4a}$ mRNA expression was measured by quantitative RT-PCR on three independent biological replicates, normalized with an internal GAPDH control, and presented as fold-change over control. A paired *t* test was used to evaluate the differences between siUbE2E1 and siControl and between siUSP7 and siControl. The significance was denoted as *, $p < 0.01$.

USP7 was also previously shown to associate with chromatin, regulate the stability of the PRC1 complex, and deubiquitinate Ring1B (18, 20). However, the effect of USP7 on H2A monoubiquitination was not investigated in USP7 knock-out cells. Therefore, we examined levels of uH2AK119 in HCT116 USP7$^{-/-}$ cells in which the *USP7* gene is knocked out and also in HeLa cells with USP7 knockdown. Decreased levels of UbE2E1, Ring1B, and uH2AK119 were observed in USP7$^{-/-}$ compared with USP7$^{+/+}$ cells (Fig. 4*A*). Similarly, USP7 knockdown resulted in decreased levels of UbE2E1, Ring1B, and uH2AK119 (Fig. 4*B*). Thus, USP7 is a key regulator as it stabilizes components of PRC1, maintains the function of the PRC1 complex, and regulates the level of uH2AK119.

*USP7 and UbE2E1 Contribute to Gene Silencing of the INK4A/ARF Locus*—H2A Lys-119 monoubiquitination leads to transcriptional repression of the *INK4A/ARF* locus (18, 30). Because both USP7 and UbE2E1 modulate the level of uH2AK119, we examined the role of USP7 and UbE2E1 in transcriptional regulation of the *INK4A/ARF* locus. Knockdown of either UbE2E1 or USP7 resulted in an increase in p16$^{INK4A}$ protein and mRNA levels (Fig. 5). It is known that p16$^{INK4a}$ plays a critical role in the induction of cellular senescence (31). Knockdown of USP7 has also been reported to induce a senescence-like proliferative arrest phenotype (18). This led us to examine the levels of senescence in the *USP7* knock-out cells. There were increased numbers of senescent cells, as indicated by senescence-associated β-galactosidase staining in HCT116 USP7$^{-/-}$ cells (18 ± 4%) compared with parental HCT116 USP7$^{+/+}$ cells (none detected) (Fig. 6*A*), correlated with decreased H2A monoubiquitination and elevated level of p16$^{INK4a}$ detected in these cells (Fig. 4*A*). To further demonstrate the role of p16$^{INK4a}$ in the growth defect observed in HCT116 USP7$^{-/-}$ cells, we examined the effect of silencing p16$^{INK4a}$ in these cells. In parental HCT116 USP7$^{+/+}$ cells, the level of p16$^{INK4a}$ is quite low, therefore silencing p16$^{INK4a}$ did not have any effect on the growth rate (Fig. 6, *B* and *D*, USP7$^{+/+}$ con siRNA *versus* USP7$^{+/+}$ p16 siRNA). However, silencing of p16$^{INK4a}$ in HCT116 USP7$^{-/-}$ cells shows increased growth rate compared with the control siRNA cells, demonstrating that the growth defect of USP7$^{-/-}$ cells is at least in part due to the increased expression of p16$^{INK4a}$ (Fig. 6, *B* and *D*, USP7$^{-/-}$ con siRNA *versus* USP7$^{-/-}$ p16 siRNA). Consistent with the increased p16$^{INK4a}$ levels shown with UbE2E1_C131A overexpression (Fig. 6*C*), altered p16$^{INK4a}$ function was demonstrated as a slower growth rate in USP7$^{+/+}$ cells transfected with UbE2E1_C131A (Fig. 6*E*, USP7$^{+/+}$ UbE2E1 *versus* USP7$^{+/+}$ UbE2E1_C131A). Together these data support the role of USP7 and UbE2E1 in determining the onset of senescence and cell growth by regulating the level of p16$^{INK4a}$, which is correlated with the role of USP7 and UbE2E1 in the regulation of uH2AK119.

Last, to confirm that the increased level of p16$^{INK4a}$ following UbE2E1 knockdown was due to transcriptional de-repression mediated by uH2AK119, a native chromatin IP was performed in UbE2E1 knockdown and control cells using an antibody against uH2AK119. Three sets of primers targeting the *INK4A/ARF* locus at specific promoter regions of *INK4A* were used in a quantitative PCR experiment to examine the occupancy of ubiquitinated H2A at these regions. UbE2E1 knockdown showed 2–4-fold decreases for all three sites within the p16$^{INK4a}$ promoter compared with the control (Fig. 6*F*). These results strongly imply

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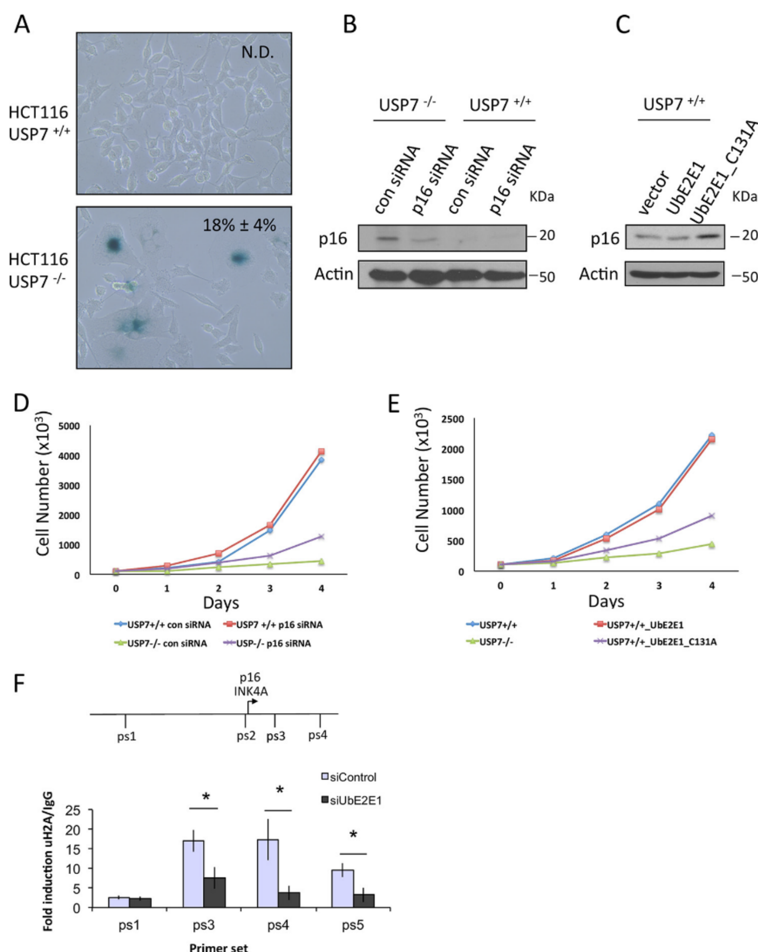


FIGURE 6. **Loss of USP7 leads to decreased H2A monoubiquitination, decreased growth rate, and increased senescence.** *A*, phase-contrast images showing senescence-associated $\beta$-galactosidase staining of HCT116 USP7$^{+/+}$ and USP7$^{-/-}$ cells. *Blue* staining is indicative of senescent cells. *B*, knockdown of p16$^{INK4a}$ in USP7$^{-/-}$ cells. HCT116 USP7$^{+/+}$ and USP7$^{-/-}$ were transfected with siControl or siRNA against p16. Lysates were immunoblotted with a p16$^{INK4a}$-specific antibody. *C*, overexpression of UbE2E1 or UbE2E1_C131A. Levels of p16$^{INK4a}$ increased in the presence of overexpressed UbE2E1_C131A. Lysates were immunoblotted with a p16$^{INK4a}$-specific antibody. *D*, cell growth of HCT116 USP7$^{+/+}$ and USP7$^{-/-}$ cells transfected with siControl or siRNA against p16$^{INK4a}$ were monitored and plotted for 5 days. *E*, the effect of UbE2E1 on cell growth of HCT116 cells. HCT116 USP7$^{+/+}$ cells transfected with UbE2E1 or UbE2E1_C131A were monitored and compared with HCT116 USP7$^{-/-}$ cells. *F*, U2OS cells were transfected with control siRNA or siRNA targeting UbE2E1 and subject to ChIP using an antibody against uH2AK119. Quantitative PCR was performed using primer sets targeting different sites (ps1–4) within the *INK4A/ARF* locus as illustrated. The amount of uH2AK119 at different locations of *INK4A/ARF* locus was normalized by the background signal obtained using IgG and is represented by fold-induction of uH2A over IgG. All amplifications were performed in triplicate, and *error bars* represent S.D. A paired *t* test was used to evaluate the difference between siUbE2E1 and siControl. The significance was denoted as *, $p < 0.01$.

that UbE2E1 contributes to the transcriptional repression of *INK4A/ARF* by regulating monoubiquitination of H2A.

## Discussion

In this study, we showed that both UbE2E1 and USP7 regulate the levels of uH2AK119. Importantly, UbE2E1 appears to be a critical E2 enzyme for *in vivo* PRC1-mediated H2A monoubiquitination. Knockdown of UbE2E1 or overexpression of the inactive UbE2E1_C131A mutant reduced the level of uH2AK119 accompanied by increased expression of its repressed target p16$^{INK4a}$. Similar effects have previously been observed by removing other subunits of the PRC1 complex (such as the E3 ligases, Ring1B, Ring1A, and BMI1) in various cell types with a suppressed p16$^{INK4a}$ locus (23, 24, 26). These results imply that UbE2E1 is an integral component of the PRC1 complex and regulates the level of uH2AK119.

Previous *in vitro* studies have reported that more than one E2 can work with Ring1B·BMI1 to catalyze H2A Lys-119 ubiquitination (9, 10, 29). UbcH5c/UbE2D2 was shown to form a ternary complex with Ring1B·BMI1 and was active in catalyzing



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Ring1B-dependent H2A ubiquitination (10). Recently, the crystal structure of the PRC1 complex including Ring1B·BMI1 and UbcH5d/UbE2D3 interacting with nucleosome revealed the importance of the E2 enzyme in stabilizing contact with the nucleosome (28). Nevertheless, several lines of evidence support UbE2E1 as a critical *in vivo* E2 for PRC1-mediated H2A Lys-119 monoubiquitination. UbE2E1 is located primarily in the nucleus (11) and our proteomic data shows that UbE2E1 interacts with components of the PRC1 complex. We were readily able to show co-immunoprecipitation of both Ring1B and Ring1A along with UBE2E1. As shown in this study, UbE2E1, but not UbE2Ds, functions in the ubiquitination of H2A Lys-119 *in vivo*, as knockdown of UbE2Ds did not result in changes in the levels of uH2AK119. This study also shows that UbE2Ds produce both mono- and polyubiquitin chains on H2A, whereas UbE2E1 only catalyzes monoubiquitination of H2A *in vitro*, which was also observed by Buchwald *et al.* (9). Because H2A can be modified by both polyubiquitination and monoubiquitination, UbE2D and UbE2E1 may have different roles in H2A ubiquitination, even though they can both assist with ubiquitination at H2A Lys-119. It is interesting to note that the UbE2E1 gene follows a similar evolutionary path to the Polycomb genes, which were initially identified in *Drosophila* and conserved in multicellular eukaryotes (32), whereas UbE2D genes are found in all eukaryotes. As uH2AK119 only occurs in multicellular eukaryotes, it is plausible that UbE2E1 and PRC1 co-evolved to catalyze this histone modification for gene silencing and transcriptional regulation (33). There are two other UbE2E members (UbE2E2 and UbE2E3), which share high sequence similarity to UbE2E1 and have redundant roles to UbE2E1 in many physiological processes. Further research is required to investigate whether UbE2E2 and UbE2E3 also contribute to regulation of H2A monoubiquitination.

Because it is well established that uH2AK119 is one of the epigenetic signatures for transcriptional repression of the *INK4A/ARF* locus, we used this locus to analyze the potential role of UbE2E1 in PRC1-dependent transcriptional regulation. We examined the levels of $p16^{INK4a}$ mRNA and protein, uH2AK119 occupancy of the *INK4A/ARF* locus, and cell growth by altering cellular UbE2E1 activity. We showed that UbE2E1 contributes to the transcriptional repression of *INK4A/ARF* by regulating monoubiquitination of H2A Lys-119. In all cases, the results are in agreement with the critical function of UbE2E1 in monoubiquitination of H2A Lys-119.

The role of USP7 in Polycomb group protein-mediated silencing has been reported previously. USP7 was shown to interact with guanosine monophosphate synthase and contribute to H2B deubiquitination and gene silencing in both humans and *Drosophila* (34, 35). In addition, USP7 and USP11 were co-purified with PRC1 complex proteins and shown to regulate the levels of $p16^{INK4a}$ (18). In this study, we further confirmed that USP7 plays an important role in maintaining the steady state level of PRC1 proteins especially UbE2E1 and Ring1B. In contrast to the study by Maertens *et al.* (18), we are able to readily and reproducibly detect a robust decrease in the level of uH2AK119 with USP7 knockdown or deletion, suggesting that USP7 also plays a role in regulating the level of uH2AK119. This is supported by Lecona *et al.* (21) who also observed decreased levels of uH2A in USP7 knock-out cells. We observed increased senescence and growth arrest in HCT116 $USP7^{-/-}$ cells, which can be partially explained by decreased uH2AK119 and increased $p16^{INK4a}$ levels in these cells.

Our study sheds new light into the regulation and function of the PRC1 complex. We identified UbE2E1 as a critical *in vivo* E2 for the PRC1-catalyzed monoubiquitination of H2A Lys-119. We also provided more evidence supporting USP7 as key regulator of the PRC1 complex by modulating PRC1 stability, activity, and function.

## Experimental Procedures

*Cell Culture and Antibodies*—Human U2OS and HCT116 cells were grown in McCoy's media. HeLa cells were grown in DMEM supplemented with 10% FBS. HCT116 $USP7^{+/+}$ and $USP7^{-/-}$ cells were kindly provided by Dr. Bert Vogelstein (John Hopkins). The antibodies used for immunoblotting and immunostaining experiments include the mouse antibody to ubiquitin (clone P4G7, MMS-258R, Covance), actin (SC-1616, Santa Cruz), Myc (clone 4A6, 05–724, Millipore), FLAG M2 (F3165, Sigma), mouse, rabbit, and goat antibodies to UbE2E1 (611218, BD, A-630, Boston Biochem and SC-475478, Santa Cruz), USP7 (A300-033, Bethyl), Histone H2A (07–146, Millipore), ubiquitin-Histone H2A Lys-119 (clone D27C4, 8240, Cell Signaling), p16 (Neomarkers), UbcH5/UbE2D (A-615, Boston Biochem), Ring1A (clone D2P4D, 13069, Cell Signaling), CBX2 (ab80044, ABCAM), BMI1 (5856, Cell Signaling), Ring1B (clone D22F2, 5694, Cell Signaling and Active Motif), and H3K4me2/3 (ab6000, ABCAM). These antibodies have previously been used in our and other studies (11, 36–40).

*Affinity Capture Proteomic Studies of UbE2E1*—FLAG-tagged UbE2E1-expressing cells were harvested and resuspended in 4× (w:v) lysis buffer (50 mM HEPES (pH 8.0), 100 mM KCl, 2 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, 1 mM PMSF, 1 mM DTT, and 1:500 protease inhibitor mixture (Sigma)). Resuspended cells were incubated with pre-equilibrated FLAG-M2-agarose beads (Sigma) for 2 h at 4 °C. Beads were pelleted by centrifugation at $1000 \times g$ for 1 min, and then washed once with 1 ml of lysis buffer, and two times with 1 ml of ammonium bicarbonate rinsing buffer (50 mM ammonium bicarbonate (pH 8.0), 75 mM KCl). Elution was performed by incubation with 150 $\mu$l of 125 mM ammonium hydroxide (pH 11.0) and repeated twice more. 1 $\mu$g of mass spectrometry grade L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-trypsin (Promega) dissolved in 70 $\mu$l of 50 mM ammonium bicarbonate (pH 8.3) was added to the eluate and incubated at 37 °C overnight. The resulting peptides were lyophilized and resuspended in buffer A (0.1% formic acid). Liquid chromatography (LC) analytical columns (75 $\mu$m inner diameter) and pre-columns (100 $\mu$m) were prepared in-house from fused silica capillary tubing from InnovaQuartz and packed with 100Å $C_{18}$-coated silica particles (Magic, Michrom Bioresources). Peptides were subjected to nanoflow LC-electrospray ionization (ESI)-tandem mass spectrometry (MS/MS), using a 120-min reversed phase LC (RPLC; 95% water-95% acetonitrile, 0.1% formic acid) buffer gradient running at 250 nl/min on a Proxeon EASY-nLC pump in-line with a hybrid LTQ-Orbitrap velos mass spectrometer (Thermo Fisher Scientific). A parent

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ion scan was performed in the Orbitrap using a resolving power of 60,000, then up to 40 of the most intense peaks were selected for MS/MS (minimum ion count of 1000 for activation), using standard CID fragmentation. Fragment ions were detected in the LTQ. Dynamic exclusion was activated such that MS/MS of the same *m/z* (within a −0.1 and +2.1 Th window; exclusion list size = 500) detected 3 times within 45 s were excluded from analysis for 60 s. For protein identification, Thermo.RAW files were converted to .mzXML format using Proteowizard (41), then searched using X!Tandem (42) against the human RefSeq (Version 37) database. X!Tandem search parameters were: complete modifications, none; cysteine modifications, none; potential modifications, +16@M and W, +32@M and W, +42@N terminus, +1@N and Q. Data were analyzed using the ProHits (43) and SAINT software tools (44).

*Immunoprecipitation*—Co-immunoprecipitations were performed using FLAG-tagged UbE2E1 and endogenous Ring1A, as well as endogenous UbE2E1 and Ring1B. Cells were harvested 48 h after transfection and lysed in RIPA buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate and protease inhibitor mixture (Roche Applied Science)). Lysates were incubated with antibodies against FLAG for FLAG-tagged UbE2E1 or Ring1B overnight at 4 °C followed by the addition of protein A beads for another 60 min. Immunoprecipitates were washed three times using RIPA buffer. The IP complexes were released by boiling the beads for 5 min in SDS sample buffer and resolved on 12% SDS-PAGE, followed by immunoblotting with specific antibodies against UbE2E1, Ring1A, or Ring1B.

*FLAG Pulldown Assay*—Cells were transfected with FLAG-tagged Ring1B and BMI1. The cells were harvested after 48 h and lysed in buffer containing 50 mM Tris (pH 8.0), 500 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate and protease inhibitor mixture (Roche Applied Science). The cell lysate was incubated with FLAG-agarose to capture the FLAG-tagged Ring1B·BMI1 complex. After extensive washing, 1 mg of UbE2E1 or GST as negative control were incubated with the FLAG-tagged Ring1B·BMI1-immobilized agarose beads. The captured proteins were eluted with 3× FLAG peptide and resolved on 12% SDS-PAGE, followed by immunoblotting with specific antibodies against Ring1B, BMI1, UbE2E1, and GST.

*In Vitro Ubiquitination Assays*—Ubiquitination of H2A was performed in a volume of 20 μl by incubating E1 (100 ng), E2 (UbE2E1 or UbE2D2, 200 ng), ubiquitin (5 μg), FLAG-tagged wild-type or mutant Ring1B·BMI1 complex, and 0.5 μg of reconstituted nucleosome in 50 mM Tris (pH 7.6), 5 mM $MgCl_2$, 2 mM ATP, and 2 mM DTT at 30 °C for 90 min. The reaction was stopped by the addition of 5 μl of SDS-PAGE sample buffer and separated by 15% SDS-polyacrylamide gels. Ubiquitinated proteins were visualized and evaluated by Western blotting using specific antibodies against Ring1B, H2A, and uH2AK119. The proteins used in this assay were prepared as follows. E1 was expressed as $His_6$-tagged fusion protein using baculovirus-infected insect cell systems. E2s and ubiquitin were expressed as $His_6$-tagged fusion protein in *Escherichia coli*. E1, E2s, and ubiquitin were purified using affinity chromatography and prepared for assay as described previously (27). Recombinant nucleosome was prepared as previously described (46). The protein concentrations were estimated using the Bradford protein assay (Bio-Rad).

*Overexpression of UbE2E1 and UbE2E1_C131A*—Cells were transfected with UbE2E1 and UbE2E1_C131A. The cells were harvested 48 h after transfection and lysed. The lysate was resolved on 12% SDS-PAGE, followed by immunoblotting with specific antibodies against uH2A, H2A, and methylated H3K4.

*siRNA Knockdown of UbE2E1, UbE2Ds, or USP7*—Cells were transfected with siRNAs for UbE2E1 (5′-GACCAAGAGA-UACGCUACA and 5′-GUGUAUUCUUUCUCGAUAU-3′, Genepharma), with siRNAs for UbE2Ds (5′-CAGUAAUG-GCAGCAUUUGUTT-3′ and 5′-GAUCACAGUGGUCGC-CUGCTT-3′, Genepharma), with siRNA for USP7 (5′-CCCAAAUUAUUCCGCGGCAAA-3′, Genepharma) or a negative control siRNA (provided by Genepharma). siRNA was transfected into cells using Lipojet (SignaGen) according to the manufacturer's protocol and cells were harvested 72 h post-transfection. The lysate was resolved on 12% SDS-PAGE, followed by immunoblotting with specific antibodies as indicated.

*Histone Extraction*—The cells were lysed in 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1.5 mM PMSF, 1 mM *N*-ethylmaleimide (NEM), and 10 μM ubiquitin aldehyde followed by the addition of hydrochloric acid (0.2 M final concentration) and incubated on ice for 30 min (34). The lysate was centrifuged and the soluble fraction was dialyzed against 0.1 M acetic acid followed by distilled water. The purified histones were analyzed by immunoblotting with antibodies against uH2AK119, H3K4me2/3, and H2A.

*$p16^{INK4A}$ mRNA Expression Analysis*—Total cellular RNA was extracted with TRIzol reagent (Invitrogen). RT-PCR was performed with 2 μg of total RNA using the ThermoScript™ RT-PCR kit (Invitrogen). The levels of $p16^{INK4A}$ mRNA and GAPDH mRNA were detected by quantitative PCR using the following primer pairs: 5′-GACCCCGCCACTCTCACC-3′ and 5′-CCTGTAGGACCTTCGGTGACTGA-3′ for $p16^{INK4A}$, and 5′-AAGGTCATCCCTGAGCTGAAC-3′ and 5′-ACGC-CTGCTTCACCACCTTCT-3′ for GAPDH. The $p16^{INK4A}$ expression levels were normalized to that of GAPDH.

*Growth Curves and Senescence-associated β-Galactosidase Assay*—Cell growth was measured by seeding equal numbers of each cell line or experimental condition in triplicate for each day of the experiment, followed by daily harvest and counting using a Coulter counter. The total numbers of cells were plotted *versus* day to generate a growth curve. Cells were prepared for β-galactosidase staining by fixation with PBS formaldehyde/glutaraldehyde equilibrated to pH 6 and stained for β-galactosidase activity as described (47).

*Chromatin Immunoprecipitation and Quantitative PCR*—Cells were either treated with UbE2E1 siRNA or its control, and harvested 72 h after transfection. Nuclear pellets were generated with 0.2% Triton X-100 and treated with 20 mM *N*-ethylmaleimide to block endogenous deubiquitinase activity. The pellets were washed twice and resuspended in cutting buffer (10 mM Tris (pH7.5), 60 mM KCl, 15 mM NaCl and 3 mM $CaCl_2$), followed by a 5-min treatment with monococcal nuclease (Worthington) stopped by the addition of EDTA. The supernatant was collected (S1) and the nuclear pellet was further incubated to release the poynucleosomal fraction (S2). The DNA

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concentration was determined using 260/280 nm of the S2 fraction and used to allow equal inputs in the ChIP. S2 extracts were incubated with anti-uH2A or rabbit IgG overnight, followed by precipitation with protein A beads and washed with PBS containing 0.5% Nonidet P-40. The IP complex was dissociated with 1% SDS and DNA was precipitated using a phenol/chloroform extraction with glycogen as a carrier. Equal amounts of DNA (8 $\mu$g) from each ChIP fraction or input DNA (DNA extracted from the S2 fraction) were used as template in quantitative PCR using So fast master mix (Bio-Rad) and the following primer sets: Primer set 1, 4.5 kb downstream of *ARF* promoter (GGAGCGATGTGATCCGTTATC and TGAAATCCCAATCGTCTTCCAC); Primer set 2, 1 kb upstream of *INK4A* promoter (CTCAAAGCGGATAATTCAAGAGC and AAGCCTTAAGAACAGTGCCACAC); Primer set 3, Exon 2 of *INK4A* (CAAGCTTCCTTTCCGTCATGC and GCCAGAGAGAACAGAATGGTCAGAGCCA); and Primer set 4, 1.5 kb downstream of exon 3 (TGTCTACCCAACACTTCCCTGC and AAGGCAAAGGTAACTGAAACGC) (45).

*Author Contributions*—K. K. W., F. S., B. S. J., H. D., O. E., and Y. S. performed the experiments, acquired and analyzed the data. L. K. contributed nucleosome. B. R. acquired and analyzed the mass spectrometry data. Y. S. and V. S. designed the study, supervised the trainees, and prepared the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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