IDENTIFICATION AND CHARACTERIZATION OF A NEW E1 THAT ACTIVATES UBIQUITIN AND FAT10

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TO MY PARENTS, CHIN-HSIN AND LI-LUNG,

AND MY HUSBAND, HON-REN

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Ubiquitination is one of many post-translational modifications in eukaryotes. Three enzymes (E1, E2, and E3) are involved in conjugating ubiquitin to protein substrates. I identified a novel E1-like protein, E1-L2, which is homologous to the ubiquitin E1 and another E1 involved in the activation of the ubiquitin-like protein ISG-15 (E1-L1). E1-L2 activates both ubiquitin and FAT10, a ubiquitin-like protein. Interestingly, E1-L2 can transfer ubiquitin to only a subset of E2 enzymes, Ubc5 and Ubc13, but not Ubc3 and E2-25K, suggesting that E1-L2 may function in certain ubiquitination reactions. E1-L2, but not E1 or E1-L1, forms a thioester with FAT10 in vitro. The formation of the thioester bond requires

the active site cysteine residue of E1-L2 and the C-terminal diglycine motif of FAT10. In addition, endogenous FAT10 forms a thioester with E1-L2 in cells stimulated with tumor necrosis factor-alpha and interferon-gamma, which induce FAT10 expression. Silencing of E1-L2 expression by RNAi impaired the formation of FAT10 conjugates in cells. Furthermore, E1-L2 deficient embryos died before embryonic day 13.5, suggesting that E1-L2 is essential for early embryonic development. Since the FAT10-deficient mice develop normally, it is likely that specific ubiquitination reactions catalyzed by E1-L2 play an important role in animal development.

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1. **Chiu, Y-H.,** and Chen, Z.J. DNA induces MAVS-mediated antiviral response through generating RNA intermediates (in preparation).
2. **Chiu, Y-H.**, Sun, Q., and Chen, Z.J. (2007) E1-L2 activates both ubiquitin and FAT10, *Molecular Cell*, **27**:1014-1023.
3. Kanayama, A., Seth, R.B., Sun, L., Ea, C-K., Hong, M., Shaito, A., **Chiu, Y-H.**, Deng, L., and Chen, Z.J, (2004) TAB2 and TAB3 activate the NF-κB pathway through binding to polyubiquitin chains, *Molecular Cell*, **15**:535-548.

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LIST OF ABBREVIATIONS

E1: the activating enzyme

E1-L1: E1-like 1 protein

E1-L2: E1-like 2 protein

E2: the conjugating enzyme

E3: the ubiquitin-like protein ligase

FAT10: HLA-F adjacent transcript 10

GST: glutathione S-transferase

HA: Hemagglutinin

IFNγ: interferon gamma

ISG15: Interferon-stimulated gene 15

NUB1: NEDD8 ultimate buster protein 1

RanGAP1: Ran GTPase-activating protein

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

TNFα: tumor necrosis factor alpha

TRAF6: tumor necrosis factor associated factor 6

Ub: ubiquitin

Ubc3: ubiquitin-conjugating enzyme 3

Ubc5: ubiquitin-conjugating enzyme 5

Ubc13: ubiquitin-conjugating enzyme 13

Ubls: Ubiquitin-like proteins

Uev1A: ubiquitin-conjugating enzyme E2 variant 1 isoform A

CHAPTER I: GENERAL INTRODUCTION

I.A Ubiquitination

I.A.1 The mechanism of ubiquitination

Ubiquitin is a 76-amino acid polypeptide that is highly conserved in all eukaryotes. There are only three amino acid differences between the yeast and human homologues. Ubiquitin works as a protein modifier by forming a covalent attachment to its substrates. This covalent modification is usually referred to as ubiquitination (Pickart 2001; Johnson 2002). Ubiquitination plays an important role in nearly all aspects of physiology including cell division, the immune response, development, endocytic trafficking, biosynthetic pathways and cancer development. The conjugation of ubiquitin to its substrate is through an isopeptide bond which is formed between the carboxyl group of the diglycine motif at the C-terminus of ubiquitin and the ε-amino group of a lysine residue on the substrate. There are three sequential steps catalyzed by activating (E1), conjugating (E2), and ligase (E3) enzymes in ubiquitination (Figure 1) (Pickart 2001). In the first step, ubiquitin interacts with ATP to form a Ub-adenylate intermediate and then reacts with the cysteine residue of ubiquitin E1 enzyme to form a E1~Ub thioester bond. In the next step, the ubiquitin is transferred to the second enzyme, E2. The interaction between ubiquitin and E2 is also mediated by a thioester bond (shown as E2~Ub in Figure 1). In the last step of ubiquitination, with the facilitation of E3 enzyme, ubiquitin forms an isopeptide linkage to a lysine residue of the substrate. Ubiquitin itself contains seven lysine residues, and therefore ubiquitin can

also serve as a substrate. One of these lysine resides on ubiquitin can be further conjugated with another ubiquitin and reiteration of this process results in the formation of a polyubiquitin chain.

Previously, only a single ubiquitin-activating enzyme (ubiquitin E1), encoded by the *UBE1* gene, was identified to promote ubiquitination reactions. Ubiquitin E1 enzyme contains two adenylation domains (A), a catalytic cysteine domain (CC) and a ubiquitin-fold domain (UFD) (Figure 2A). There is an ATP motif embedded in the second adenylation domains. Ubiquitin E1 uses ATP hydrolysis to provide the chemical potential needed for the subsequent transfer steps in ubiquitination (Huang, Paydar et al. 2005; VanDemark and Hill 2005). There are two steps involved in the ubiquitin-activating process. First, ubiquitin is adenylated at the C terminus. Followed by the activation step, ubiquitin E1 catalyzes a second reaction to form a covalent thioester intermediate between the catalytic cysteine in the catalytic cysteine domain of ubiquitin E1 and the C terminus of ubiquitin. Therefore each fully loaded E1 holds two activated ubiquitins. One is adenylated and the other forms a thioester with ubiquitin E1 enzyme (Figure 2B) (Pickart 2001). Structural and biochemical studies of the activating enzyme for the ubiquitin-like protein NEDD8 (NEDD8 E1) provide insights into the mechanism of the E1 reaction cycle. The E1 for NEDD8 is a heterodimer of APPBP1 and UBA3. This NEDD8 E1 has been shown to recognize the E2 enzyme for NEDD8 specifically. The catalytic core domain of NEDD8 E2 interacts with the C terminus of UBA3 where the ubiquitin-fold domain is located (Huang, Paydar et al. 2005). Thereby the ubiquitin-fold domain is also named E2-binding domain based on its function. This model of E1-E2 complex might be able to apply to ubiquitin and it defines the relative

position of the E1 and E2 cysteine residues where the transfer of ubiquitin is taking place. The final product of this E1-catalyzed reaction is a covalent E2~Ub thioester intermediate.

E2 enzymes have a catalytic core domain (UBC domain) which contains around 150 amino acid residues. The catalytic core domain is a common feature of E2s for different ubiquitin-like proteins. Usually, the E2 proteins consist exclusively of a catalytic core domain (class I E2). However, some E2 enzymes have additional N-, C-, or N- and C-terminal extensions beyond the core domain (class II, III, and IV E2s), which may have some effects on E2 activity regulation, E3 association or substrate binding (Figure 3) (Pickart 2001; Plafker, Plafker et al. 2004). The catalytic core domain is sufficient for binding E1, forming an E2~Ub thioester intermediate, associating with E3-target complex, and transferring ubiquitin (Huang, Paydar et al. 2005). The structures of several E2 proteins have been solved. These studies reveal an overall similarity between those E2 proteins, and there is no dramatic conformational change after E2s form complexes with other proteins (VanDemark and Hill 2002). The ultimate function of E2 is to ligate ubiquitin to the lysine residue of its target proteins. Some E2 proteins can directly transfer ubiquitin to the substrate, but usually a third step including E3 enzymes is necessary for adding ubiquitin to the target proteins. The E1 and E3 binding sites on an E2 might be overlapping. The unconjugated form of E2 has low affinity for free ubiquitin and unloaded E1, but has high affinity for loaded E1 (Hershko, Heller et al. 1983; Haas, Bright et al. 1988; Miura, Klaus et al. 1999). Some studies suggest that E3s show high affinity to E2~Ub thioester intermediate and low affinity to unconjugated E2 (Kawakami, Chiba et al. 2001; Siepmann, Bohnsack et al. 2003). Therefore, once E1 is loaded with ubiquitin, the affinity between E1 and E2 is increased, bringing these two

enzymes in close proximity and allowing the transfer of ubiquitin. The E3 proteins are attracted to the E2~Ub thioester intermediate and compete with loaded E1. The inferred competitive binding might set up the sequence of the reactions in ubiquitination and enhance the efficiency of transferring E2 from E1 to an E3 complex (Huang, Paydar et al. 2005).

While E1, E2 and deubiquitination enzymes can be recognized by the highly conserved domains, E3 enzymes are structurally diverse. Basically, E3s can be classified into three groups by the conserved domains, homology to the E6-AP carboxyl terminus (HECT), really interesting new gene (RING) and UFD2 homology (U-box) domains (Pickart and Eddins 2004). The HECT domain contains 350 amino acids, including a conserved cysteine residue. This cysteine residue is capable of forming a thioester intermediate with ubiquitin in the presence of E1 and E2 enzymes and then transferring ubiquitin to the substrate. HECT E3s transfer ubiquitin to the target proteins not only through the interaction of binding with E2 and the substrates, but also via catalysis. The first identified HECT E3 is E6 associated protein (E6-AP). The N-terminus of E6-AP recruits the target proteins while the HECT domain of E6AP binds to the E2~Ub intermediate and transfers ubiquitin from E2 to the conserved cysteine residue at ~35 residues upstream of the C-terminus of E6-AP (Pickart and Eddins 2004). This process results in an E6AP~Ub thioester intermediate. On the other hand, none of the RING E3s are reported to be capable of forming a thioester intermediate with ubiquitin. The RING E3s feature the RING finger domain and are the largest one among the three groups of E3s. The RING finger domain contains a short motif rich in conserved cysteine and histidine residues ($C\text{-}X_2\text{-}C\text{-}X_{9\text{-}39}\text{-}C\text{-}X_{1\text{-}3}\text{-}H\text{-}X_{2\text{-}3}\text{-}C/H\text{-}X_2\text{-}C\text{-}X_{4\text{-}48}\text{-}C\text{-}X_2\text{-}C$) (Saurin, Borden et al. 1996). The conserved cysteine and histidine residues in this motif form

a unique cross-brace arrangement to chelate two zinc ions (Saurin, Borden et al. 1996). The role of the RING finger domain in ubiquitination was first recognized in the observation of a plant N-end rule E3 Prt1. The RING finger domain of plant N-end rule E3 Prt1 is similar to yeast proteins Ubr1p, Hrd1p/Der3p, Rad18p and Apc11p which are known to be involved in ubiquitination (Potuschak, Stary et al. 1998). The RING finger domain was found to be capable of binding to E2s (Lorick, Jensen et al. 1999; Seol, Feldman et al. 1999; Zheng, Schulman et al. 2002). Some RING finger domain containing proteins need to form a protein complex with other molecules in order to obtain their ubiquitin ligase activity. These RING E3s include the Skp1, cullin, F-box (SCF) E3, the von Hippel-Lindau (VHL) E3 and the anaphase promoting complex (APC) (Jackson, Eldridge et al. 2000). For example, a subtype of RING E3s, such as Rbx1/Roc1, is known as the core component of multi-subunit E3 complexes named the SCF complexes which contain Skp1, Cul1, and an F-box protein that recognizes a specific protein substrate (Figure 4) (Deshaies 1999). Skp1 and Cul1 serve as bridge to link RING finger protein to F-box protein. Therefore the E2~Ub intermediate and the substrate can be brought together by binding to RING finger proteins and F-box proteins, respectively. Other RING E3s, like tumor necrosis factor associated factors (TRAFs), are capable of promoting ubiquitination in vivo without association with other complexes. Working alone, these E3 proteins can bring together the target proteins and the E2~Ub intermediate. The U-box domain consists of ~70 amino acids and is found in proteins from yeast to humans. The predicted three-dimensional structure of the U-box is similar to that of the RING-finger except that the structure of the U-box domain lacks the metal-chelating residues of the RING finger domain (Hatakeyama and Nakayama 2003). Yeast UFD2, for

example, binds to its substrates with only a few ubiquitin molecules and promotes multiubiquitin-chain assembly in the presence of E1, E2, and E3 enzymes. Therefore, UFD2 is also considered as an "E4" enzyme (Hoppe 2005). However, several U-box proteins have been shown to mediate ubiquitination in conjunction with E1 and E2 and in the absence of other E3 proteins. These findings define U-box proteins as a third type of E3 enzyme, in addition to the HECT and RING E3s. The E4 activity might be a specialized type of E3 activity that targets oligoubiquitinated proteins as substrates (Hoppe 2005).

I.A.2 The function of ubiquitination

Ubiquitination and another covalent modification named phosphorylation are similar in certain respects (Wong, Parlati et al. 2003). Phosphorylation is one of the best-known regulatory processes in biology. First, like phosphorylation, ubiquitination is also reversible. Phosphorylation can be reversed by a set of enzymes referred to as protein phosphatases. This process is called dephosphorylation, while the similar reaction in ubiquitination is named deubiquitination. After ubiquitination, ubiquitin can be removed from its substrate by members of a large family of enzymes known as isopeptidases or deubiquitination enzymes (Dubs) (Wong, Parlati et al. 2003). Second, both phosphorylation and ubiquitination happen rapidly, usually within seconds or minutes after stimulation. In the presence of ATP, the enzymes involved in these two modifications work efficiently in response to the stimuli. Third, these two protein modifications are very specific and are strongly regulated by a very diverse set of enzymes. After the completion of the human genome project, more than 500 protein kinases and 120 protein phosphatases have been revealed. For ubiquitination, there

are more than 40 E2s, 500 different E3s and 80 different Dubs predicted in the human genome. These findings suggest that in order to promote or reverse either protein modification of a target protein efficiently, a specific combination of enzymes are required. Fourth, both phosphorylation and ubiquitination can be recognized by specialized domains which bind to phospho-amino acids or to ubiquitin. Proteins containing phosphotyrosine can be detected by SH2 and PTB domains (Schlessinger and Lemmon 2003). The phophoserine or phosphothreonine in specific sequences can be recognized by a FHA and a WD repeat domain (Yaffe and Elia 2001). For ubiquitin and polyubiquitin chains, several different domains have been identified to be used for recognition. These domains include UIM (Ub-interacting motifs), CUE (Cue1-homologous), UBA (Ub-association), UEV (Ub E2 variant), PAZ (polyubiquitin-associated zinc finger), NZF (novel zinc finger), GLUE (GRAM-like ubiquitin binding in Eap45), GAT (Gga and TOM1), and Vps27/HRS/STAM (VHS) (Schnell and Hicke 2003; Hicke, Schubert et al. 2005). The variety of these ubiquitin-binding domains suggests that like phosphorylation, ubiquitination may be involved in many different cellular regulatory mechanisms which are not fully yet discovered.

Based on these four points, ubiquitin can be considered as a phosphate-like group which can be rapidly covalently conjugated to other proteins. Moreover, ubiquitination is reversible and tightly regulated. The target proteins modified by ubiquitin would change their stability, localization and activity. In some cases, phosphorylation and ubiquitination can occur in the same signal transduction pathway and even operate on the same protein. For example, nuclear factor kappa B (NF-κB) is inhibited by the inhibitor of NF-κB (IκB) in the resting state. When cells receive certain stimuli, such as tumor necrosis factor-α (TNFα),

interleukin-1β (IL-1β), or pathogen-associated molecular patterns (Pumps), several cellular molecules are activated which leads to the activation of IκB kinase complex (IKK). The IKK kinase complex contains two catalytic subunits, IKKα and IKKβ, and the regulatory subunit, IKKγ. After IKK is activated, IKKβ rapidly phosphorylates IκB at two serine residues in the N-terminus, which enhances the ubiquitination of IκB. The ubiquitinated IκB is then brought to the 26S proteasome for degradation. Therefore the inhibitory effect of NF-κB is abolished and NF-κB enters the nucleus to work as a transcription factor to activate a large set of target genes (Figure 5) (Ghosh, May et al. 1998; Silverman and Maniatis 2001).

Ubiquitin contains seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) (Figure 6A). Each lysine can be used as ubiquitination sites for further conjugation by another ubiquitin through ubiquitination machinery, thereby forming a polyubiquitin chain (Figure 6B) (Pickart 2001; Peng, Schwartz et al. 2003). These seven polyubiquitin linkages named K6, K11, K27, K29, K33, K48 and K63-linked polyubiquitin chains have been found in the budding yeast proteome (Peng, Schwartz et al. 2003). Different linkages of polyubiquitin chains may play different roles in cellular regulatory mechanisms including protein degradation, endocytosis and sorting, histone modification, transcription, DNA repair, DNA replication, kinase activation and signal transduction (Schnell and Hicke 2003; Sun and Chen 2004; Welchman, Gordon et al. 2005). With the technology of mass spectrometry, the ubiquitination sites of the target proteins could be accurately and directly identified, which could facilitate understanding of the regulatory mechanism of the target proteins.

The best-characterized function of ubiquitin is its role in protein degradation. Polyubiquitinated proteins with K48 linkage are directed to the 26S proteasome, a large ATP-dependent protease complex consisting of a 20S catalytic core and two 19S regulatory complexes, and then the proteins are degraded to peptides. The ubiquitin conjugated to the protein is modified by another ubiquitin through the isopeptide bond between the lysine 48 residue of one ubiquitin and the C-terminal carboxyl group of the next ubiquitin in chain. This ubiquitin-proteasome machinery is the basic mechanism for turnover of normal short-lived proteins and a single K48-linked polyubiquitin chain modification is sufficient to bring the substrate to the 26S proteasome (Chau, Tobias et al. 1989). The K48-linked ubiquitination-mediated protein degradation works in destruction of misfolding proteins, turnover of short-lived proteins and also in the degradation of the molecules in signal transduction pathway. By degrading the activators or the inhibitors, K48-linked polyubiquitin can change the state of cells upon stimulation.

K63-linked polyubiquitin chains have different functions than protein degradation. It has been shown that K63-linked polyubiquitin chain is involved in kinase activation, vehicle trafficking and DNA repair (Sun and Chen 2004). For example, K63-linked ubiquitination can regulate IKK activation in a proteasome-independent manner (Sun and Chen 2004). K63-linked ubiquitination chains are synthesized by the Ubc13 and Ubc-like protein Uev1A, which are E2 enzymes, and TRAF6, which serves as a ubiquitin ligase (Deng, Wang et al. 2000). One of the targets of these K63-linked polyubiquitination chains is TRAF6 itself. The ubiquitination of TRAF6 is induced by dimerization or oligomerization and then the ubiquitinated TRAF6 binds to a protein kinase complex consisting of TAK1, TAB1 and

TAB2, which leads to the activation of this protein kinase complex and the downstream molecules (Wang, Deng et al. 2001). TAB2 binds preferentially to K63-linked polyubiquitination chain (Kanayama, Seth et al. 2004). The polyubiquitination chain might serve as a scaffold to bring proteins with the ubiquitin binding domain together and then activate those proteins.

Although proteins are usually polyubiquitinated, some are modified by a single ubiquitin on one or multiple lysines. Monoubiquitination is not normally involved in targeting proteins for degradation by the proteasome. Instead, it is a signal for DNA repair, gene silencing, receptor internalization, vehicle sorting, and lysosomal degradation of plasma membranes proteins (Hicke 2001). Ubiquitination has been reported to be involved in several steps in endocytosis (Dupre, Urban-Grimal et al. 2004). During endocytosis, cargos enter into primary endocytic vesicles and then bud from the plasma membrane. It has been shown that ubiquitination of the cargo is required for proper vesicle sorting (Katzmann, Odorizzi et al. 2002; Raiborg, Rusten et al. 2003). Ubiquitination also works in transcriptional regulation. Monoubiquitination of histone H2B is involved in transcriptional initiation and elongation, like regulating chromatin structure and enabling a series of lysine methylations on another core histone, histone H3 (Xiao, Kao et al. 2005), while polyubiquitination is required for activating a subset of transcriptional activators (Lipford and Deshaies 2003; Muratani and Tansey 2003). Another example is DNA repair. Several proteins, like the proliferating cell nuclear antigen (PCNA), are ubiquitinated upon DNA damage. PCNA is a protein that assembles into a trimeric ring that encircles DNA and recruits DNA polymerase during DNA replication and post-replicative DNA repair (Hoege, Pfander et al. 2002). PCNA contains an

accessible lysine residue for ubiquitination on the outside rim of the trimeric ring, which is a good position for recruiting distinct factors of the DNA synthesis and repair machinery. It is reported that the monoubiquitination of PCNA catalyzed by Rad6 (an E2) and Rad18 (a RING-domain E3) is required for error-prone DNA repair and the K63-linked polyubiquitination of PCNA generated by Ubc13/Mms2 (E2) and Rad5 (a RING-domain E3) is essential for error-free repair of the damage DNA (Hoege, Pfander et al. 2002).

Even though the current model of ubiquitination shows the modification is mediated through the lysine residue of the target protein, some studies report that when the protein lacks lysine residues, other amino acid residues can be targeted as ubiquitination sites (Ikeda, Ikeda et al. 2002; Cadwell and Coscoy 2005). For example, even when all the lysine residues in the MHC I intracytoplasmic tail are mutated to arginines, these mutant proteins can still be ubiquitinated and down-regulated in the presence of the specific E3 enzyme, the modulators of immune recognition 1 (MIR1) (Cadwell and Coscoy 2005). This kind of ubiquitination requires a cysteine residue in the intracytoplasmic tail of MHC I molecules. Thus, when the lysine resides are not available, ubiquitination may still occur on other amino acid residues of the target proteins.

I.B Ubiquitin-like modification

I.B.1 Ubiquitin-like proteins

Besides ubiquitin, several ubiquitin-like proteins (Ubls) have been identified. These proteins structurally resemble ubiquitin and also share similar mechanisms of substrate

conjugation with ubiquitin. Ubls have a wide range of functions, which are usually via substrate modification (Table 1 and Figure 7) (Kerscher, Felberbaum et al. 2006). For example, ISG15 is involved in antiviral interferon response (Haas, Ahrens et al. 1987; Loeb and Haas 1992; Narasimhan, Potter et al. 1996; Nicholl, Robinson et al. 2000; Yuan and Krug 2001; MacQuillan, Mamotte et al. 2003). SUMOs are linked to cell division, nuclear transport, the stress response and signal transduction (Hay 2001; Johnson and Gupta 2001; Muller, Hoege et al. 2001; Takahashi, Kahyo et al. 2001; Tatham, Jaffray et al. 2001; Pichler, Gast et al. 2002; Schmidt and Muller 2002). NEDD8 (Rubl in *Saccharomyces cerevisiae*) can activate SCF E3 complex and is involved in cell cycle control, signaling and embryogenesis (Lammer, Mathias et al. 1998; Liakopoulos, Doenges et al. 1998; Pozo, Timpte et al. 1998; Jones and Candido 2000; Tateishi, Omata et al. 2001; Kurz, Pintard et al. 2002). Atg8 (also called Aut7 and Cvt5) modifies the lipid phosphatidylethanolamine to regulate membrane dynamics (Mizushima, Noda et al. 1998; Ichimura, Kirisako et al. 2000). Hub1 plays a role in cell polarization by modifying cell polarity factors (Dittmar, Wilkinson et al. 2002; Huang, Walden et al. 2004). Ubls are linked to their substrates by the isopeptide bond between the diglycine residues of the C-terminal carboxyl group of the Ubls and the lysine residue of the target proteins. The conjugation machinery for Ubls are also mediated by E1, E2, and E3 enzymes. The E1 enzymes for ubiquitin, ISG15, NEDD8 and SUMOs are highly related to each other. These E1 proteins are either a single protein, like the E1 for ubiquitin and ISG15 (Ube1 and Ube1L), or a heterodimeric complex, like the ones for NEDD8 and SUMO (APPBP1/UBA3 and SAE1/SAE2) (Figure 8). One subunit of the heterodimeric E1 complex corresponds to the N-terminal half of the single-chain E1, while the other subunit

corresponds to the C-terminal half. Both single-chain and heterodimeric E1s contain a ATP-binding motif and a conserved cysteine residue which is involved in forming the thioester bond with their cognate Ubls (Huang, Walden et al. 2004).

Interferon (IFN)-stimulated gene 15 (ISG15) is a 15kDa protein induced by interferon, lipopolysaccharide (LPS) stimulation or viral infection. It is also called ubiquitin cross-reactive protein (UCRP) due to its cross-reactivity with ubiquitin antibodies (Staub 2004). Unlike ubiquitin which can be found from yeast to human, ISG15 only exists in vertebrates and is about 47% conserved among mammalian species. ISG15 contains two domains, each of which shares around 30% identity to ubiquitin (Staub 2004). ISG15 is synthesized as a 17 kDa precursor. In order to work as a modifier, ISG15 needed to be processed into a 15 kDa protein by a specific protease to expose the diglycine motif at the C-terminus, which is used to conjugate its substrate. ISG15 functions as a modifier like other Ubls (Loeb and Haas 1992) or as a free peptide with immunoregulatory properties (Recht, Borden et al. 1991). The process of ISG15 modifying its target proteins is referred as ISGylation. Several enzymes involved in ISGylation have been identified, such as UbE1L (the activating enzyme, E1), UbcH8 (the conjugating enzyme, E2), Herc5, Efp (ISG15 ligase, E3) and UBP43 (deconjugating enzyme). UbE1L was cloned as a protein homologous to the ubiquitin-activating enzyme and is expressed in normal lung cells, but not in 14 human lung cancer cell lines that have been tested (McLaughlin, Helfrich et al. 2000). Ube1L was shown to be an ISG15 interacting protein by GST-ISG15 pull-down experiments. The interaction between Ube1L and ISG15 is mediated by a thioester bond. Ube1L specifically catalyzes the activation step in ISGylation, but not in ubiquitination, and functions as E1 enzyme for

ISG15 (Yuan and Krug 2001). The other evidence for Ube1L being the E1 for ISG15 has been shown in K562 cells (Malakhova, Yan et al. 2003). ISG15 is induced in K562 cells upon interferon stimulation as unconjugated monomers. However, ISG15 conjugation is not detected in these cells. Reconstitution of Ube1L can restore protein ISGylation in K562 cells. UbcH8 was purified as an E2 enzyme by a biochemical approach using radiolabeled ISG15 (Zhao, Beaudenon et al. 2004). UbcH8 is also involved in ubiquitination. In the in vitro assay, ISG15 can be transferred to UbcH8 in the presence of Ube1L to form a UbcH8~ISG15 thioester intermediate, whereas UbcH8~Ub was observed when ubiquitin and ubiquitin E1 were used in the assay. RNA interference experiments show that UbcH8 is the major ISG15 conjugating enzyme in HeLa cells treated with interferon-β. The similarity of ubiquitination and ISGylation by sharing UbcH8 suggests that the E3 enzymes involved in ISGylation might be the ubiquitin E3 which is known to be associated with UbcH8. A HECT protein, Herc5, and a RING E3, Efp, have been reported as the E3 enzymes for ISG15 (Dao and Zhang 2005; Dastur, Beaudenon et al. 2006). These two enzymes are interferon-β inducible. Although Herc5 and Efp have been shown to function with ubiquitin, both E3 enzymes are able to conjugate ISG15 to target proteins in the presence of Ube1L and UbcH8.

Even though the E1, E2, and some of the E3 enzymes involved in the ISGylation machinery have been identified, a physiological role for ISGylation is still not clear. ISG15, Ube1L, and UbcH8 are all induced by interferon treatment. Therefore, ISGylation can be observed predominantly upon interferon-β stimulation and may be involved in the antiviral pathway as one of the interferon inducing proteins. Uncovering the substrates for ISG15 might provide some insights into the function of ISGylation. Around 158 proteins have been

identified as candidates for the target proteins of ISG15 by using modified tandem affinity purification (TAP)-tagged ISG15 (Zhao, Denison et al. 2005). Some of the proteins are interferon-α/β inducible antiviral proteins, like PKR, MxA, HuP56, and RIG-I. Most of the proteins are constitutively expressed and function in diverse pathways, including RNA splicing, chromatin remodeling/polymerase II transcription, cytoskeleton organization and regulation, stress responses, and translation (Zhao, Denison et al. 2005). Another implication of ISGylation being involved in an antiviral pathway is the study of influenza B virus NS1 protein (NS1B). Free ISG15 is strongly induced by influenza B virus, but not by influenza A virus. However, a specific region of NS1B protein, which contains a part of its effector domain, inhibits the conjugation of ISG15 to its substrates. The inhibitory effect of NS1B is mediated by its binding to free ISG15 proteins and blocking ISGylation at the first activation step by interrupting the thioester bond formation between ISG15 and Ube1L (Yuan and Krug 2001).

After interferon stimulation, ISG15 is found in cells both in the free and conjugated forms. ISG15 can be detected in the extracellular environment, which suggests that ISG15 can be secreted or released from the cells (Knight and Cordova 1991). This property of ISG15 is unique to other Ubls. It has been reported that free ISG15 can stimulate interferon-γ production by $CD3^+$, but not $CD14^+$ or $CD56^+$ cells (Recht, Borden et al. 1991; D'Cunha, Ramanujam et al. 1996). With $CD3^+$ cells, ISG15 promotes the proliferation of $CD56^+$ natural killer cells and increases non-major histocompatibility complex-restricted cytotoxicity (D'Cunha, Ramanujam et al. 1996). In this case, ISG15 can be considered as a cytokine. Another studies show that ISG15 is constitutively expressed and secreted from

melanoma cells, which causes E-cadherin expression on tumor dendritic cells and likely changes the migratory behavior of dendritic cells to escape tumor immune surveillance as a negative regulator (Padovan, Terracciano et al. 2002). These reports support the idea that without being conjugated to other proteins, ISG15 itself can work as a functional protein.

Small ubiquitin-related modifier (SUMO) is one of the most well studied Ubls. The conjugation of SUMO (SUMOylation) is involved in several cellular pathways including cell-cycle control, nuclear transport, chromosome segregation, and transcriptional regulation. Three SUMO isoforms are found in human: SUMO-1, -2 and -3. SUMO-2 and SUMO-3 share 97% identity and are distinct from SUMO-1. There is a C-terminal extension after the diglycine motif in SUMOs, which can be processed. Like other Ubls, the exposure of this diglycine motif is required for protein conjugation. It is believed that the sumoylation motif ([I/V/L]-K-X-[D/E], X: any amino acid) around Lys11 in SUMO-2 and -3 is essential for polySUMO-chain formation. This sumoylation motif is lacking in SUMO-1, therefore SUMO-1 is incapable of forming polymers (Welchman, Gordon et al. 2005). The identified enzymes involved in SUMOylation are SAE1/SAE2 (activating enzyme, E1), UbcH9 (conjugating enzyme, E2), RanBP1, PIAS, the poly comb protein Pc2 (SUMO ligase, E3) and Ulp1 (deconjugating enzyme) (Welchman, Gordon et al. 2005). Ulp1 is not only involved in deSUMOylation, but also in processing of SUMO precursors (Welchman, Gordon et al. 2005). Ran GTPase-activating protein (RanGAP1) is the first identified substrate for SUMO-1, which is essential for nucleocytoplasmic trafficking (Schwartz and Hochstrasser 2003). The SUMOylation of RanGAP1 by RanBP1 is required for its localization to the nuclear pore complex and facilitates nuclear protein import. The substrates

for SUMO-2 and SUMO-3 include CCAAT/enhancer-binding protein-β1 (C/EBPβ1) and topoisomerase II (Recht, Borden et al. 1991; D'Cunha, Ramanujam et al. 1996; Azuma, Arnaoutov et al. 2003; Eaton and Sealy 2003). SUMOylation of C/EBPβ1 negatively regulates its transcriptional activity from cyclin-D promoter. The modification of topoisomerase II has no effect on its enzyme activity, but plays an important role on repositioning topoisomerase II on mitotic chromosomes at the metaphase-anaphase transition. In contrast to those well studied Ubls, the activation and conjugation machinery of some Ubls, including FAT10, FUBI, and Hub1, is still unknown (Schwartz and Hochstrasser 2003).

I.B.2 The FAT10 protein

FAT10 was first termed as diubiquitin. FAT10 is one of the nonclass I genes which are encoded in the HLA-F locus at the telomeric end of the human major histocompatibility complex (MHC) class I (Fan, Cai et al. 1996). The domain structure of FAT10 is similar to ISG15. It also contains two ubiquitin-like domains, which share 30% and 41% identify to ubiquitin, respectively (Figure 7). There is a diglycine motif in the second ubiquitin-like domain at the the C-terminus of FAT10. Unlike Ubiquitin, ISG15, and SUMOs, there is no extension at the C-terminus after the diglycine motif of FAT10. Therefore processing of the precursor by specific protease is not necessary for FAT10 conjugation (Figure 7). Both ubiquitin-like domains of FAT10 contain conserved lysine residues including the one equivalent to Lys48 of ubiquitin (Figure 9). These lysine residues might be capable of polymeric chain formation. However, poly-FAT10 chain formation has not been reported (Liu, Pan et al. 1999). According to Northern blot analysis, the expression level of FAT10

mRNAs is high in thymus and spleen, and is low or untraceable in other tissues, including prostate, testis, ovary, small intestine, colon ic mucosa lining, and peripheral blood leukocytes (Liu, Pan et al. 1999). FAT10 mRNAs are expressed in mature B cells and dendritic cells along with several other human cell lines following stimulation with interferon-gamma (IFNγ) and tumor necrosis factor-alpha (TNFα), but not with interferon-α (Bates, Ravel et al. 1997; Raasi, Schmidtke et al. 1999). Although the mRNA level of FAT10 is upregulated in those cell lines after TNFα and IFNγ treatment, the protein level of FAT10 is difficult to detect by immunoblot. The low level of FAT10 protein expression might be due to its rapid degradation. After IFNγ treatment, the expression of FAT10 proteins in JY cells (EBV transformed human B lymphoblastoid cells) is enhanced in the presence of a proteasome specific inhibitor (ALLN) (Liu, Pan et al. 1999). Without further stimulation, hepatocellular carcinoma (HCC) tissue from affected patients shows that FAT10 mRNA is highly upregulated and is primarily expressed in HCC cells with low and scattered signals in other surrounding cells, including immune cells, by *in situ* hybridization. FAT10 proteins can be detected in HCC cells with immunohistochemistry utilizing anti-FAT10 antibodies. FAT10 proteins are localized in the nuclei of HCC cells. The normal adjacent liver tissues from the same patients show undetectable level of both FAT10 mRNAs and protein (Lee, Ren et al. 2003). However, subcellular fractionation of JY cells shows that FAT10 proteins are mainly present in cytoplasm. This result has been confirmed by *in situ* hybridization on normal mouse spleen and thymus tissue sections (Liu, Pan et al. 1999). The different observations of FAT10 localization in different systems might be due to the different types of tissue samples used. It is also possible that in the normal cells, FAT10 is

located in cytoplasm, and the nuclear localization of FAT10 is somehow related to the pathogenesis of hepatocellular carcinoma. Upregulated expression of FAT10 has also been observed in other gastrointestinal and gynecological cancers. Furthermore, most of the genes involved in translation initiation, elongation or post-translational modification processes have no significant difference in samples from HCC patients than in samples from non-HCC patients. These finding imply that the upregulation of FAT10 expression in HCC cells is not due to the general increase in protein synthesis (Lee, Ren et al. 2003).

FAT10 forms covalent conjugates with cellular proteins through its C-terminal diglycine motif when exogenous hemagglutinin (HA) tagged FAT10 was introduced and expressed in mouse fibroblasts (B8 cells) in a tetracycline-repressible manner (Raasi, Schmidtke et al. 2001). Besides free FAT10, a FAT10 conjugation product around 35kDa was observed in the absence of tetracycline. Further analysis with two-dimensional NEPHGE/SDS-PAGE indicates that the FAT10 conjugation product consists of a number of different proteins. There proteins might include both FAT10 dimer and its substrates. However, the substrates of FAT10 conjugation have not been identified. Without discovering either the enzymes involved in FAT10 conjugation or the protein substrate, the biochemical and cellular functions of FAT10 have remained a mystery.

Although the definitive function of FAT10 is still unclear, some studies of FAT10 might give us hints about its function. Yeast two-hybrid screening of a human lymphocyte library revealed a FAT10 binding protein, mitotic arrest deficient-2 (MAD2), which is a spindle assembly check-point protein (Liu, Pan et al. 1999). The interaction was further confirmed by immunoprecipitation experiments. However, the interaction between FAT10

and MAD2 is not mediated with a covalently isopeptide bond, which is one of the requirements for being the target proteins for Ubls. Therefore, MAD2 serves as a binding partner, but not the substrate of FAT10. The biological significance of this interaction is not clear.

When wild-type, but not diglycine mutant, FAT10 was overexpressed in cells, apoptosis was observed by annexin V cell surface staining and DNA fragmentation. Apoptosis caused by FAT10 overexpression was blocked with general caspase inhibitor (z-VAD-fmk). Therefore, this apoptotic signaling event is mediated by caspases (Raasi, Schmidtke et al. 2001). Since FAT10 is induced by TNFα, it might be involved in TNFα induced programmed cell death. However, FAT10 deficient mice exhibited only minimal phenotypic differences with wild-type mice (Canaan, Yu et al. 2006). FAT10 deficient mice are viable and fertile and show no lesions or obvious histological differences with wild-type mice. The lymphocyte populations and dendritic cells from FAT10 deficient spleen, thymus, and bone marrow had no abnormalities. A minimal difference was observed when analyzing lymphocytes of FAT10 deficient mice with flow cytometry analysis. It showed a slight increase of spontaneous apoptotic death in mutant mice. FAT10 knockout mice were more sensitive to low doses of endotoxin challenge. Fifty percent of FAT10 knockout mice were lethal upon 50μg of endotoxin treatment, whereas in wild-type mice, 150 μg of endotoxin was required to cause 50% death. There was no deficiency in apoptosis observed in FAT10 knockout mice. Lymphocytes purified from FAT10 deficient mice showed even more spontaneous apoptosis, which suggests that FAT10 may function as a survival factor. These findings in FAT10 deficient mice conflict with the FAT10 overexpression experiments. It is

possible that the effects of FAT10 may be dependent on the cell type and physiological state and the level of expression of FAT10. Therefore, the role of FAT10 in apoptosis remains to be further tested.

FAT10 itself is a labile protein. N-terminal fusion of FAT10 to a long-lived GFP led to a reduction in the half-life of GFP as potently as ubiquitin fused GFP (Hipp, Kalveram et al. 2005). This FAT10 mediated protein degradation was not due to the ubiquitination of FAT10 protein itself since mutating all the lysine residues of FAT10 to prevent ubiquitination did not affect the degradation of FAT10. NEDD8 ultimate buster-1L (NUB1L) is identified as another binding partner for FAT10 by yeast two-hybrid screen (Hipp, Raasi et al. 2004). NUB1L is a longer splice variant of NUB1 (Tanaka, Kawashima et al. 2003). NUB1 is an interferonβ/γ-inducible protein that has been reported to interact with the ubiquitin-like protein, NEDD8 (Kito, Yeh et al. 2001). The interaction between FAT10 and NUB1L is non-covalent and is stronger than the interaction between FAT10 and NEDD8. Co-expressing NUB1L and FAT10 in cells led to an 8-fold enhanced degradation rate of FAT10. NUB1 has been reported to bind to the proteasome subunit RPN10 in vitro (Kamitani, Kito et al. 2001). NUB1L might function as a bridge to target FAT10 for degradation by the 26S proteasome. Therefore, FAT10 might serve as a ubiquitin-independent signal for proteasomal degradation.

In the course of identifying ubiquitin-binding proteins, I identified a novel E1-like protein termed E1-like 2 (E1-L2). I found that E1-L2 could activate ubiquitin to form E1-L2~Ub thioester intermediate and transfer the ubiquitin to a subset of ubiquitin E2s, suggesting that E1-L2 is a new ubiquitin E1 involved in some ubiquitination reactions. E1-L2

could also activate FAT10 by forming a thioester between the active site cysteine of E1-L2 and the C-terminal diglycine motif of FAT10. The endogenous E1-L2 and FAT10 also formed a thioester in cells stimulated with TNFα and IFNγ. Furthermore, RNA interference experiments showed that decreasing the expression of E1-L2 significantly reduced FAT10 conjugation in cells. These results uncover a novel E1 that can activate both ubiquitin and FAT10.

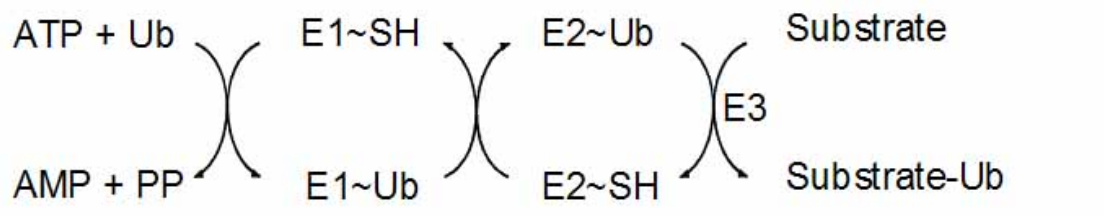


Figure 1. Ubiquitination machinery.
There are three steps in the ubiquitination cascade. E1 activates ubiqutin by ATP hydrolysis. Then, ubiquitin is transferred to the E2 enzyme to form an E2~Ub thioester intermediate. Some E2s can directly add ubiquitin to a substrate, but usually a third step catalyzed by an E3 enzyme is required for transferring the ubiquitin from E2 to the substrate. Ub, ubiquitin; E1, ubiquitin activating enzyme; E2, ubiquitin conjugating enzyme; E3, ubiquitin ligase; PP, pyrophosphate.

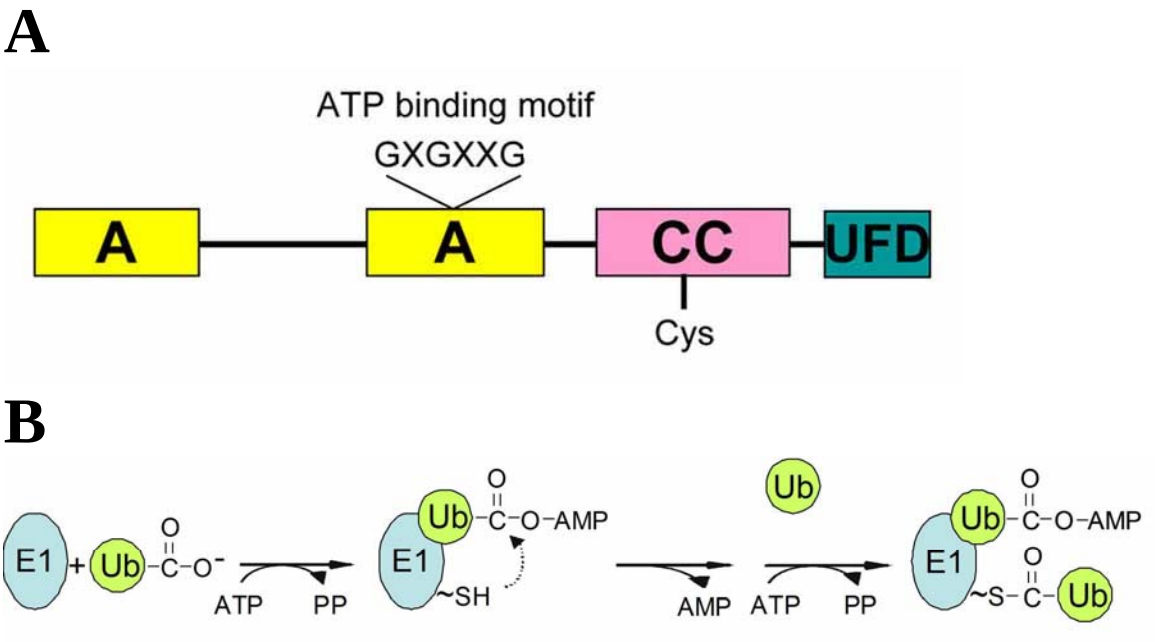
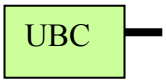


Figure 2. Domain structures of E1 and the mechanism of E1-catalyzed reaction.
(A) Schematic illustration of E1 domain structures. The adenylation domain of E1 contains an ATP binding motif. There is a conserved cysteine in the catalytic cysteine domain. A: adenylation domain; CC: catalytic cysteine domain; UFD: ubiquitin-fold domain. (B) Two steps of the ubiquitin activation. Ubiquitin is adenylated first and then transferred from adenylate to cysteine. The fully loaded E1 harbors two ubiquitins.

Class I: UBC domain only

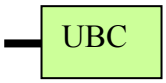
UbcH5, UbcH7, Ubc13

Class II: with C-terminal extension



RAD6, CDC34, E2-25K

Class III: with N-terminal extension



UbcH6, UbcH8, UbcH9, UbcM2

Class IV: with both N- and C- terminal extensions

Figure 3. The E2 family contains four classes of enzymes.
All E2 enzymes contain a catalytic core domain (UBC domain). E2 enzymes can be divided into four classes. Class I E2s contain only an UBC domain. Class II E2s have a C-terminal extension. There is an N-terminal extension of class III E2s. The E2s with both N- and C-terminal extensions are classified as the class IV. The representative E2 proteins of each class are listed on the right.

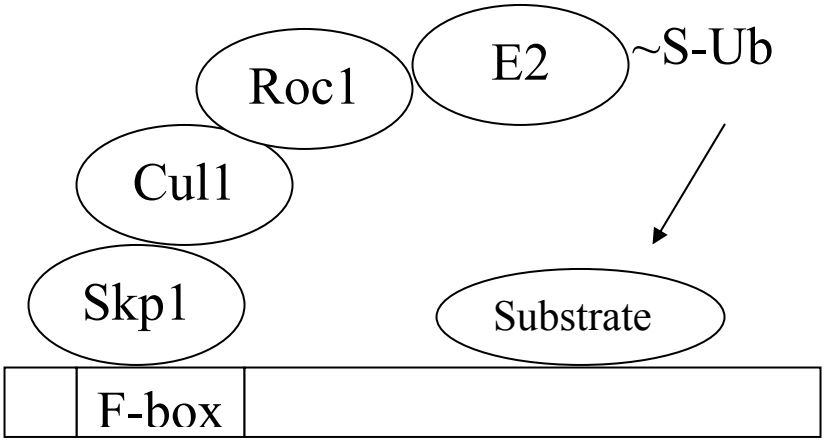


Figure 4. The model of SCF complex.
Substrate is recognized by the F-box protein. Skp1 interacts with F-box domain and recruits Cul1. Cul 1 is associated with the RING finger protein Rbx1/Roc1, which binds to the E2~Ub thioester intermediate. Then ubiquitin is transferred to the substrate.

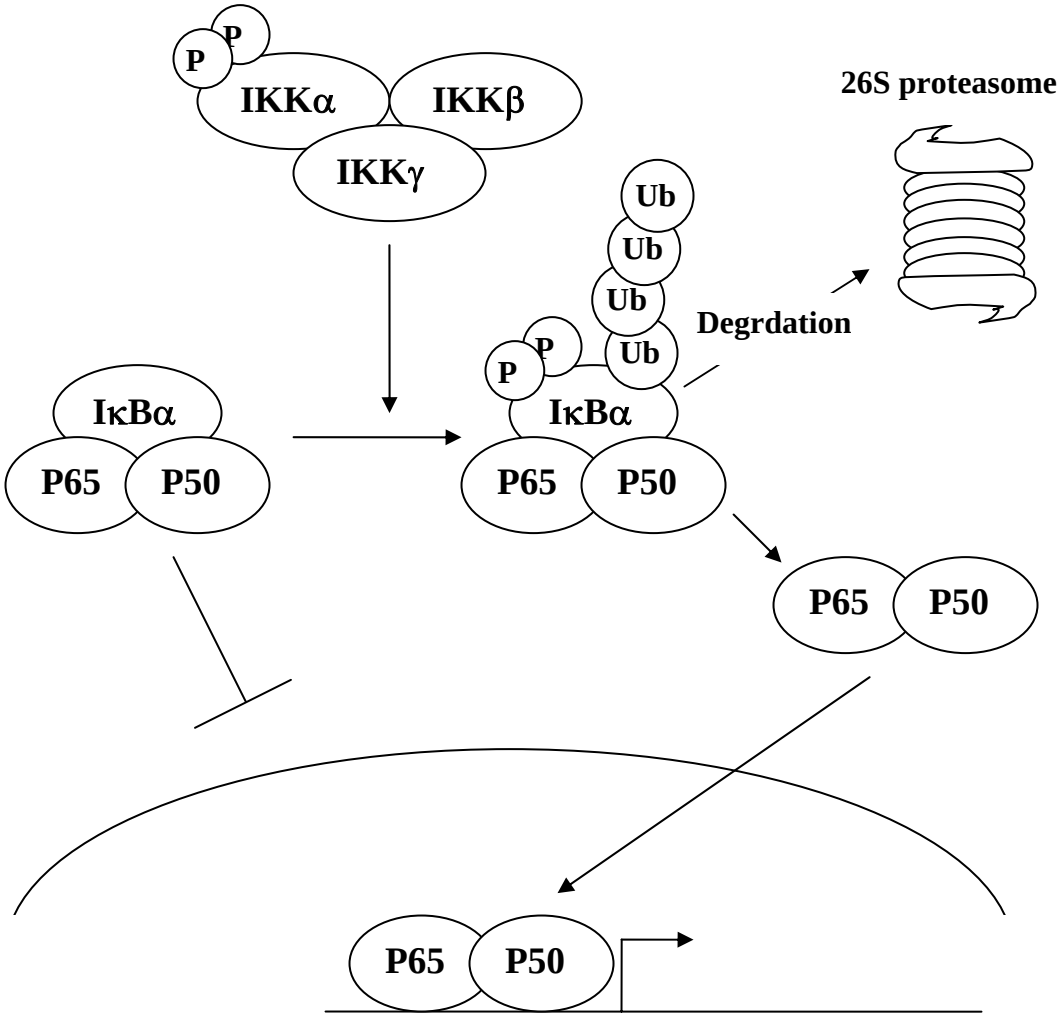


Figure 5. Phosphorylation and ubiquitination in NFκB activation.
Both phosphorylation and ubiquitination are required for NFκB activation. Activated IKK complex (IKKα, IKKβ, and IKKγ) phosphorylates IκB, which is the inhibitor of NFκB (p65 and p50). Phosphorylation of IκB results in its polyubiquitination and subsequent degradation by the 26S proteasome. In the absence of the IκB inhibitor, NFκB enters the nucleus and activates transcriptions.

A

Ubiquitin

MQIFV**K**TLTG**K**TITLEVEPSDTIENV**K**A**K**IQD**K**EGIPPDQQRLI
FAG**K**QLEDGRTLSDYNIQ**K**ESTLHLVLRLRGG

B

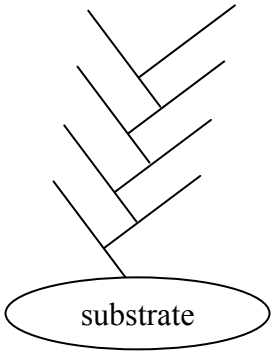


Figure 6. The primary structure of ubiquitin and polyubiquitin chain.
(A) The amino acid sequence of ubiquitin. Seven lysine residues are shown in bold and the diglycine motif is underlined. (B) Polyubiquitin chain formation on the substrate. One straight line represents one ubiquitin molecule. One lysine residue on the ubiquitin can be further conjugated by another ubiquitin to form a polyubiquitin chain.

Table 1. Ubiquitin and ubiquitin-like proteins

Modifier	E1	E2	E3	Function
Ubiquitin	Ube1	UbcH1-8, 10, 11, 13, Mms2	HECT E3s, RING E3	Protein degradation, kinase activation, DNA repair
SUMOs	SAE1/ SAE2	UbcH9	Siz1, Siz2, Mms21	Nuclear transport, chromosome segregation, transcriptional regulation
NEDD8	APPBP1 /UBA3	UbcH12	Dcn1	Activation of SCF complex, meiosis-to-mitosis transition
ISG15	Ube1L	UbcH8	Herc, Efp	Antiviral reponse (?)
Atg8	Atg7	Atg3	-	Autophagy
Atg12	Atg7	Atg10	-	Autophagy
Urm1	Uba4	-	-	Budding, nutrient sensing, oxidative-stress reponse
UFM1	Uba5	Ufc1	-	Unknown
FAT10	-	-	-	Protein degradation (?)
FUBI	-	-	-	T cell activation
Hub1	-	-	-	Cell polarity

(Adapted from Annu Rev. Cell Dev. Biol. 2006, 22, 159-180.)

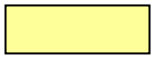
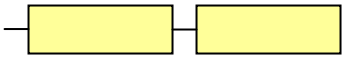
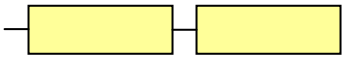
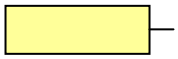
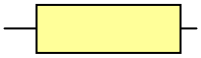
Ubls	Domian structure	% identity with ubiquitin	Protease Processing
Ubiquitin		100	yes
ISG15		32/36	yes
FAT10		30/41	no
NEDD8		55	yes
SUMOs		16-20	yes

Figure 7. Comparison of ubiquitin, ISG15, FAT10, NEDD8, and SUMOs.
The domain structures of ubiquitin, ISG15, FAT10, NEDD8, and SUMOs are shown. Ubiquitin-like domains are indicated by yellow blocks. The percentage of identity with uiquitin and the requirement for processing are listed. ISG15 and FAT10 have two ubiquitin-like domains. The identity of each domain with ubiquitin is indicated respectively.

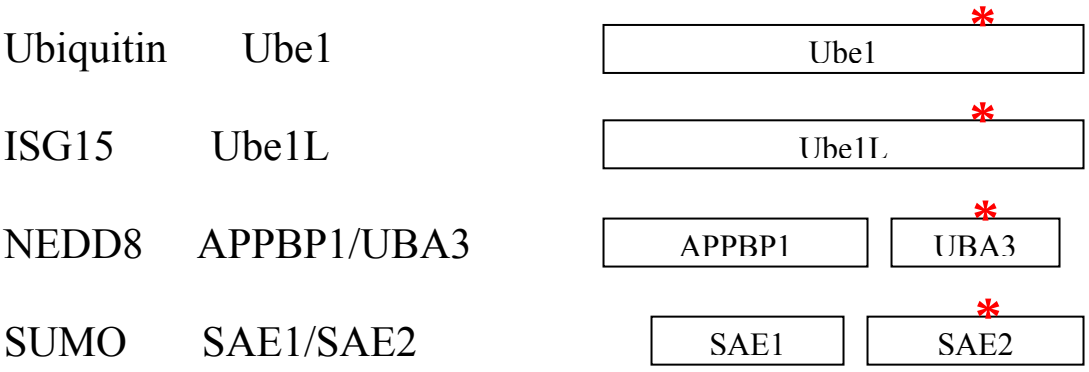


Figure 8. E1s for ubiquitin-like proteins.
The activating enzymes for ubiquitin and ISG15 are single proteins with the molecular weight between 110 kDa to 120 kDa. E1 enzymes of NEDD8 and SUMO are heterodimeric complexes. Ubls: ubiquitin-like proteins; E1s: activating enzymes.

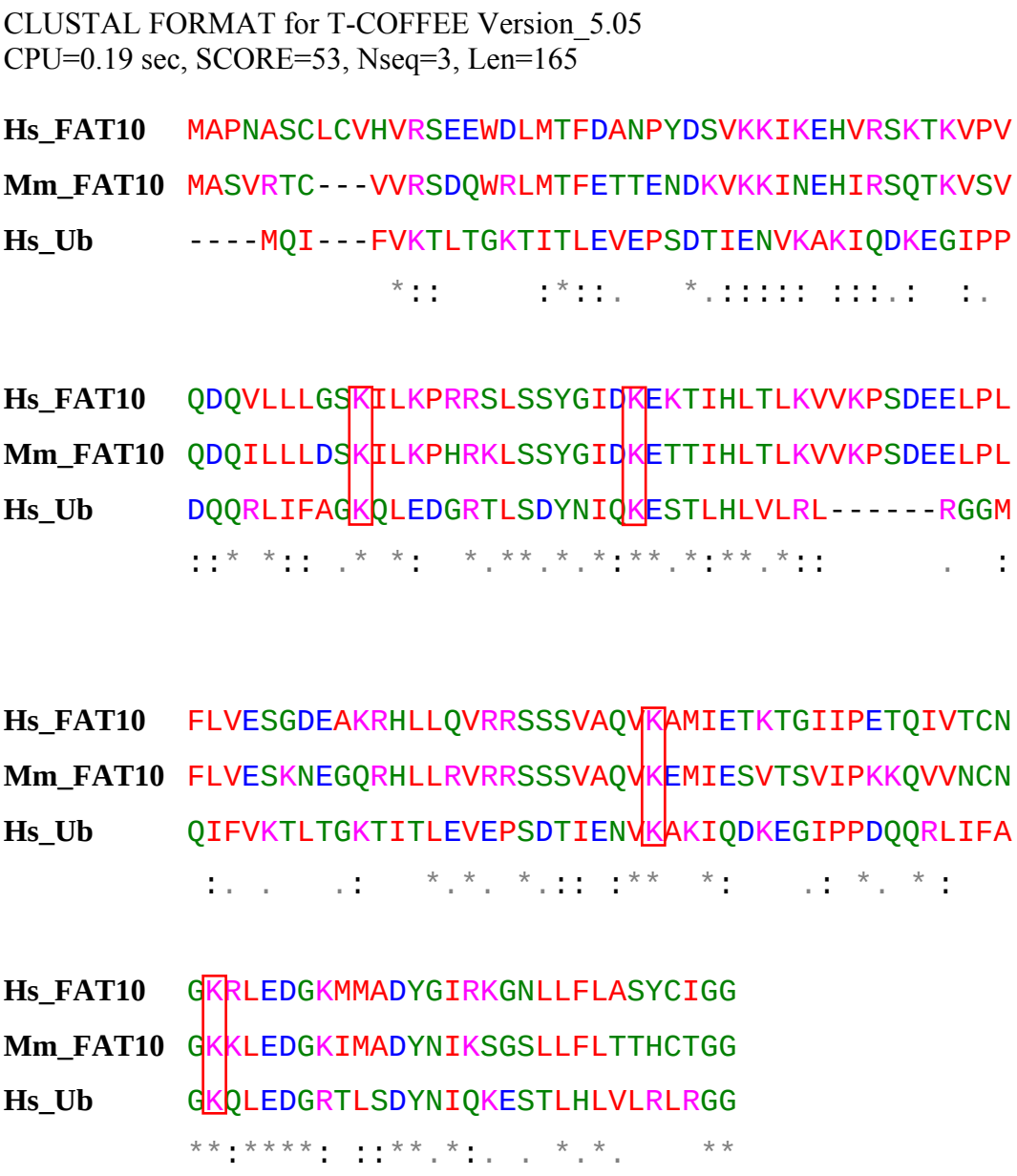


Figure 9. Alignment of FAT10 and Ubiquitin.
T-COFFEE program was used to align the amino acid sequences of human FAT10 (Hs_FAT10), mouse FAT10 (Mm_FAT10), and human ubiquitin (Hs_Ub). The conserved lysine residues are marked by red boxes. The color of the letter indicates the property of the amino acid residue. (Red: small or hydrophobic, Blue: acidic, Magenta: basic, and Green: hydroxyl, amine, and basic-Q). "*"indicates that the residues in the column are identical among the homologs. ":" means that conserved substitutions have been observed according to the color. "." shows that semi-conserved substitutions are observed.

CHAPTER II: MATERIALS AND METHODS

II.A Reagents

II.A.1 Plasmids

Human E1, E1-L1, FAT10 and mouse E1-L2 coding sequences were amplified by Polymerase Chain Reaction (PCR) and then subcloned into pFastBac (Invitrogen) in frame with an N-terminal His_6 tag for expression in Sf9 cells using the baculovirus system. Site-directed mutagenesis of E1-L2 and FAT10 was carried out using the QuickChange kit (Stratagene). The open reading frames of wild-type and mutant FAT10 sequences were subcloned into the mammalian expression vector pcDNA3 in frame with an N-terminal FLAG tag or another N-terminal tag containing His_6 and followed by three tandem copies of FLAG (His_6-3xFLAG). Sequences encoding residues 1-368 of human E1-L2 and residues 1-163 of human ISG15 were subcloned into the bacterial expression vector pET14b to generate N-terminal His_6 tagged proteins (Novagen). pET14b-ISG15 construct was generated by Dr. Gabriel Pineda. In order to generate N-terminal glutathione S-transferase (GST) tagged proteins, the full-length human FAT10 sequence was subcloned into a modified bacterial expression vector pGEX-4T1 in which the multiple cloning sites were different from the original pGEX-4T1 vector (Sheffield, Garrard et al. 1999). pGEX-4T-Ubc3 was kindly provided by Dr. Hongtao Yu (UT Southwestern Medical Center) and pGEX-E2-25K was a gift from Dr. Cecile Pickart (Johns Hopkins University).

II.A.2 Baculovirus

The method used for preparing baculovirus is based on the protocol from Invitrogen. A pFastBac containing the gene of interest served as a donor plasmid and then was transformed into the *Escherichia coli* DH10Bac competent cells (Invitrogen). The gene of interest was inserted into bacmid by recombination, which interrupted the expression of lacZα peptide. The transformed DH10Bac cells were grown on LB agar plate in the presence of kanamycin, gentamycin, tetracycline, Bluo-gal and IPTG. White colonies represent recombinant bacmids were isolated and cultured in LB media with kanamycin, gentamycin and tetracycline. Bacmid DNAs were extracted from the bacteria culture and the gene insertion was confirmed by PCR using one primer against the sequence of bacmid and another primer against the sequence of inserted gene. Recombinant bacmid DNAs were transfected into monolayer Sf9 cells by CellFECTIN reagent (Invitrogen). After 72 hours of incubation at 27^{o}C, supernatant from transfected Sf9 cells was collected as the P1 viral stock. The infected Sf9 cells were harvested and lysed in TBS buffer (20 mM Tris, pH7.5 and 150 mM NaCl) containing 0.5% NP-40. The cell lysate was analyzed by immunoblotting analysis to confirm protein expression. In order to get better quality and quantity of virus, P1 baculovirus were added into Sf9 cell culture at a ratio of 1 to 10 and incubated for another 96 to 120 hours with proper shaking (around 150 rpm) at 27^{o}C. Infected cell cultures were centrifuged to deplete cells and cellular debris. The supernatant containing recombinant baculovirus particles was harvested as the P2 viral stock. Both P1 and P2 viral stocks were filter-sterilized by vacuum filtration through 0.22 μm low-protein-binding filters and stored at 4^{o}C in dark.

II.A.3 Purification of Recombinant Proteins

Bacterial expression vectors were transformed into *Escherichia coli* strain BL21 (DE3). Protein expression was induced by addition of 0.1 mM IPTG for 4 hours at 25^{o}C. His_6-tagged proteins were purified by nickel affinity chromatography (Qiagen) according to manufacturer's instruction. Transformed bacteria were lysed in lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 10 mM imidazole). The bacteria lysate was centrifuged to pellet cellular debris. Ni-NTA resins were added to supernatant and mixed at 4^{o}C for 2 hours. The mixture was loaded into a column to eliminate unbound proteins. The column was washed with lysis buffer followed by wash buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 20 mM imidazole). His_6-tagged proteins were eluted with elution buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 250 mM imidazole) and dialyzed in 20 mM Hepes, pH 7.5, 20 mM NaCl, 0.5 mM DTT and 10% glycerol. All the proteins generated by baculovirus system have an N-terminal His_6 tag. P2 viral stock was added to Sf9 cells and incubated for 48 hours. Infected cells were lysed at 4^{o}C and His_6-tagged proteins were purified by the procedure mentioned above. GST-tagged proteins were purified by glutathione-affinity chromatography according to manufacturer's instruction (GE Healthcare). After IPTG induction, bacteria lysate was prepared by sonication in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8m M KH_2Po_4, pH 7.3) containing 1% Triton X-100. Supernatant containing GST-tagged proteins was generated by centrifugal force. Glutathione Sepharose 4B was added into supernatant and incubated at 4 ^{o}C for 2 hours. The mixture was loaded into a column to collect GST-tagged proteins bound to Glutathione Sepharose 4B. The column was washed with PBS buffer. GST-tagged proteins were eluted with elution

buffer (50 mM Tris-HCl, pH 8.0, 10 mM reduced glutathione) at 25 ^{o}C and dialyzed in 20 mM Hepes, pH 7.5, 20 mM NaCl, 0.5 mM DTT and 10% glycerol at 4 ^{o}C. Bovine ubiquitin was purchased from Sigma and dissolved in water at a concentration of 10mg/ml stock. The preparation of recombinant TRAF6, Ubc5, Ubc13 and Uev1A proteins have been described previously (Deng, Wang et al. 2000) and generated by Dr. Zong-Ping Xia. All the proteins were stored at -80 ^{o}C.

II.A.4 Preparation of protein affinity column

E1-L2 affinity column was generated by covalently conjugating a recombinant N-terminal fragment of human E1-L2 to NHS (N-hydroxysuccinimide)-activated Sepharose (GE). Residues 1-368 of human E1-L2 was expressed in *E. coli* with an N-terminal His_6 tag and purified as mentioned in Section II.A.3. E1-L2 proteins were dialyzed in 20 mM Hepes, pH 7.5, 20 mM NaCl, 0.5 mM DTT and 10% glycerol to remove primary amine in the protein solution. Two ml of NHS-Sepharose slurry was loaded into a column to remove isopropanol from the solution. In order to activate the matrix, NHS-Sepharose was washed with 10 ml of cold HCl followed by 50 ml of cold distilled water. NHS-Sepharose was then transferred to a 15 ml conical tube containing E1-L2 protein solution and mixed gently for coupling by rotating at 25^{o}C for 90 minutes. After incubation, a 50 μl aliquot of the mixture was taken and centrifuged. The protein concentration of the supernatant was measured to make sure there were almost no unbound proteins presented in the mixture. Longer incubation might be needed if necessary. Tris buffer (1 M Tris-HCl, pH 8.0) was added to the mixture at a final concentration of 0.1 M and incubated for another 60 minutes to block

reactive sites of NHS-Sepharose. The mixture was loaded into a clean column to capture E1-L2-Sepharose. The column was washed with 5 bed-volume of column wash solution I (50 mM Ammonium acetate, pH 5.5, 0.5 M NaCl) followed by 5 bed-volume of column wash solution II (50 mM Tris-HCl, pH 8.0, 0.5 M NaCl). After 3 cycles of column wash, 5 bed-volume of column storage buffer (50 mM Tris-HCl, pH 7.5, 0.02% Na-azide) was added for final wash. Store the column in the column storage buffer at 4 ^{o}C. FAT10 affinity column was generated through the same method. The full-length FAT10 proteins were expressed in *E. coli* as GST fusion proteins and purified as mentioned in Section II.A.3. However, in instead of eluting GST-tagged FAT10 with elution buffer from the glutathione sepharose, His_6-tagged Tev protease was used to cleave FAT10 protein from its GST tag which bound to the glutathione sepharose. After incubation, FAT10 proteins and Tev protease were collected as the outflow from the column. His_6-tagged Tev protease was then depleted by nickel-sepharose and FAT10 proteins without the GST tag were used for making the affinity column. Ubiquitin affinity column was made by Dr. Zhijian 'James' Chen.

II.A.5 Affinity purifications of antibodies

In order to generate polyclonal antibodies against human E1-L2, the same recombinant protein containing residues 1-368 of human E1-L2 mentioned in Section II.A.4 was used as antigen. For generating polyclonal antibodies against human FAT10, the full-length FAT10 protein without any tag prepared by the procedures mentioned in the previous methods was used. The E1-L2 and FAT10 proteins were used to immunize rabbits (Rockland), and the resulting antibodies were affinity purified using specific antigen

columns. Ten ml serum was diluted in 20 ml of buffer A (50 mM Hepes, pH 8.0, 150 mM NaCl) and centrifuged to remove debris. The diluted serum was mixed respectively with 2 ml of E1-L2 or FAT10 affinity sepharose prepared by the method in Section II.A.4. The mixture was incubated at 25^{o}C for 1 hour by rotating and then loaded into a column. The outflow was collected and reapplied to the column. The second outflow was collected and frozen at -20 ^{o}C for further purification in the future. The column was washed with 10 ml of buffer A and eluted with 15 ml of Na-citrate buffer, pH6.0 (40 mM Na-citrate, 20 mM NaCl) followed by 15 ml of Na-citrate buffer, pH 5.0. Five ml of 1 M Hepes, pH 8.0 was added in the collection tubes to adjust the pH value. Fifteen ml of Na-citrate buffer, pH 4.0 was used for further elution. The antibodies were finally eluted with 15 ml of Na-citrate buffer, pH 3.2 from the column. Five ml of 1 M Hepes, pH 8.5 was added in the collection tubes to adjust the pH value of pH 4.0 and pH 3.2 eluates. All the antibody eluates were concentrated by concentrators and stored at -20 ^{o}C. An aliquot of E1-L2 and FAT10 antibodies was diluted to 0.2 mg/ml in dilution buffer (50 mM Hepes, pH 7.5, 0.02% Na-azide) as working solution and stored at 4 ^{o}C. The antibody for human E1 was kindly provided by Dr. Alan Taylor (Tufts University). The antibodies for Ubc5 and ubiquitin were purchased from Boston Biochem and Santa Cruz Biotech, respectively. The antibodies of His (Qiagen) and FLAG (M2, Sigma) were purchased from the respective commercial sources.

II.A.6 HeLa S100 cell lysate preparation

HeLa S3 cells from 50 liters culture were purchased from National Cell Culture Center. All procedures were carried out at 4°C. The cell pellet was washed by gently

resuspending with 4 packed cell volume of cold PBS buffer followed by centrifuging at 1,000 x g for 5 minutes. The pellet was quickly washed again by cold hypotonic buffer (10 mM Tris-HCl, pH 7.5, 10 mM KCl, 0.5 mM EGTA, and 1.5 mM $MgCl_2$) with the same procedure. These wash steps were done gently and quickly to avoid the breakage of HeLa cells. HeLa cells were resuspended in 2 packed cell volume of cold hypotonic buffer containing 0.5 mM DTT and EDTA-free protease inhibitor cocktail (Roche). After sitting on ice for 10 minutes, cells were homogenized and lysed by a 40 ml glass dounce homogenizer with at least 20 strokes. The cell lysate was transferred to 50 ml conical tubes and centrifuged at 1,000 x g for 10 minutes to remove nuclei. The supernatant was transferred to a new tube as the crude cytosolic extract and centrifuged again at 100,000 x g for 45 minutes. The second supernatant was collected and filtered through 0.22 μm, low protein binding filters to deplete lipid particles from the solution. The clear filtrate was collected as the HeLa S100 cell lysate. Various sizes of aliquots were made and frozen at -80°C for long-term storage.

II.B Cell culture and Experimental Methods

II.B.1 Cell culture, transfection and cytokine stimulation

Human embryonic kidney (HEK) 293 cells and U2OS cells (osteosarcoma cell line) were cultured in DMEM supplemented with 10% fetal calf serum (FCS), penicillin G (100 μg/ml), and streptomycin (100 μg/ml). Human HeLa S3 cells were purchased from National Cell Culture Center (Minneapolis) and cultured in DMEM supplemented with 10% fetal

bovine serum (FBS), penicillin G (100 μg/ml), and streptomycin (100 μg/ml). Human Namalwa cells (human Burkitt lymphoma) were a gift from Dr. Tom Maniatis (Harvard University) and JY cells (EBV transformed human B lymphoblastoid) were kindly provided by Dr. Jack Strominger (Harvard University). Namalwa, JY, and U937 (monoblast) cell lines were both cultured in RPMI medium supplemented with 10% FBS, penicillin G (100 μg/ml), and streptomycin (100 μg/ml). All the cell lines were incubated at 37 ^{o}C with 5% CO_2. Transfection of HEK293 cells was carried out by calcium phosphate precipitation or Lipofectamine 2000 reagent (Invitrogen). For transfection with calcium phosphate precipitation, plasmid DNAs or siRNAs were added in 0.25 M $CaCl_2$ solution followed by an equal volume of 2x HBS buffer (50 mM Hepes, pH 7.05, 1.5 mM Na_2HPO_4, 140 mM NaCl, 10 mM KCl, and 12 mM Dextrose). The sample was quickly mixed by vortex. Calcium phosphate precipitation occurred during mixing. Immediately, the DNA or siRNA/calcium phosphate suspension was added dropwisely to monolayer HEK293 cells. The transfection reagents were removed on the next day and replaced with fresh medium. The method used for transfection with Lipofectamine 2000 reagent was based on the manufacture's instruction from Invitrogen. The induction of endogenous FAT10 proteins were tested in HEK293, HeLa, Namalwa, JY, U2OS and U937 cells with TNFα (0.2 μg/ml) and IFNγ (3000 U/ml) stimulation for 16 hours, and the protein lysates were analyzed by SDS-PAGE followed by immunoblotting analysis.

II.B.2 siRNA preparation and RNA interference

The RNA oligos were synthesized at the UT Southwestern Center for Biomedical Invention (CBI) facility. Sense and antisense oligos were dissolved separately in annealing buffer (30 mM Hepes-KOH, pH 7.4, 100 mM potassium acetate, and 2 mM magnesium acetate). An aliquot of oligos were diluted to the concentration of 20 μM. Equal volume of 20 μM sense and antisense oligos for the same gene were mixed together. The mixture of oligos were heated at 95°C for 5 minutes and then cooled down slowly till the temperature of the sample reached 25°C. Several aliquots of 20 μM annealed siRNA duplex were made and stored at -20°C. For RNA interference experiments, siRNA oligos were transfected into HEK293 cells at a final concentration of 20 nM using the calcium phosphate precipitation method mentioned in Section II.B.1. The transfection procedure was repeated on the next day. On the third day, cells were transfected with expression plasmids using Lipofectamine 2000 reagent (Invitrogen). Cells were harvested on the fourth day for analysis.

The sequences of the siRNA oligos are listed as follows (only the sense strands are shown):

GFP (471-489), GCAGAAGAACGGCAUCAAG;

E1-L1 (2652-2670), CUACCUACAUCUGGCUGAA;

E1-L2a (996-1014), GGACCAGUUUCAGGAGAAA;

E1-L2b (1732-1750), GACAGUCGUUGCUUAGCAA;

FAT10a (103-121), GAACATGTCCGGTCTAAGA;

FAT10b (347-365), AAGCAATGATCGAGACTAA.

II.B.3 Ubiquitin affinity purification and identification of E1-L2

One ml of Ubiquitin-Sepharose (6 mg/ml resin) described in Section II.A.4 was washed by buffer A (50 mM Tris pH 7.2, 0.2 mM DTT, 2 mM ATP and 5 mM $MgCl_2$). Ten ml of HeLa S100 cell lysate (7.2 mg/ml) generated by the method in Section II.A.6 were incubated with Ubiquitin-Sepharose at room temperature for 1 hour in the presence of 5 mM $MgCl_2$ and 2 mM ATP. After incubation, the slurry was packed into a column. The column was washed with 4 column volume of buffer A and eluted with 4 column volume of high salt buffer (50 mM Tris pH 7.2, and 1 M KCl). The high salt eluate might contain E3 enzymes. The column was washed with 4 column volume of TE buffer (50 mM Tris, pH 7.6, and 0.1 mM EDTA). DTT buffer (50 mM Tris pH 7.6, 0.1 mM EDTA, and 10 mM DTT) was used to elute E1 and E2 enzymes from the Ubiquitin-Sepharose. The column was further eluted with high pH buffer (50 mM Tris pH 9.0, 0.1 mM EDTA, and 5 mM DTT). The high pH eluate might contain E3 enzymes and isopeptidase which is a deubiquitination enzyme. High salt/high pH buffer (50 mM Tris pH 9.0, and 1 M KCl) was used in the last elution step. 100 μg of BSA was added into all collection tubes to minimize nonspecific absorption to the tubes. All the eluates were collected on ice and concentrated. TED buffer (50 mM Tris, pH 7.2, 0.1 mM EDTA, and 0.5 mM DTT) was used for buffer exchange. The salt concentration of each eluate was below 0.1 M before stored at -80°C. Proteins in these fractions were resolved by SDS-PAGE and visualized by Coomassie blue staining. The high salt/high pH fraction was digested with trypsin in solution, and the peptide masses were acquired by nano-HPLC/MS/MS (UT Southwestern Protein ID core facility). Similar affinity purification procedures were used to detect the thioester formation between E1-L2 and FAT10. In this

experiment, ubiquitin- or FAT10-Sepharose were used to incubate with recombinant His_6-tagged E1 or E1-L2 proteins in the presence of 5 mM $MgCl_2$ and 2 mM ATP. After the same sequential steps of elution, the presence of E1 or E1-L2 proteins was detected by immunoblotting analyses.

II.B.4 Thioester assay

For detecting the thioester formation between E1 enzymes and ubiquitin-like proteins, 0.26 μM of recombinant His_6-tagged E1, E1-L1, or E1-L2 proteins purified from Sf9 cells through the baculovirus systems (see Section II.A.3) was incubated with 6.25 μM ubiquitin, 1.67 μM ISG15 or 1.67 μM FAT10 proteins and 2mM ATP at 37^{o}C for 15 minutes. In the time course experiments, various time points from 0 to 15 minutes were used for the assays. The reaction without ATP was also performed for testing the dependence of ATP in thioester formation. SDS sample buffer (10mM Tris, pH6.8, 0.8% SDS, 10% glycerol, and 0.05% bromophenol blue) was added into each sample to stop the reaction. The reaction mixtures were resolved by SDS-PAGE in the presence or absence of 86 mM β-mercaptoethanol which represented reducing or non-reducing conditions, respectively. Three E1 proteins and their thioester intermediates were detected by immunoblotting with antibodies against His_6 peptide. For detecting E2~ubiquitin thioester, the ubiquitin E2 enzymes, Ubc3 (4.7 μM), Ubc5 (15 μM), Ubc13/Uev1A (16.3 μM) or E2-25K (4.9 μM) was incubated with ubiquitin (12.5μM) and E1 or E1-L2 (0.52μM) in the presence or absence of 2mM ATP at 37^{o}C for 15 minutes. The samples were subjected to SDS-PAGE under reducing or non-reducing conditions followed by Coomassie blue staining or immunoblotting with antibodies against

His_6, GST, or Ubc5 proteins. Similar experimental procedures were performed in order to determine the ability of FAT10 proteins to form thioester intermediate with those ubiquitin E2 enzymes. FAT10 proteins instead of ubiquitin were incubated with E1-L2, E2 enzymes and ATP in these experiments.

II.B.5 Subcellular fractionation

HEK293 cells were treated with TNFα and IFNγ for 16 hours as mentioned in Section II.B.1. Stimulated or unstimulated HEK293 cells were wash with PBS buffer once and then lysed in cold HMEKS buffer (20 mM Hepes, pH 7.5, 2 mM $MgCl_2$, 2 mM EDTA, 10 mM KCl and 250 mM sucrose) by douncing with a glass dounce homogenizer. Subcellular fractionation was carried out by differential centrifugation in a three-step process using a modified protocol (Mumby 2002). In the first step, crude cell lysates were carefully layered over a 1 M sucrose cushion and centrifuged at 1,600 x g for 10 minutes at 4 ^{o}C. The first pellet (P1) contained nuclei. In the second step, the supernatant collected from the first step (S1) was further centrifuged at 15,000 x g for 10 minutes at 4 ^{o}C. The second pellet (P2) was saved as heavy membranes. In the last step, the supernatant collected from the second step (S2) was centrifuged again at 200,000 x g for 30 minutes at 4 ^{o}C. The third pellet (P3) was saved as light membranes and the final supernatant (S3) was collected as the cytosolic fraction. To investigate the distribution of endogenous E1-L2 protein, SDS sample buffer with β-mercaptoethanol was added to the starting material (crude cell lysate) and all the subcellular fractions including nuclei, heavy membranes, light membranes, and cytosol.

These protein samples were then resolved by SDS-PAGE and analyzed by immunoblotting with the antibodies against endogenous E1-L2 proteins.

II.B.6 RT-PCR

HEK293, HeLa, U2OS, U937, and Namalwa cells were treated with TNFα, IFNγ, or TNFα and IFNγ as mentioned in Section II.B.1. TRIZOL reagent (Invitrogen) was used to isolate total RNAs. Cells were homogenized and lysed in 1 ml Trizol at 25°C for 5 minutes. 0.2 ml chloroform was added into the sample and vigorously shaken for 15 seconds. After incubation for 3 minutes, the samples were centrifuged at 12,000 x g for 15 minutes at 4 °C. The aqueous phase was carefully collected and mixed with 0.5 ml isopropyl alcohol. The samples were incubated at 25°C for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4 °C. The pellet was washed with 1 ml 75% ethanol and air-dry for 5 minutes and dissolved in RNase-free water. The RNA samples were treated with TURBO DNA-free kit (Ambion) to eliminate the contamination from genomic DNA. cDNAs were synthesized by using the iScript cDNA Synthesis Kit (Bio-Rad). 1μg of total RNA was mixed with iScript Reverse Transcriptase and 5x iScript Reaction Mix. The reaction was carried out in a RCR machine (Applied Biosystems) and incubated at 25 °C for 5 minutes, 42 °C for 30 minutes, and 85 °C for 5 minutes. cDNA products were used as a template for PCR reaction with Pfu polymerase and gene-specific primers. RT-PCR products were separated in 1.8% agarose gel. The sequences of the primers are listed as belowed:

FAT10 (sense), TGATCGAGACTAAGACGGGTATAA

FAT10 (antisense), GAAGAGTAAGTTGCCCTTTCTGAT

GAPDH (sense), ATGACATCAAGAAGGTGGTG

GAPDH (antisense), CATACCAGGAAATGAGCTTG

II.B.7 Immunoprecipitation

For immunoprecipitation of exogenous FAT10, HEK293 cells were transfected with FLAG-tagged wild-type or mutant FAT10 by calcium phosphate precipitation (see Section II.B.1). Forty eight hours later, cells were lysed in lysis buffer (0.5% NP-40 in TBS buffer) and centrifuged at 13,000 x g to collect cell lysate. Cell lysate was incubated with the ANTI-FLAG M2 affinity gel at 4°C for 2hr. The mixture was centrifuged at 10,000 x g for 5 minutes at 4°C to spin down the gel. The M2 beads were washed with lysis buffer and then eluted with DTT buffer (10 mM DTT in TBS buffer). After DTT elution, FLAG-FAT10 was eluted with the FLAG peptide. SDS sample buffer with β-mercaptoethanol was added to the elution fractions. The samples were analyzed by immunoblotting analysis with antibodies against FLAG peptide or E1-L2 proteins.

For co-immunoprecipitation of endogenous ubiquitin and FAT10, HEL293 cells were stimulated with TNFα and IFNγ for 16 hours (see Section II.B.1). Treated or untreated cells were lysed in lysis buffer and centrifuged for collecting cell lysate. Endogenous E1-L2 proteins were immunoprecipitated with the E1-L2-specific antibodies. Cell lysate was incubated with the antibodies for 1 hour at 4°C followed by one-hour incubation with 10 μl protein A/G beads (Pierce). The immunocomplexes were washed with lysis buffer and then eluted with DTT buffer. SDS sample buffer with β-mercaptoethanol was added to the beads to disrupt the interaction between E1-L2 proteins and anti-E1-L2 antibodies and collected as

SDS eluate. The fractions were resolved by SDS-PAGE followed by immunoblotting with the antibodies against E1-L2, ubiquitin and FAT10 proteins.

II.B.8 Ubiquitination assay

To determine the ability of E1-L2 for polyubiquitin chain formation, various amount of E1 or E1-L2 (from 3 to 300ng), His_6-tagged Ubc13/Uev1A (16.3 μM), TRAF6 (approximately 0.1 μM), ubiquitin (12.5 μM) and ATP (2 mM) were incubated at 37^{o}C for 15 minutes. The reaction products were resolved by SDS-PAGE under reducing conditions and analyzed by immunoblotting with an antibody against ubiquitin to observe polyubiquitin chain. Another aliquot of the reaction products were analyzed by SDS-PAGE under non-reducing condition followed by immunoblotting. The antibodies against His tag were used to detect the Ubc13/Uev1A~Ub intermediate.

II.B.9 Identification of substrates

To identify the substrates for FAT10, HEK293 cells were transfected with His_6-3xFLAG-tagged wild-type or mutant FAT10 by calcium phosphate precipitation (see Section II.B.1). Forty eight hours later, cells were lysed in lysis buffer (0.5% NP-40 in TBS buffer) and centrifuged at 13,000 x g to collect cell lysate. Cell lysate was incubated with the ANTI-FLAG M2 affinity gel at 4°C for 2hr. The mixture was centrifuged at 10,000 x g for 5 minutes at 4°C to sediment the gel. The agarose was subsequently washed with lysis buffer, TBS buffer containing 1M KCl, RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS), and lysis buffer again. The proteins were then eluted with 8 M Urea buffer at

pH 4.5 (8 M Urea, 100 mM NaH_2PO_4, and 10 mM Tris-Cl, pH 4.5). The pH value of eluate was adjusted to 8.0 by 1 M Tris buffer (pH9.0). FAT10 conjugates were further purified with nickel affinity chromatography. The beads were washed with 8.0 M Urea buffer at pH 8.0 and eluted with SDS sample buffer containing β-mercaptoethanol. The samples were resolved by SDS-PAGE followed by silver staining or immunoblotting with antibodies against FLAG peptide. The bands of interesting were excised and treated with trypsin for in-gel digestion, and the peptide masses were acquired by nano-HPLC/MS/MS (UT Southwestern Protein ID core facility).

CHAPTER III: ACTIVATION OF UBIQUITIN BY E1-L2

III.A Identification of E1-L2

I carried out ubiquitin-affinity chromatography in an effort to identify new ubiquitin-binding proteins (Figure 10A). HeLa S100 cell lysates were incubated with Ubiquitin-Sepharose in the presence of ATP at room temperature for 1 hour and then loaded into an open column (Ciechanover, Elias et al. 1982). Most of proteins bound non-covalently to ubiquitin were eluted extensively with a buffer containing 1M KCl from the column. This high salt eluate might contain potential E3 enzymes. Then DTT buffer was used to elute proteins from the column. DTT was able to reduce the thioester bond between proteins. The ubiquitin thioester intermediates were dissociated under that condition and therefore, ubiquitin E1 and E2s were present in this fraction. Some E3s and isopeptidase T (ubiquitin deconjugating enzyme) were eluted from the column with high pH buffer (pH9.0). Proteins that remained bound to the column, which are candidate ubiquitin-binding proteins, were eluted with a high pH buffer (pH 9.0) containing 1 M KCl. Proteins in these fractions were resolved by SDS-PAGE and visualized by Coomassie blue staining. BSA was added as carriers in each fraction except the last one to minimize nonspecific absorption by the tubes (Figure 10B). Proteins in high pH/1 M KCl eluate were digested with trypsin in solution and then identified by mass spectrometry. One of these proteins was identified as an uncharacterized protein highly homologous to the ubiquitin E1 and another activating enzyme, Ube1L. Ube1L is the activating enzyme which activates the ubiquitin-like protein ISG-15 (Yuan and Krug 2001). In this dissertation, Ube1L is referred to E1-like 1 (E1-L1),

whereas the novel E1 identified in high pH/1 M KCl fraction is named E1-like 2 (E1-L2). E1-L2 consists of 1052 amino acid residues and contains all the structural elements characteristic of a functional activating enzyme (Walden, Podgorski et al. 2003), including the adenylation domains (A), a catalytic cysteine domain (CC) and a ubiquitin-fold domain at the very C-terminus (UFD; Figure 11A). There is a conserved ATP-binding motif located between amino acid residues 467 and 472 in the second adenylation domain of E1-L2 and a conserved cysteine residue at position 625 in the catalytic cysteine domain. Cys625 is predicted to be the putative active site which might be involved in thioester formation with ubiquitin-like proteins. E1-L2 shares a 42% identity with the ubiquitin E1 (herein referred to as E1; Figure 11B), and a 36% identity with E1-L1. There is 89% identity between human and mouse E1-L2 proteins (Figure 12A and 12B). Interestingly, E1-L2 is evolutionarily conserved and its orthologs can be found in the invertebrates including the sea urchin *S. purpuratus* (Figure 12A). However, yeast, *Drosophila*, and *C. elegans* do not have apparent orthologs of E1-L2. The genes for the human and mouse E1-L2 are located in the chromosomes 4 and 5, respectively. E1-L2 is widely expressed in different human tissues based on the expression profile suggested by analysis of expressed sequence tags (EST) counts (NCBI UniGene database). A splice variant of E1-L2 gene named *nUBE1L* has been reported (Zhu, Zhou et al. 2004). nUBE1L was discovered by comparing genes expressed in fetal testis with the ones expressed in adult testis through cDNA microassay. The first adenylation domain of E1-L2 is missing in nUBE1L. Expression profile revealed that nUBE1L was predominantly expressed in testis and highly expressed in adult testis compared to other developmental stages of testis. The function of nUBE1L remains unclear. In order to

determine the subcellular localization of E1-L2, HEK293 cells were gently lysed and subsequently separated into several subcellular fractions by differential centrifugation. Nuclei, heavy membrane, light membrane, and cytosol fractions were collected. Immunoblotting of these subcellular fractions using an E1-L2 specific antibody showed that E1-L2 is predominantly present in the cytoplasm (Figure 13A; lane 10). The specificity of E1-L2 antibodies was further confirmed by RNA interference knock down experiments. After transfecting small interfering RNA (siRNA) oligos against E1-L2 or GFP into HEK293 cells, cell lysates were resolved by SDS-PAGE followed by immunoblotting. The protein expression level of endogenous E1-L2 was undetectable in cells transfected with siRNA against E1-L2, whereas the expression level of E1-L2 remained unchanged in the control experiments by transfecting siRNA against GFP (Figure 13B; compare lane 3 to lane 1 and lane 2 to lane 1). Thess results show that the 118kDa protein detected in the subcellular fractions by immunoblotting with E1-L2 specific antibodies is indeed the endogenous E1-L2 protein. Furthermore, to calculate the protein concentration of endogenous E1-L2 in cells, different amount of HEK293 cell lysates were resolved by SDS-PAGE. Endogenous E1 and E1-L2 proteins were detected with E1 and E1-L2 specific antibodies by immunoblotting, respectively, and compared with different amounts of recombinant proteins on the same immunoblots (Figure 14). There is about 6.3 ng of E1 present in 0.6 μg of cell lysate, while it requires around 2.5 μg of cell lystae to detect the same amount of E1-L2 (Figure 14; compare lane 5 to lane 12 in the E1 sample and lane 3 to lane 12 in the E1-L2 sample). The concentration of E1-L2 is less than 25% of the concentration of E1 in HEK293 cells. Based

on semi-quantitative immunoblotting, I estimated that the concentration of E1-L2 in HEK293 cells is approximately 0.25 μM, or $6x10^5$ molecules per cell.

III.B E1-L2 forms a thioester with ubiquitin.

Since E1-L2 contains all the structural elements characteristic of a functional activating enzyme and is 42% identical to the ubiquitin E1, I decided to test whether E1-L2 can activate ubiquitin. One of the properties of activating enzyme is forming a thioester intermediate with its ubiquitin-like proteins. To investigate the biochemical functions of E1-L2, recombinant E1-L2 proteins were generated and used in the in vitro biochemical assays. I expressed His_6-tagged E1-L2 as well as His_6-tagged E1 in Sf9 insect cells using the baculovirus system (see Section II.A.2 for generating baculovirus). Both proteins were purified by using nickel affinity chromatography (see Section II.A.3 for protein purification). E1 and E1-L2 were purified to apparent homogeneity shown in SDS-PAGE followed by Coomassie blue staining (Figure 15; upper panel). Both recombinant proteins were used to test the specificity of E1 and E1-L2 antibodies. E1 antibodies can only recognize recombinant E1 proteins but not E1-L2 proteins, whereas E1-L2 antibodies can only recognize E1-L2 proteins and did not cross-react with E1 proteins (Figure 15; lower two panels). In the other words, there is no cross-reaction between E1 and E1-L2 for these two antibodies.

These two recombinant proteins were used to determine if E1-L2 could form a thioester bond with ubiquitin. Ubiquitin-Sepharose was incubated with E1 or E1-L2 in the presence of ATP at room temperature for 1 hour and then loaded into a column. A buffer

containing 1M KCl was used to wash the column. Proteins that form thioesters with ubiquitin were eluted with 10 mM DTT. Subsequently, proteins that bound to the column non-covalently were eluted with a high pH buffer (pH 9.0) followed by a high pH buffer (pH 9.0) containing 1M KCl. The eluted proteins in each fraction were collected and concentrated. The concentrated samples were analyzed by SDS-PAGE followed by immunoblotting with a His_6 antibody to detect E1 or E1-L2. E1 is known to form a thioester with ubiquitin in the presence of ATP and therefore, serves as a positive control. As expected, recombinant E1 proteins were detected in the DTT eluate but not in the samples collected from high salt wash, pH 9.0 or pH 9.0/1 M KCl eluates after incubating with Ubiquitn-Sepharose (Figure 16; top panel). Recombinant E1-L2 was incubated with Ubiquitn-Sepharose in the presence of ATP and eluted with the aforementioned protocol for E1. E1-L2 was detected in the DTT eluate by immunoblotting. In addition, E1-L2 also bound to the ubiquitin column non-covalently and was eluted with the pH 9.0/ 1 M KCl buffer, explaining why E1-L2 was identified in this fraction in my earlier experiment (Figure 10). As shown in Figure 16, both E1 and E1-L2 were eluted from the ubiquitin column with DTT, suggesting that E1 and E2 formed thioester bonds with ubiquitin (lane 8 and lane 9 in the upper two panels).

To detect the E1-L2~Ubiquitin thioester directly, I incubated the purified N-terminal His_6-tagged E1-L2 and ubiquitin in the presence or absence of ATP for 15 minutes at 37^{o}C. After incubation, the reactions were stopped by adding SDS sample buffer in the absence of β-mercaptoethanol (β-ME), a reducing agent. The reaction mixture was separated the proteins by SDS-PAGE under the non-reducing conditions followed by immunoblotting with His specific antibodies. As shown in Figure 17A, E1-L2 and ubiquitin formed a slower

migrating band on the non-reducing gel and the formation of this slower migrating band was dependent on ATP (compare lane 13 to lane 14). This band was absent when the samples were treated with β-ME, indicating that E1-L2 and ubiquitin formed a thioester bond (Figure 17B; lane 7). E1 was used as control. A slower migrating band was observed when E1 and ubiquitin were incubated together with ATP and analyzed under the non-reducing conditions (Figure 17A; compare lane 2 to lane 1). The thioester formation between E1 and ubiquitin was also confirmed by the β-ME treatment (Figure 17B; lane 1).

Two different approaches have been independently used to determine the thioester formation between E1-L2 and ubiquitin. In the first experiment, after incubating with Ubiquitin-Sepharose and ATP, E1-L2 was eluted with DTT buffer after high salt wash. In addition, E1-L2 and ubiquitin form a slower migrating band on the non-reducing gel after analyzing the reactions by immunoblotting under the non-reducing condition. This slower migrating band is sensitive to a reducing agent (β-ME) and ATP is required for the formation of this band. Taken together, these results strongly suggest that E1-L2 is able to catalyze the formation of a thioester bond between itself and ubiquitin in vitro in an ATP-dependent manner.

III.C E1-L2 activates ubiquitin in cells.

The thioester bond formation between E1-L2 and ubiquitin was shown and confirmed by the in vitro assays described in the previous section. However, it is important to determine whether the thioester formation between these two proteins also takes place in cells.

Therefore, I further tested whether endogenous E1-L2 forms a thioester bond with endogenous ubiquitin. Overexpression of E1-L2 or ubiquitin was avoided in these experiments to minimize the artificial effects caused by high levels of the exogenous proteins. In order to study the endogenous E1-L2, the ability of E1-L2 specific antibodies to immunoprecipitate the endogenous E1-L2 protein was tested first. HEK293 cell lysates were incubated with E1-L2 antibodies or rabbit IgG at 4^{o}C for 1 hour followed by mixing with protein A/G beads for an another hour. As shown in Figure 18A, endogenous E1-L2 was immunoprecipitated by E1-L2 antibodies, but not by control rabbit IgG (compare lane 7 to lane 4).

To determine if endogenous E1-L2 and ubiquitin forms a thioester, HEK293 cells were lysed in TBS buffer containing 0.5% NP-40. Cell lysates were then immunoprecipitated using an antibody against E1-L2. Rabbit IgG was used as a negative control. After washing with lysis buffer, proteins were eluted with 10 mM DTT buffer. The condition used for elution with DTT buffer did not disrupt the interaction between E1-L2 antibodies and E1-L2 proteins (Figure 18A; lane 6). Because potential thioester bonds were cleaved with DTT, the proteins associated with E1-L2 through thioester bond were eluted in the DTT fraction. The remaining proteins were eluted with SDS sample buffer containing β-ME. The eluted proteins were resolved by SDS-PAGE and immunoblotted with an antibody against ubiquitin (Figure 18B). Ubiquitin was detected in the DTT eluate from the E1-L2 immunoprecipitates, but not from the control IgG precipitates (Figure 18B; lane 3 and lane 4 in the lower panel). This result suggests that E1-L2 forms a thioester with ubiquitin in cells. The low steady state levels of E1-L2~ubiquitin thioester intermediates may be due to the rapid transfer of

ubiquitin to the downstream enzymes (e.g, E2 enzymes) in the cascades (Figure 18B; compare lane 4 to lane 1 in the lower panel). In addition, the low steady state levels of E1-L2~ubiquitin may also be due to the competition between E1-L2 and ubiquitin E1.

In this section, I have shown that endogenous E1-L2 and endogenous ubiquitin were co-immunoprecipitated with E1-L2 specific antibodies in HEK293 cells. Furthermore, the interaction between these two proteins could be disrupted by a reducing agent, DTT, which suggested that these two proteins bound to each other through a thioester. Together with the results in the previous section, these finding indicate that E1-L2 forms a thioester with ubiquitin both in vivo and in vitro.

III.D E1-L2 transfers ubiquitin to select E2s

One of the properties of a functional activating enzyme is to transfer ubiquitin-like proteins to its cognate conjugating enzymes (E2). The interaction between the conjugating enzymes and ubiquitin-like proteins are mediated by a thioester bond. Since E1-L2 can form a thioester with ubiquitin (Figure 16 and 17), I examined whether E1-L2 could transfer ubiquitin to several known ubiquitin E2s, including Ubc5, Ubc13, Ubc3 and E2-25K. The similar thioester assay mentioned in the Section III.B was used to detect the E2~ubiquitin thioester intermediate. E1 or E1-L2 was incubated with E2 and ubiquitin in the presence or absence of ATP at 37^{o}C for 15 minutes. SDS sample buffer was added to stop the reaction. The samples were then resolved by SDS-PAGE followed by Coomassie blue staining or immunoblotting with Ubc5, His (for detecting Ubc13), or GST (for detecting Ubc3 and E2-25K) specific antibodies under the non-reducing condition. E1 was shown to transfer

ubiquitin to both Ubc5 and Ubc13 in the presence of ATP (Figure 19A; lane 2 and lane 6) and the E2~ubiquitin thioester intermediates were disrupted under the reducing conditions as expected (Figure 19B; lane 1 and lane 3). E2s and ubiquitin formed slower migrating bands on the non-reducing gel in the presence of E1-L2 and ATP. These results indicate that these two ubiquitin E2 enzymes were able to accept ubiquitin form E1-L2 in an ATP-dependent manner (Figure 19A; compare lane 4 and lane 8 to lane 3 and lane 7). The slower migrating bands were absent when the samples were analyzed under the reducing conditions (Figure 19B; lane 2 and lane 4). Therefore, in the presence of E1 or E1-L2, ubiquitin formed thioesters with Ubc5 and Ubc13 (Figure 19A). In the case of Ubc3 and E2-25K, E1 transferred ubiquitin to these two E2 enzymes as anticipated (Figure 20A; lane 2 and lane 6). Interestingly, E1-L2 could not support the transfer of ubiquitin to GST-Ubc3 or GST-E2-25K (Figure 20A; lane 4 and lane 8). GST is a 26kDa protein which is larger than Ubc3 and is about the same size of E2-25K. It is possible that GST tag might interfere with the ubiquitin transfer from E1-L2 to GST-Ubc3 and GST-E2-25K. To rule out this possibility, GST tags were removed from GST-Ubc3 and GST-E2-25K by thrombin protease. Then these two E2 enzymes lacking the epitope tag were used in the same assay and the same results as using GST-Ubc3 or GST-E2-25K were observed. Therefore, the absence of ubiquitin transfer was not due to the presence of the GST tag, as Ubc3 and E2-25K lacking the tag could accept the activated ubiquitin from E1 but not from E1-L2 (Figure 20B; lane 2 and lane 6 for E1; lane 4 and lane 8 for E1-L2). The E1-L2~ubiquitin thioester intermediate could still be observed in these reactions, hence the inability of ubiquitin transfer to Ubc3 and E2-25K from E1-L2 was not due to the absence of E1-L2~ubiquitin formation. According to the in vitro thioester

assay, E1-L2 transferred ubiquitin to Ubc5 and Ubc13, but not to Ubc3 and E2-25K, whereas E1 could transfer ubiquitin to each of these four E2 enzymes. These results showed that although E1-L2 is capable of activating ubiquitin, it can only transfer ubiquitin to a specific subset of E2s.

III.E E1-L2 promotes polyubiquitination.

In addition to forming a thioester with ubiquitin-like protein and then transferring ubiquitin-like protein to its cognate E2s, another property of the activating enzyme is to promote ubiquitin-like modification. Since E1-L2 formed a thioester with ubiquitin and transferred ubiquitin to a subset of ubiquitin E2 enzymes, it is likely that E1-L2 could promote certain type of polyubiquitination. I have shown that E1-L2 transferred ubiquitin to Ubc5 and Ubc13. Therefore, I chose Ubc13 as the E2 enzyme to test the formation of polyubiquitin chain by E1-L2. The in vitro ubiquitination assay reported by our laboratory was used for detecting the polyubiquitin chain (Deng, Wang et al. 2000). I tested the ability of E1-L2 to stimulate the synthesis of polyubiquitin chains by Ubc13/Uev1A, a heterodimeric E2 complex, and TRAF6, a RING domain E3. Various amounts of E1 or E1-L2 (from 3 ng to 300 ng) were incubated with E2 complex, E3 enzyme, ubiquitin and ATP at 37^{o}C for 15 minutes (Figure 21; lower panel). After incubation, the samples from each reaction were divided into two aliquots. One aliquot of the samples was added with SDS sample buffer in the presence of β-ME. These samples were resolved by SDS-PAGE and immunoblotted with ubiquitin specific antibodies under reducing conditions. As shown in Figure 21, even at low concentration, E1 stimulated the synthesis of polyubiquitin chains efficiently (lane 13 in upper panel). E1-L2 was able to promote polyubiquitination in the

presence of Ubc13/Uev1A and TRAF6 (Figure 21; even lanes in upper panel). Although E1-L2 could support polyubiquitin chain synthesis, its activity was weaker than that of E1 (Figure 21; compare even lanes to odd lanes in upper panel). With 12 ng of E1-L2, the synthesis of polyubiquitin chain was barely observed (Figure 21; lane 10 in upper panel). Another aliquot of each sample was mixed with SDS sample buffer in the absence of β-ME, and these samples were analyzed by immunoblotting with His specific antibodies to observe the His_6-Ubc13/Uev1A~ubiquitin thioester intermediate (Figure 21; middle panel). The ability of E1-L2 to transfer ubiquitin to Ubc13/Uev1A was also weaker than that of E1 (Figure 21; compare even lanes to odd lanes in middle panel). Therefore, the reduced polyubiquitin chain synthesis by E1-L2 was due, at least in part, to the less efficient formation of Ubc13~Ub thioester in the presence of E1-L2 than in the presence of E1. These results suggest that even though E1-L2 can promote the synthesis of polyubquitin chain, it may not be as efficient as E1 in catalyzing certain ubiquitination reactions.

III.F Summary

In this chapter, I have demonstrated that E1-L2 ia able to activate ubiquitin. E1-L2 was identified as an E1-like protein in the course of identifying ubiquitin-binding proteins with ubiquitin-affinity chromatography and contains all the essential elements of a functional activating enzyme. E1-L2 shares 42% identity with ubiquitin E1 and is evolutionarily conserved. However, unlike ubiquitin E1, E1-L2 cannot be found in the genomes of yeast, *Drosophila* or *C. elegans*. E1-L2 is predominantly located in cytosol based on subcellular fractionation. The concentration of E1-L2 in HEK293 cells is less than 25% of the

concentration of E1. Like ubiquitin E1, E1-L2 also forms a thioester with ubiquitin both in vivo and in vitro in an ATP-dependent manner. In the in vivo assay, the E1-L2~ubiquitin thioester intermediate is clearly detected in HEK293 cells, although the steady state level of E1-L2~ubiquitin is low. In the in vitro thioester assay using ubiquitin affinity column, a fraction of E1-L2 binds to ubiquitin-sepharose noncovalently, explaining why E1-L2 was identified as a ubiquitin-binding protein in the pH 9.0/1 M KCl fraction at the beginning. E1-L2 can only transfer ubiquitin to a subset of E2 enzymes, including Ubc5 and Ubc13, but not Ubc3 and E2-25K, whereas E1 transfers ubiquitin to Ubc5, Ubc13, Ubc3 and E2-25K. Furthermore, E1-L2 can support the synthesis of polyubiquitin chain in the presence of Ubc13/Uev1A and TRAF6. However, the efficiency of E1-L2 in polyubiquitin chain synthesis is weaker than the efficiency of ubiquitin E1. These results indicate that E1-L2 is involved in the activating step of certain types of ubiquitination, but the physiological implication of having two ubiquitin-activating enzymes remain a mystery. E1-L2 may play a role in other ubiquitin-like modifications.

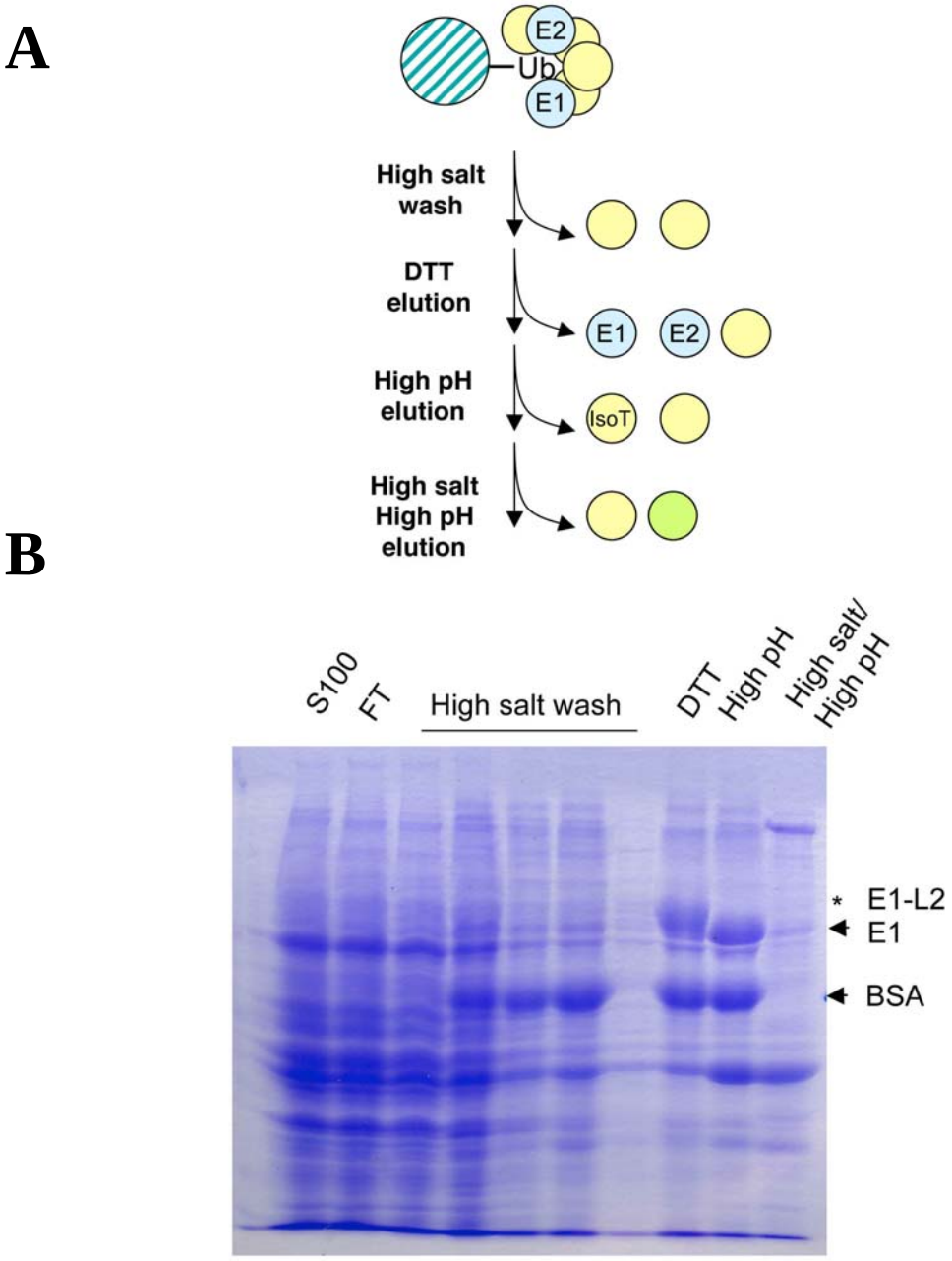
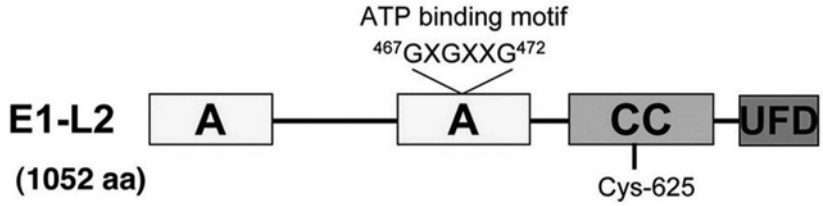


Figure 10. Identification of E1-L2 by ubiquitin affinity chromatography.
(A) HeLa S100 was incubated with Ub-Sepharose in the presence of ATP at 25^{o}C for 1 hour, and then the slurry was poured into a column. The column was eluted sequentially with a buffer containing 1M KCl (high salt wash), 10 mM DTT, a pH 9.0 buffer (high pH elution) and finally a pH 9.0 buffer containing 1M KCl (high salt / high pH elution). The collection tubes for the DTT and high pH elutions contain BSA as a carrier protein. (B) Aliquots of the eluted fractions were analyzed by SDS-PAGE followed by Coomassie blue staining. IsoT: isopeptidase T.

A



B

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E1          1 MSSSPLSKKRRVSGPDPKPGSNCSPAQSVLSEVPSVPTNGMAKNGSEADIDEGLYSRQLY
E1-L2       1 -----------MEGSEPVAAHQGEEASCSSWGTGSTNKNLPIMSTASVEIDDALYSRQRY

E1         61 VLGHEAMKRLQTSSVLVSGLRGLGVEIAKNIILGGVKAVTLHDQGTAQWADLSSQFYLRE
E1-L2      50 VLGDTAMQKMAKSHVFLSGMGGLGLEIAKNLVLAGIKAVTIHDTEKCQAWDLGTNFFLSE

E1        121 EDI--GKNRAEVSQPRLAELNSYVPVTAYTGPLVE----DFLSGFQVVVLTNTPLEDQLR
E1-L2     110 DDVVNKRNRAEAVLKHIAELNPYVHVTSSSVPFNETTDLSFLDKYQCVVLTEMKLPLQKK

E1        175 VGEFCHNR--GIKLVVADTRGLFGQLFCDFGEEMILTDSNGEQPLSAMVSMVTKDNPGVV
E1-L2     170 INDFCRSQCPPIKFISADVHGIWSRLFCDFGDEFEVLDTTGEEPKEIFISNITQANPGIV

E1        233 TCLDEARHGFESGDFVSFSEVQGMVELNGNQPMEIKVLGPYTFSICDTSNFSDYIRGGIV
E1-L2     230 TCLENHPHKLETGQFLTFREINGMTGLNGSI-QQITVISPFSFSIGDTTELEPYLHGGIA

E1        293 SQVKVPKKISFKSLVASLAEPDFVVTDFAKFSRPAQLHIGFQALHQFCAQHGRPPRPRNE
E1-L2     289 VQVKTPKTVFFESLERQLKHPKCLIVDFSNPEAPLEIHTAMLALDQFQEKYSRKPNVGCQ

E1        353 EDAAELVALAQAVNARALPAVQQNNLDEDLIRKLAVVAAGDLAPINAFIGGLAAQEVMKA
E1-L2     349 QDSEELLKLATSIS-ETLL--EEKPDVNADIVHWLSWTAQGFLSPLAAAVGGVASQEVLKA

E1        413 CSGKFMPIMQWLYFDALFCLPEDKEVLTEDKCLQRQNRYDGQVAVFGSDLQEKLGKQKYF
E1-L2     406 VTGKFSPLCQWLYLDAAADIVESLGKPECEE-FLPRGDRYDALRACIGDTLCQKLQNLNIF

E1        473 LVGAGAIGCELLKNFAMIGLGCG-EGGEIIVTDMDTIEKSNLNRQFLFRPWDVTKLKSDT
E1-L2     465 LVGCGAIGCEMLKNFALLGVGTSKEKGMITVTDPDLIEKSNLNRQFLFRPHHIQKPKSYT
                ▲  ▲  ▲
E1        532 AAAAAVRQMNPHIRVTSHQNRVGPDTERIYDDDFFQNLDGVANALDNVDARMYMDRRCVYY
E1-L2     525 AADATLKINSQIKIDAHLNKVCPTTETIYNDEFYTKQDVIITALDNVEARRYVDSRCLAN

E1        592 RKPLLESGTLGTKGNVQVVIPFLTESYSSSQDPPEKSIPICTLKNFPNAIEHTLQWARDE
E1-L2     585 LRPLLDSGTMGTKGHTEVIVPHLTESYNSHRDPPEEEIPFCTLKSFPAAIEHTIQWARDK
                                                  *
E1        652 FEGLFKQPAENVNQYLTDPKFVERTL-RLAGTQPLEVLEAVQRSLVLQRPQTWADCVTWA
E1-L2     645 FESSFSHKPSLFNKFWQTYSSAEEVLQKIQSGHSLEGCFQVIK-LLSRRPRNWSQCVELA

E1        711 CHHWHTQYSNNIRQLLHNFPPDQLTSSGAPFWSGPKRCPHPLTFDVNNPLHLDYVMAAAN
E1-L2     704 RLKFEKYFNHKALQLLHCFPLDIRLKDGSLFWQSPKRPFSPIKFDLNEPLHLSFLQNAAK

E1        771 LFAQTYGLTG-SQDRAAVA--TFLQSVQVPEFTPKSGV-KIHVSDQELQSANASVDDSR-
E1-L2     764 LYATVYCIPFAEEDLSADALLNILSEVKIQEFKPSNKVVQTDETARKPDHVPISSEDERN

E1        826 --LEELKATLPSPDKLPGFKMYPIDFEKDDDSNFHMDFIVAASNLRAENYDIPSADRHKS
E1-L2     824 AIFQLEKAILSNEATKSDLQMAVLSFEKDDDHNGHIDFITAASNLRAKMYSIEPADRFKT

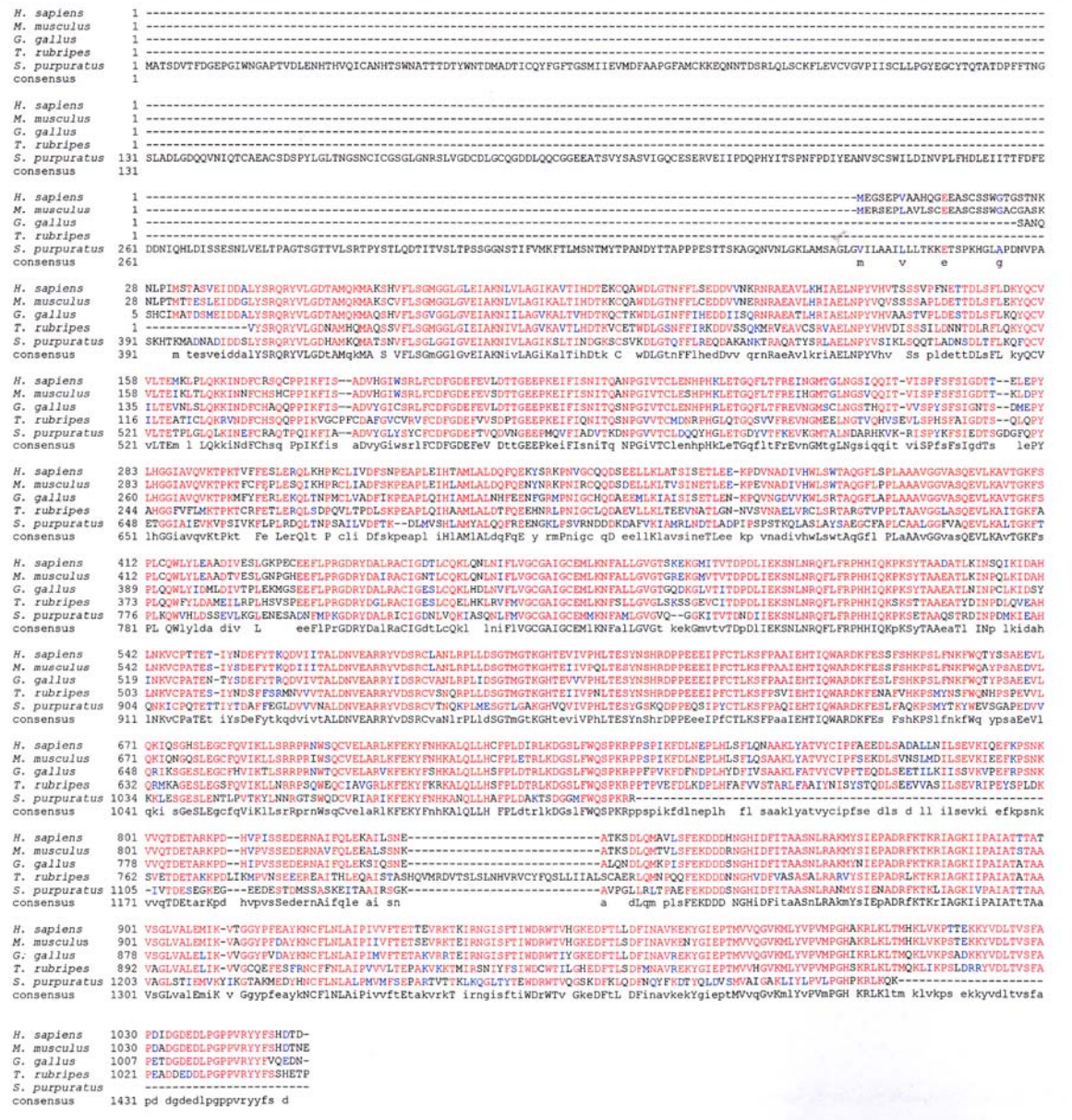
E1        884 KLIAGKIIPAIATTTAAVVGLVCLELYKVVQGHRQLDSYKNGFLNLALPFFGFSEPLAAP
E1-L2     884 KRIAGKIIPAIATTTATVSGLVALEMIKVTGGY-PFEAYKNCFLNLAIPIVVFTETTEVR

E1        944 RHQYYNQ-EWTLWDRFEVQGLQPNGEEMTLKQFLDYFKTEHKLEITMLSQGVSMLYSFFM
E1-L2     943 KTKIRNGISFTIWDRWTVHG----KEDFTLLDFINAVKEKYGIEPTMVVQGVKMLYVPVM

E1       1003 PAAKLKERLDQPMTEIVSRVSKRKLGRHVRALVLELCCNDESGEDVEVPYVRYTIR----
E1-L2     999 PGH--AKRLKLTMHKLVKPTTEKK---YV-DLTVSFAPDIDGDEDLPGPPVRYYFSHDTD
```

Figure 11. Identification of E1-L2.
(A) Schematic illustration of the domain structure of E1-L2. E1-L2 contains two adenylation domain, a catalytic cysteine domain, and a ubiquitin-fold domain. The ATP-binding motif is found in the second adenylation domain and a conserved cysteine residue is predicted in the catalytic cysteine domain. A: adenylation domain; CC: catalytic cysteine domain; UFD: ubiquitin-fold domain. (B) Alignment of E1 and E1-L2. The putative catalytic cysteine (Cys625) is indicated by an asterisk (*). The glycine triad of the ATP binding motif is indicated by the triangles. The UFD domain is underlined.

A



B

Identity (%)	*H. sapiens*	*M. musculus*	*G. gallus*	*T. rubripes*	*S. purpuratus*
H. sapiens	**100**	**89**	**80**	**54**	**56**
M. musculus		**100**	**79**	**65**	**49**
G. gallus			**100**	**66**	**56**
T. rubripes				**100**	**53**
S. purpuratus					**100**

Figure 12. Evolutionary conservation of E1-L2.
(A) Alignment of E1-L2 protein sequences from human (*H. sapiens*), mouse (*M. musculus*), chicken (*G. gallus*), pufferfish (*T. rubripes*) and sea urchin (*S. purpuratus*). E1-L2 orthologs cannot be found in yeast, *Drosophila* and *C. elegans*. (B) Sequence identity scores of E1-L2 proteins from different species.

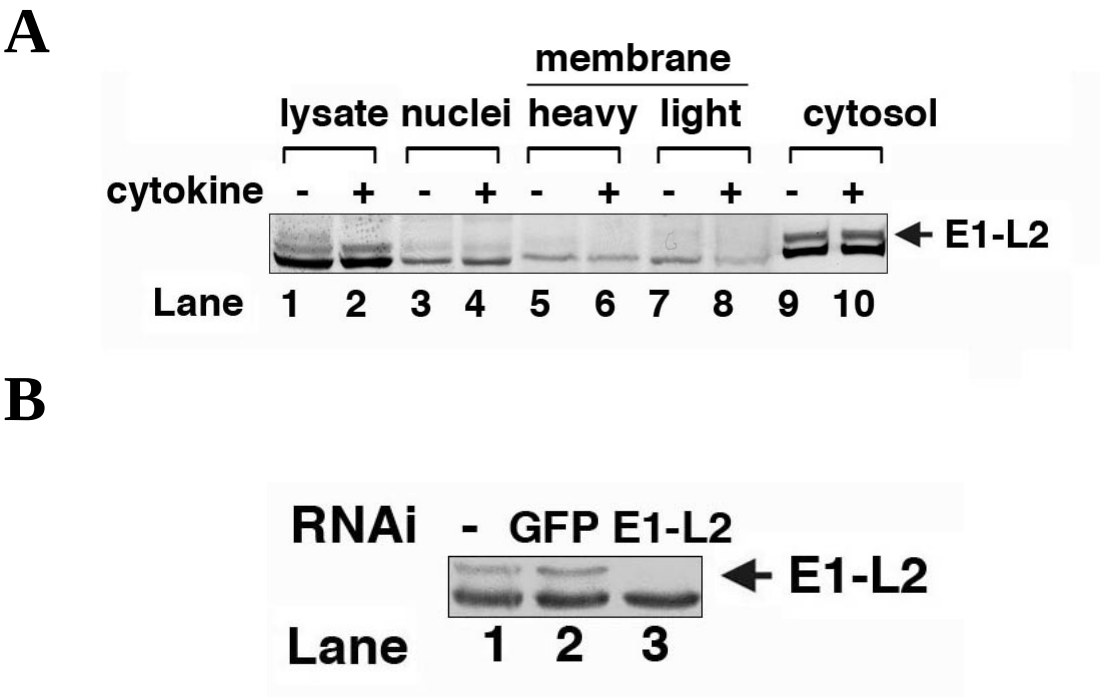


Figure 13. Subcellular localization of E1-L2.
(A) Crude lysates from HEK293 cells were fractionated into nuclei, heavy membranes, light membranes, and cytosol by differential centrifugation as described in the Section II.B.5. Proteins in these fractions were analyzed by immunoblotting with an antibody against E1-L2. (B) The specificity of the E1-L2 antibody was verified by RNAi using siRNA oligos against E1-L2 or GFP (control).

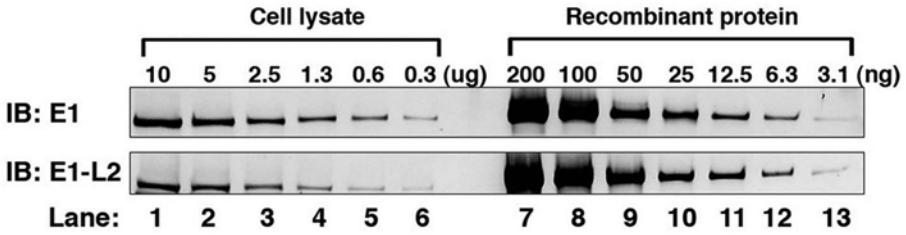


Figure 14. Semi-quantitative immunoblotting of E1 and E1-L2 in HEK293 cells.
Different amounts of cytosolic extracts of HEK293 cells were immunoblotted with an antibody against E1 or E1-L2. For quantitation, different amounts of purified E1 or E1-L2 protein were immunoblotted with the indicated antibodies.

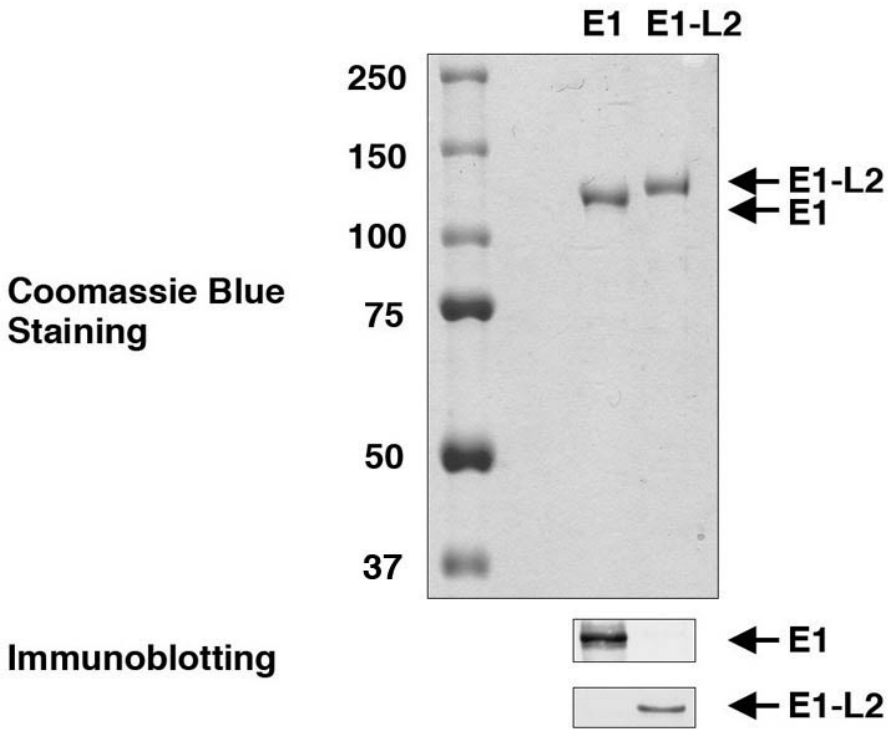


Figure 15. Purified recombinant E1 and E1-L2 proteins.
Purified recombinant E1 and E1-L2 proteins were resolved with SDS-PAGE followed by Coomassie blue staining or immunoblotting with E1 or E1-L2 specific antibodies. Both recombinant proteins were fused to an N-terminal His_6 tag.

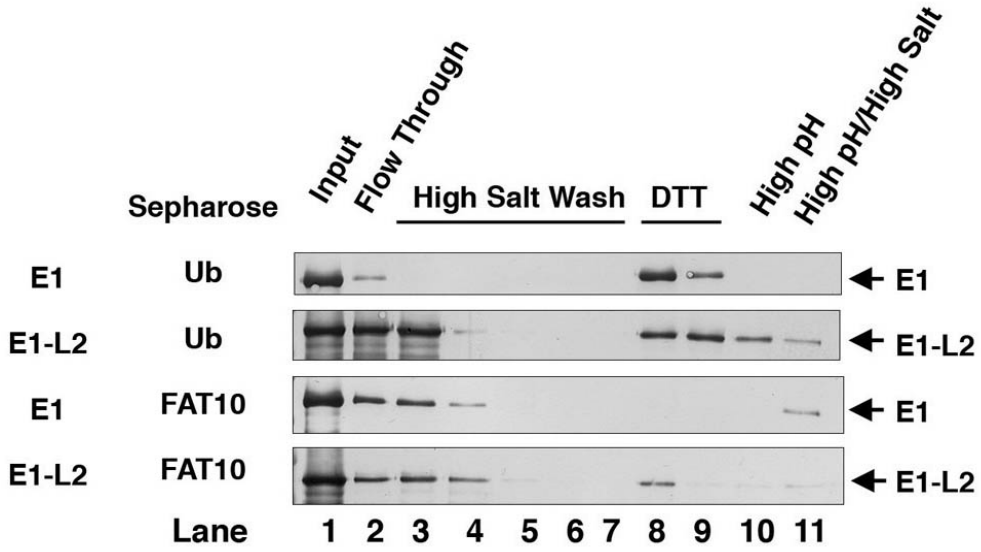
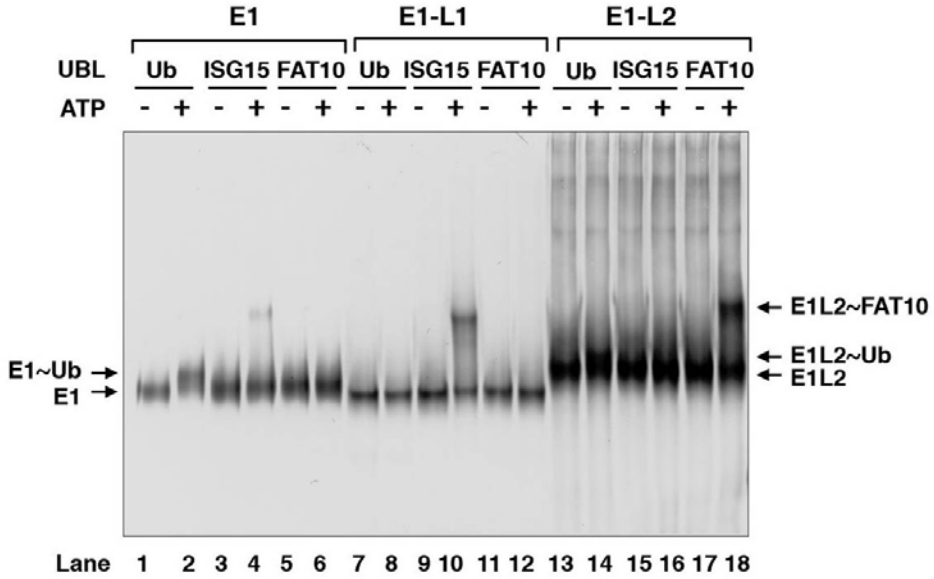


Figure 16. E1-L2 forms a thioester with ubiquitin and FAT10 *in vitro*.
Purified E1 (upper two panels) or E1-L2 (lower two panels) was incubated with Ub- or FAT10-Sepharose in the presence of ATP. The beads were washed with a buffer containing 1M KCl before elution with 10 mM DTT. Subsequently, the beads were eluted with a pH9.0 buffer followed by elution with a pH9.0 buffer containing 1M KCl. These fractions were immunoblotted with a His_6-specific antibody to detect E1 and E1-L2.

A



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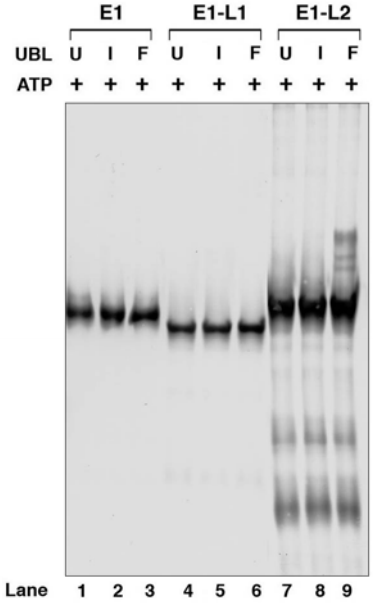
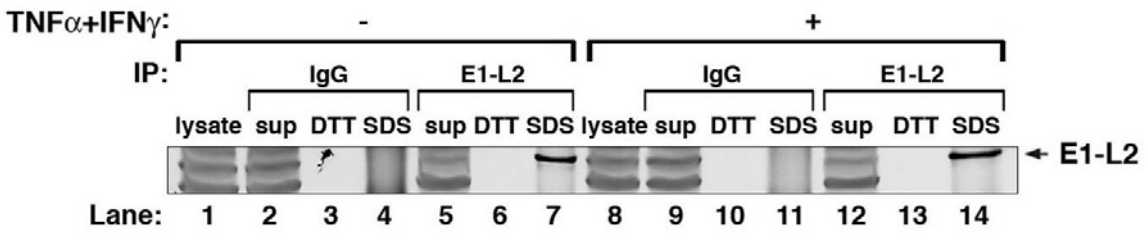


Figure 17. The thioester assay for E1, E1-L1 and E1-L2 proteins.
(A) Purified E1, E1-L1 or E1-L2 was incubated with ubiquitin, ISG15 or FAT10 in the presence or absence of ATP as indicated. The samples were resolved by SDS-PAGE in the absence of β-mercaptoethanol. (B) The same experiment described in (A) was carried out except the samples were resolved by SDS-PAGE in the presence of β-mercaptoethanol. U: ubiquitin; I: ISG15; F: FAT10.

A



B

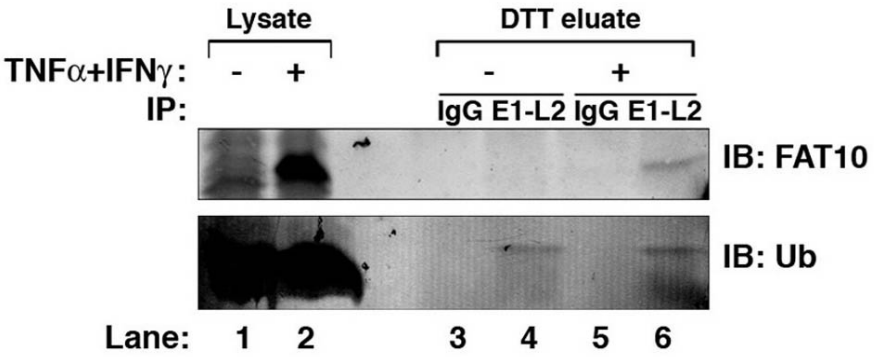
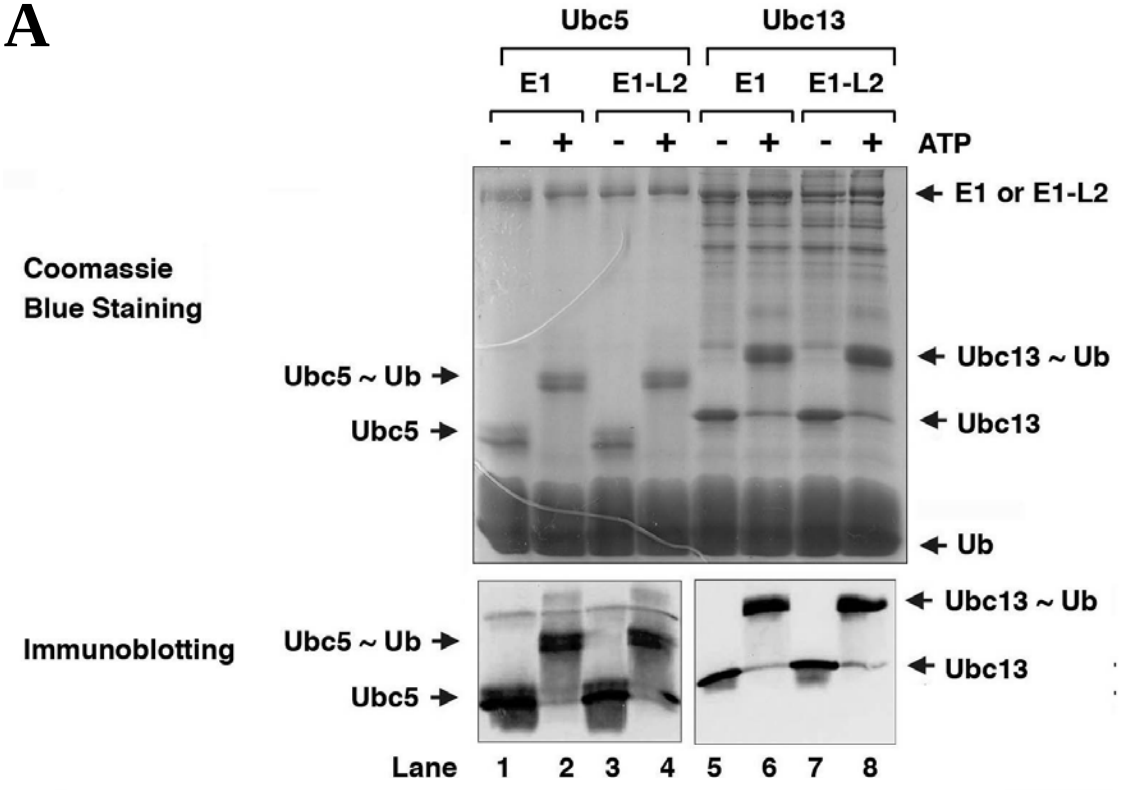


Figure 18. E1-L2 forms a thioester with ubiquitin and FAT10 in cells.
(A) HEK293 cells were stimulated with TNFα (0.2μg/ml) and IFNγ (3000U/ml) for 16 hours to induce FAT10 expression. The cell lysates were immunoprecipitated with an E1-L2 antibody or control IgG. Proteins form thioester with E1-L2 were eluted with DTT after washed with lysis buffer. The remaining proteins were eluted with SDS. The fractions were immunoblotted with the E1-L2 antibody. (B) The DTT eluates were resolved by SDS-PAGE and immunoblotted with an antibody against ubiquitin (lower panel) or FAT10 (upper panel).


A
Ubc5
Ubc13
E1
E1-L2
E1
E1-L2
- + - + - + - +
ATP
Coomassie
Blue Staining
E1 or E1-L2
Ubc13 ~ Ub
Ubc5 ~ Ub
Ubc13
Ubc5
Ub
Immunoblotting
Ubc5 ~ Ub
Ubc13 ~ Ub
Ubc5
Ubc13
Lane 1 2 3 4 5 6 7 8

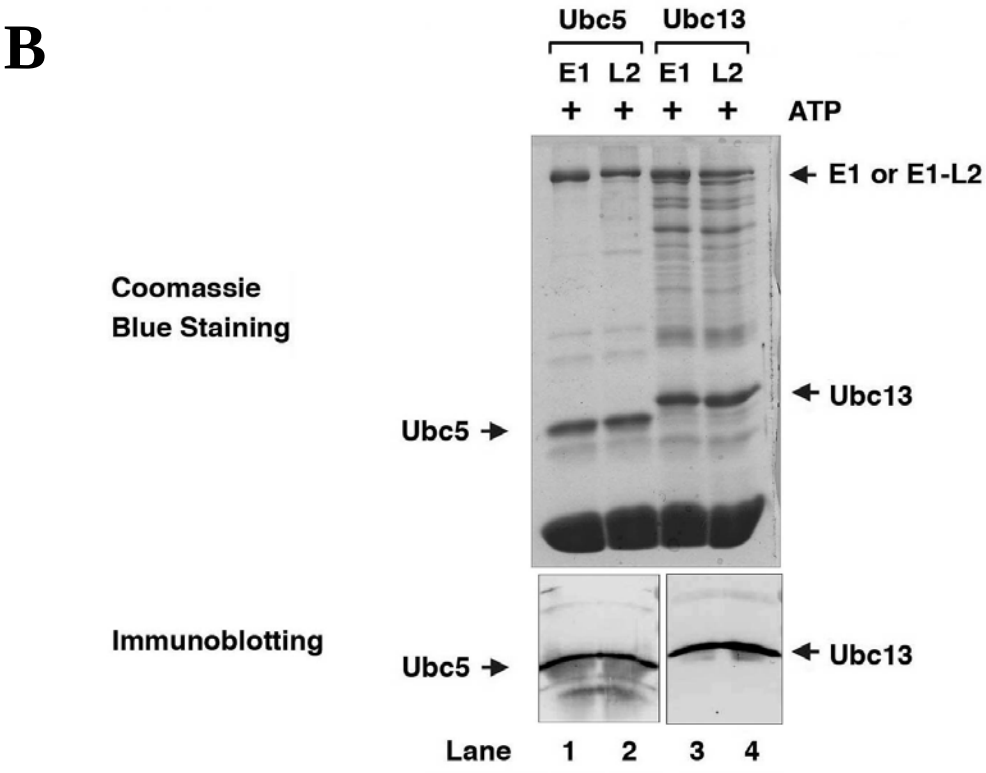
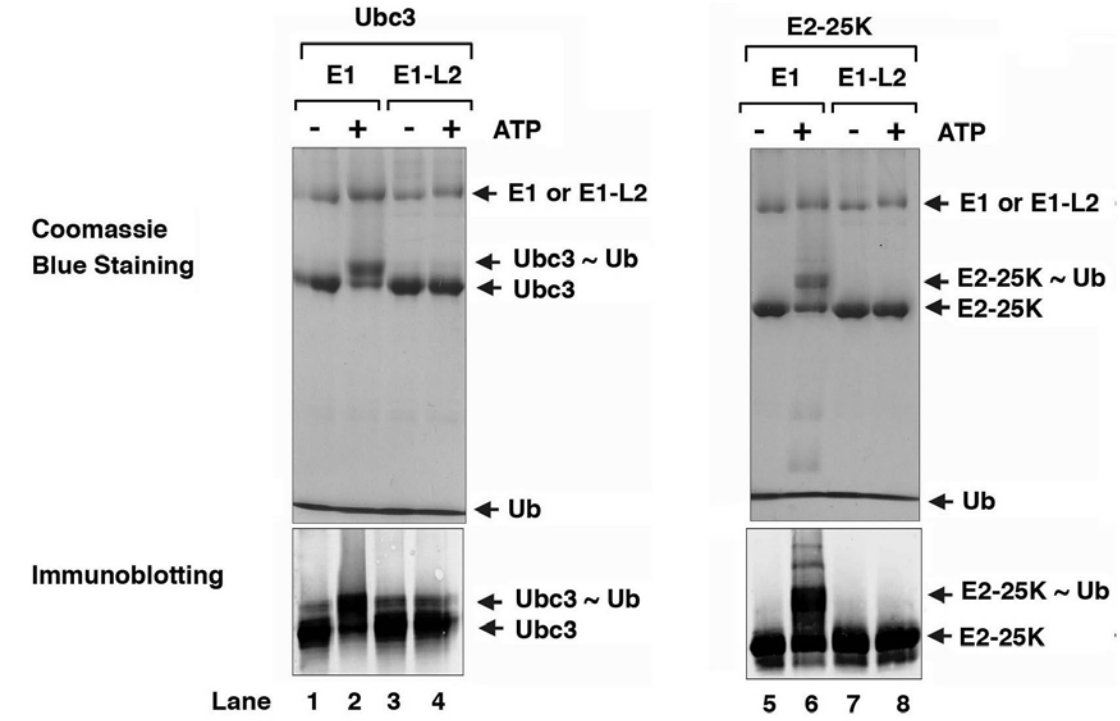

B
Ubc5
Ubc13
E1 L2 E1 L2
+ + + +
ATP
Coomassie
Blue Staining
E1 or E1-L2
Ubc13
Ubc5
Immunoblotting
Ubc5
Ubc13
Lane 1 2 3 4

Figure 19. E1-L2 transfers ubiquitin to Ubc5 and Ubc13.
(A) Purified E1 or E1-L2 was incubated with Ubc5 or Ubc13 in the presence of ubiquitin and ATP. The thioester assays were carried out as described in the Section II.B.4. The proteins were visualized by Coomassie staining as well as by immunoblotting using antibodies against Ubc5 (lower left) or His_6 (for Ubc13; lower right). (B) The same experiments were carried out as described in (A), except the samples were resolved by SDS-PAGE in the presence of β-mercaptoethanol. L2: E1-L2.

A


Ubc3
E1
E1-L2
- + - + ATP
E2-25K
E1
E1-L2
- + - + ATP
Coomassie
Blue Staining
E1 or E1-L2
Ubc3 ~ Ub
Ubc3
Ub
E1 or E1-L2
E2-25K ~ Ub
E2-25K
Ub
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Ubc3 ~ Ub
Ubc3
E2-25K ~ Ub
E2-25K
Lane 1 2 3 4
5 6 7 8

B

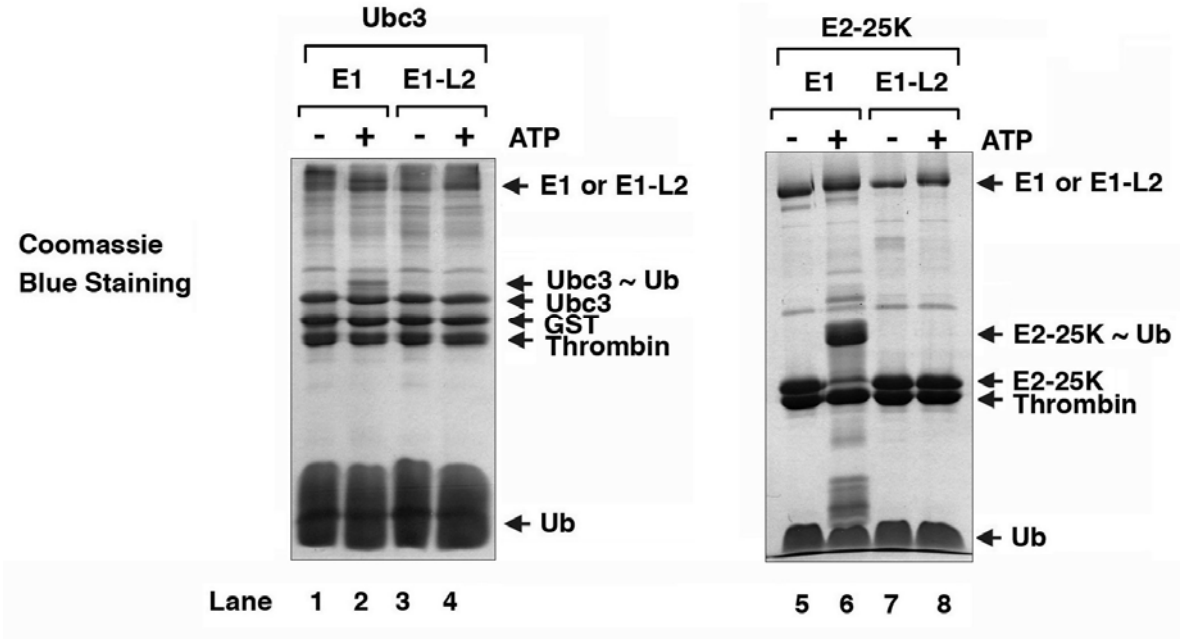

Ubc3
E1
E1-L2
- + - + ATP
E2-25K
E1
E1-L2
- + - + ATP
Coomassie
Blue Staining
E1 or E1-L2
Ubc3 ~ Ub
Ubc3
GST
Thrombin
Ub
E1 or E1-L2
E2-25K ~ Ub
E2-25K
Thrombin
Ub
Lane 1 2 3 4
5 6 7 8

Figure 20. E1, but not E1-L2, transfers ubiquitin to Ubc3 and E2-25K.
(A) Thioester assays were carried out as described in Figure 19, except that GST-Ubc3 and GST-E2-25K were used as the E2s. Immunoblotting was carried out using an antibody against GST. In the lower left panel, the weak band above Ubc3 in lanes 1, 3 and 4 might represent a contaminant that cross-reacted with the GST antibody. (B) GST-Ubc3 and GST-E2-25K were cleaved with thrombin to remove the GST tag, and the E2s were incubated with ubiquitin and E1 or E1-L2 in the presence or absence of ATP. The reaction mixtures were resolved by non-reducing SDS-PAGE and the gels were stained with Coomassie blue.

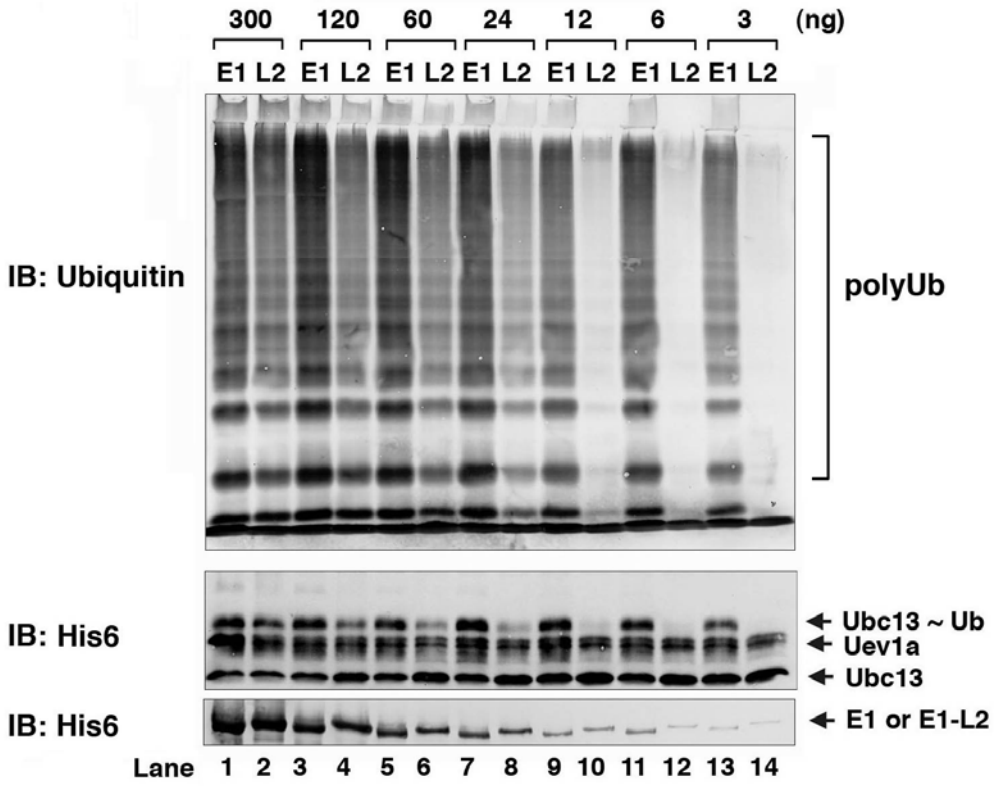


Figure 21. E1-L2 promotes polyubiquitination.
Different amounts of E1 or E1-L2 (3-300 ng) was incubated with Ubc13/Uev1A (E2), TRAF6 (E3), ubiquitin and ATP. An aliquot of the reaction mixtures was separated by SDS-PAGE under reducing conditions and immunoblotted with a ubiquitin antibody (top panel). The other aliquot was separated by non-reducing SDS-PAGE and immunoblotted with a His_6 antibody to detect Ubc13 and Uev1A (middle panel). The amount of E1 or E1-L2 proteins used in the reactions was monitored by immunoblotting with a His_6 antibody (bottom panel).

CHAPTER IV: ACTIVATION OF FAT10 BY E1-L2

IV.A E1-L2 forms a thioester with FAT10.

The domain structure of E1-L2 is similar to E1 and E1-L1. These three activating enzymes all consist of two adenylation domains, a catalytic cysteine domain and a ubiquitin-fold domain. E1 is involved in activating step of ubiquitination and E1-L1 activates ISG-15, which contains two ubiquitin-like domains. Another ubiquitin-like protein, FAT10, is also composed of two ubiquitin-like domains (Liu, Pan et al. 1999). The conjugation machinery of FAT10, including the activating enzyme had not been identified. Based on the resemblance between E1-L1 and E1-L2 and the similarity between ISG15 and FAT10, I speculated that E1-L2 might be involved in the conjugation of FAT10 and function as the activating enzyme for FAT10 (Figure 22). To test this possibility, the similar in vitro assays with affinity column and the thioester assays mentioned in the Section III.B were performed. I generated recombinant FAT10 proteins from both *E. coli* and baculovirus expression systems (Figure 25A). FAT10 proteins were conjugated to Sepharose beads as described in the Section II.A.4 and used for determining whether E1-L2 could bind to FAT10-Sepharose in a manner that is sensitive to the DTT treatment. His_6-tagged E1 or E1-L2 was incubated with FAT10-Sepharose in the presence of ATP at room temperature for 1 hour followed by subsequent wash and elution. The fractions were analyzed by immunoblotting with His specific antibodies to detect E1 or E1-L2 (Figure 16; lower two panels). Indeed, E1-L2 bound to FAT10-Sepharose and was eluted with the DTT buffer (Figure 16; lane 8 in the bottom panel). Although a small fraction of E1 was found in the pH 9.0/1 M KCl eluate, E1

was not detected in the DTT fraction eluted from FAT10-Sepharose (Figure 16; lane 8 and lane 9 in the third panel). These results indicate that E1-L2, but not E1, can form a thioester bond with FAT10.

To detect the E1-L2~FAT10 thioester intermediate directly, the thioester assay was performed. I incubated the purified His_6-tagged E1-L2 and FAT10 proteins in the presence or absence of ATP for different lengths of time (from 1 minute to 15 minutes) at 37^{o}C. Then the proteins were separated by SDS-PAGE in the presence or absence of β-mercaptoethanol followed by immunoblotting with His specific antibodies. As shown in Figure 23, E1-L2 and FAT10 formed a slower migrating band on the non-reducing gel as early as 1 minute following the addition of ATP (lane 4, 6, 8, 10 and 12). This band was absent when the samples were treated with β-ME, indicating that E1-L2 and FAT10 formed a thioester bond (Figure 23; lower panel). Upon longer incubation, a small fraction of the slower migrating band became resistant to cleavage by β-ME, suggesting that a small amount of E1-L2 might form a covalent bond, which is not a thioester, with FAT10 after prolonged incubation (Figure 23; lane 20, 22, and 24). There is a predicted catalytic cysteine residue at position 625 (Cys625) in E1-L2 protein. To further investigate whether this cysteine residue is involved in the formation of a thioester, Cys625 of E1-L2 was mutated to alanine (C625A). Moreover, the diglycine motif at the C-terminus of FAT10, which is predicted to be involved in thioester formation and substrate modification, was mutated to dialanine (AA). Wild-type as well as mutant E1-L2 and FAT10 proteins were incubated together in the presence or absence of ATP at 37^{o}C for 15 minutes and immunoblotted with His specific antibodies under non-reducing condition (Figure 24). The slower migrating band was observed only

when both wild-type E1-L2 and FAT10 were present in the assay (Figure 24; lane 2). The formation of the E1-L2~FAT10 thioester intermediate was abolished by mutating cysteine 625 of E1-L2 (C625A) or the C-terminal diglycine motif of FAT10 (AA), indicating that the thioester bond was formed between Cys625 of E1-L2 and the C-terminus of FAT10 (Figure 24; compare lane 4, 6, and 8 to lane 2). Recombinant FAT10 proteins purified from *E. coli* expression system had an N-terminal GST tag and then Tev protease was used to remove GST tags from GST-FAT10 (Figure 25A). Next, GST-FAT10 and FAT10 lacking the tag together with His_6-FAT10 purified from baculovirus expression systems were tested in the thioester assay. I found that GST-FAT10, but not FAT10 lacking the tag or His_6-FAT10, did not form a thioester with E1-L2, suggesting that the large GST tag may interfere with FAT10 activation by E1-L2 (Figure 25B; compare lane 2 to lane 4 and 6).

In the next step, I tested the specificity of E1, E1-L1 and E1-L2 in activating ubiquitin-like proteins by the in vitro thioester assay. His_6-tagged E1, E1-L1 or E1-L2 was incubated with ubiquitin, ISG15 or FAT10 in the presence or absence of ATP at 37^{o}C for 15 minutes and analyzed by immunoblotting with His specific antibodies under non-reducing (Figure 17A) or reducing condition (Figure 17B). The slower migrating bands shown under the non-reducing conditions were absent or decreased in the presence of β-ME, indicating that the slower migrating bands represented the thioester intermediate consisting of activating enzymes bound to ubiquitin-like proteins. E1 formed a thioester with ubiquitin and more weakly with ISG15 in an ATP-dependent manner, but it did not form a thioester with FAT10 (Figure 17A; lane 2, 4 and 6). E1-L1 could only form a thioester with ISG15, but not ubiquitin and FAT10 (Figure 17A; lane 8, 10 and 12). E1-L2 formed thioesters with ubiquitin

and FAT10, but not ISG15, in the presence of ATP (Figure 17A; lane 14, 16 and 18). Since the formation of thioester bond between E1-L1 and ubiquitin was not be observed, the ability of E1 or E1-L2 to form thioesters with ubiquitin is neither a general feature of all the activating enzymes nor an artificial reaction caused by using recombinant proteins in in vitro assay. More importantly, among the E1s tested, only E1-L2 can activate FAT10 in vitro. Taken together, these results demonstrate the specificity between E1-L2 and FAT10.

In the FAT10 affinity purification experiment, E1-L2 is eluted with DTT buffer from FAT10-Sepharose after high salt wash. In addition, E1-L2 and FAT10 formed a slower migrating band on the non-reducing gel in an ATP-dependent manner, which is sensitive to a reducing agent (β-ME). Furthermore, I have also shown that FAT10 forms thioester specifically with E1-L2. These findings strongly suggest that E1-L2 functions as the activating enzyme for FAT10 in vitro.

IV.B E1-L2 activates FAT10 in cells.

IV.B.1 E1-L2 forms a thioester with exogenous FAT10.

I have shown the thioester formation between E1-L2 and FAT10 by the in vitro assay in the previous section. Next, I wanted to test whether the thioester formed between these two proteins also occurs in cells. To determine if E1-L2 forms a thioester with FAT10 in cells, I transfected expression constructs encoding FLAG-FAT10 (WT) or a FAT10 mutant carrying the C-terminal diglycine mutations (AA) into HEK293 cells. The cells were lysed in TBS buffer containing 0.5% NP-40 and the cytosolic extracts were collected 48 hours after

transfection,. The FAT10 proteins were immunoprecipitated with ANTI-FLAG M2 affinity gel at 4^{o}C for two hours. After washing with lysis buffer, the proteins that formed a thioester with FAT10 were eluted with DTT buffer. The remaining proteins were eluted with FLAG peptides (Figure 26). SDS sample buffer with β-ME was added to the fractions. The DTT eluate was resolved by SDS-PAGE and immunoblotted with an E1-L2 specific antibody. As shown in Figure 26, endogenous E1-L2 was detected in the DTT fractions eluted from wild-type, but not mutant, FLAG-FAT10 immunoprecipitates (upper panel). The presence of E1-L2 in the DTT eluate suggests that the endogenous E1-L2 protein forms a thioester bond with the wild-type, but not mutant, FLAG-FAT10. The final eluate was immunoblotted with a FLAG specific antibody to detect FLAG-FAT10 proteins (Figure 26; lower panel). The expression levels of wild-type and mutant FLAG-FAT10 were comparable. Therefore, the lack of E1-L2~FAT10 thioester intermediate in cells overexpressed mutant FAT10 was not due to the lower expression level of the mutant proteins. These finding suggest that endogenous E1-L2 forms a thioester with FLAG-FAT10 through the diglycine motif at the C-terminus of FAT10 in HEK293 cells.

IV.B.2 FAT10 is induced by TNF-alpha and IFN-gamma in cells.

Endogenous E1-L2 forms thioester with exogenous wild-type, but not mutant, FAT10 in HEK293 cells. In the next step, I wanted to test whether the thioester bond is formed between endogenous E1-L2 and endogenous FAT10 in cells. According to the literature, FAT10 mRNA is highly inducible by TNFα and IFNγ treatment (Bates, Ravel et al. 1997; Raasi, Schmidtke et al. 1999). However, it is difficult to detect endogenous FAT10 proteins

by immunoblotting analysis in the absence of cytokine induction (Liu, Pan et al. 1999; Raasi, Schmidtke et al. 2001). In order to investigate whether endogenous E1-L2 and FAT10 form a thioester bond in cells, several different types of cell lines were tested for the expression level of endogenous FAT10 protein. HEK293 (transformed embryonic kidney cell), HeLa (malignant epithelial cell), U2OS (osteosarcoma cell), Namalwa (human Burkitt lymphoma), and U937 (monoblast) cells were stimulated with TNFα and IFNγ for 24 or 48 hours. Total RNAs were then extracted from the stimulated or unstimulated cells for RT-PCR analysis with the primer pairs specific for FAT10 and a housekeeping gene, GAPDH. PCR products were resolved on 1.8% agarose and visualized with ethidium bromide under UV light. PCR products of GAPDH were served as loading controls to normalize the expression level of FAT10 mRNA. As shown in Figure 27A, the level of FAT10 mRNA was upregulated in most of the cell lines. Furthermore, different cell lines needed different kinds of cytokines to induce FAT10 mRNA. In HEK293 cells, after 24 and 48 hours of stimulation, FAT10 mRNA was induced by TNFα alone, but not IFNγ, and the mRNA level was further increased by the combination of TNFα and IFNγ (Figure 27A; lane 1 to lane 8). In contrast with HEK293 cells, TNFα had no effect on inducing FAT10 mRNA in HeLa cells. However, FAT10 mRNAs were slightly increased by IFNγ and further enhanced after 24 hours stimulation with TNFα and IFNγ (Figure 27A; lane 8 to lane 16). The overall signals in Namalwa cells were weak, indicating low levels of FAT10 mRNA upon stimulation (Figure 27A; lane 17 to lane 24). TNFα was the major cytokine to induce FAT10 mRNA in U937 cells and the mRNA level remained unchanged with the combination of TNFα and IFNγ (Figure 27A; lane 25 to lane 32). Both TNFα and IFNγ were needed to induce FAT10

mRNA in U937 cells after 24 hours treatment even thought the signals were weak (Figure 27A; lane 33 to lane 40). These results suggest that mRNA level of FAT10 is upregulated by cytokines in those cells. The expression of endogenous FAT10 protein in those cells was further analyzed by immunoblotting.

Since FAT10 mRNAs were induced by TNFα and IFNγ in most of the cell lines, these two cytokines were used for determining the level of endogenous FAT10 protein It has been reported that FAT10 proteins were expressed in JY cells (EBV transformed human B lymphoblastoid) by cytokine treatment (Liu, Pan et al. 1999). Therefore, I also tested JY cells together with the other cell lines. After stimulating with TNFα and IFNγ, those cell lines were lysed in SDS sample buffer with β-ME. The samples were resolved by SDS-PAGE followed by immunoblotting with FAT10 specific antibodies (Figure 27B). Among the cell lines tested, endogenous FAT10 proteins were only observed in HEK293 cells after cytokine stimulation. FAT10 proteins could not be detected in other cell lines including JY cells, even though FAT10 mRNA was induced under the same condition in those cells. In the following in vivo experiments, HEK293 cells were used for inducing endogenous FAT10 and for detecting the thioester formation between E1-L2 and FAT10.

IV.B.3 E1-L2 forms a thioester with endogenous FAT10.

To determine if endogenous E1-L2 and FAT10 forms a thioester, I stimulated HEK293 cells with TNFα and IFNγ to induce the expression of endogenous FAT10 protein. The protein level and the subcellular localization of endogenous of E1-L2 remained the same after cytokine stimulation in HEK293 cells (Figure 13A; compare lane 2 to lane 1 and lane

10 to lane 9). Unlike FAT10, E1-L2 is expressed constitutively and is not further induced by TNFα and IFNγ in HEK293 cells. Cell lysates were generated from stimulated or unstimulated cells and then immunoprecipitated using an antibody against E1-L2 or a rabbit IgG for a negative control followed by mixing with protein A/G beads at 4^{o}C. After washing with lysis buffer, the beads were eluted with the DTT buffer. Then remaining proteins were further eluted with SDS sample buffer containing β-ME (Figure 18A). The potential thioester bonds were then cleaved with DTT. Therefore, proteins that formed thioesters with E1-L2 were present in the DTT eluate fractions. The eluted proteins from the DTT fractions were analyzed by SDS-PAGE and immunoblotted with an antibody against FAT10 as well as an antibody against ubiquitin (Figure 18B). Endogenous FAT10 was eluted with DTT from the E1-L2 immunoprecipitates, but not from the control IgG precipitates after cytokine treatment (Figure 18B; compare lane 6 to lane 5 in the upper panel). Like E1-L2~ubiquitin, the steady state levels of E1-L2~FAT10 is also low. It may be also due to the rapid transfer of FAT10 from E1-L2 to the downstream enzymes (e.g E2) in the cascades. This data provides strong evidence that E1-L2 activates FAT10 through a thioester intermediate in vivo. Furthermore, the formation of the E1-L2~FAT10 thioester intermediate was induced by TNFα and IFNγ, whereas E1-L2 formed a thioester with ubiquitin constitutively. These results (Figures 18 and 26) indicate that despite apparently high concentration of ubiquitin in the cells, E1-L2 is capable of activating both FAT10 and ubiquitin.

To further determine if E1-L2 could activate FAT10 in the presence of competing ubiquitin, I carried out in vitro thioester assays in the presence of FAT10 and ubiquitin at varying ratios (Figure 28). Both FAT10 and ubiquitin were incubated with His_6-tagged E1-

L2 and ATP at 37 ^{o}C for 15 minutes and immunoblotted with His specific antibodies to observe the presence of E1-L2~ubiquitin and E1-L2~FAT10 thioester intermediates under the non-reducing condition. When FAT10 and ubiquitin were present in an equimolar ratio, E1-L2 preferentially formed a thioester with ubiquitin (Figure 28; lane 10). E1-L2~FAT10 thioester was observed together with E1-L2~uniquitin thioester when the molar concentration of FAT10 is two times higher than that of ubiquitin in the assay (Figure 28; lane 11). When the ratio of FAT10 to ubiquitin was increased to 4 to 1 in the reaction, both E1-L2~FAT10 and E1-L2~ubiquitin thioester intermediates were detectable. However, the amount of E1-L2~FAT10 thioester intermediate was not further enhanced when the concentration of FAT10 was increased (Figure 28; compare lane 12 to lane 11). This result suggests that E1-L2 does not appear to have a higher intrinsic affinity for FAT10 than for ubiquitin. Hence, the activation of FAT10 by E1-L2 observed in cells (Figure 18) may be due to the induction of FAT10 by cytokines, which drastically increases FAT10 concentration. In addition, the competition of ubiquitin by the traditional ubiquitin E1 may lower the chance of E1-L2~ubiquitin formation and then benefit the activation of FAT10 by E1-L2 in the presence of TNFα and IFNγ.

IV.C E1-L2 cannot transfer FAT10 to several ubiquitin E2s.

I have shown that E1-L2 forms a thioester with FAT10 both in vivo and in vitro. In Chapter III, I have demonstrated that E1-L2 was able to activate ubiquitin and then transfer ubiquitin to a subset of ubiquitin E2 enzymes, including Ubc5 and Ubc13, but not Ubc3 and E2-25K (Figure 19 and 20). Therefore, it would be of interest to test whether these ubiquitin

E2 enzymes are involved in FAT10 modification as conjugating enzymes by accepting FAT10 from E1-L2.

Ubc5 and Ubc13/Uev1A complex were tested first for their participation in FAT10 conjugation. Ubc5 or His_6-tagged Ubc13/Uev1A was incubated with ubiquitin or FAT10 together with E1-L2 in the presence or absence of ATP at 37^{o}C for 15 minutes. The reactions were stopped by adding SDS sample buffer and then the proteins were resolved by SDS-PAGE under the non-reducing condition followed by Coomassie blue staining or immunoblotting with Ubc5 or His (for detecting Ubc13/Uev1A) specific antibodies (Figure 29A). As shown in Chapter III, ubiquitin was transferred from E1-L2 to Ubc5 and Ubc13/Uev1A to form a slower migrating band in an ATP-dependent manner (Figure 29A; lane 2 and lane 6). These slower migrating bands represent the Ubc5~ubiquitin and Ubc13/Uev1A~ubiquitin thioester intermediates. However, this kind of slower migrating bands were not detected by either Coomassie blue staining or immunoblotting when FAT10 was used in the assay (Figure 29A; lane 2 and lane 6). The lack of E2~FAT10 thioesters was not due to the inactivation of FAT10 since E1-L2~FAT10 thioester intermediates were still observed under these conditions. These results suggest that even though FAT10 is activated by E1-L2, the activated FAT10 still could not be transferred to Ubc5 or Ubc13/Uev1A for further conjugation.

Ubc3 and E2-25K were then tested for FAT10 transfer. E1 was used as a positive control for thioester assay. Ubc3 and E2-25K formed a thioester with ubiquitin in the presence of E1, but not E1-L2 as expected (Figure 29B; lane 2 and lane 8 for E1; lane 4 and lane 10 for E1-L2). None of the slower migrating bands were observed when Ubc3 or E2-

25K was incubated with E1-L2 and FAT10 (Figure 29B; lane 6 and lane 12). These results suggest that neither Ubc3 nor E2-25K formed thioesters with FAT10 in the presence of ATP. Even though E1-L2 is able to activate both ubiquitin and FAT10 and transfer ubiquitin to Ubc5 and Ubc13, it cannot transfer FAT10 to those E2 enzymes. It is likely that both E1-L2 and FAT10 contribute to the selection and the recognition of a cognate E2. The identity of the conjugating enzymes for FAT10 remains unknown. It is still possible that there is a conjugating enzyme which can catalyze with both ubiquitin and FAT10 in the presence of E1-L2.

IV.D E1-L2 is required for FAT10 conjugation in cells.

E1-L2 formed a thioester with FAT10 both in vivo and in vitro. The thioester was formed between the catalytic cysteine (Cys635) of E1-L2 and the diglycine motif at the C-terminus of FAT10. I wanted to further test whether E1-L2 is involved in FAT10 conjugation as an activating enzyme. Since other enzymes (e.g. E2s and E3s) involved in FAT10 conjugation are still unknown, it is difficult to address FAT10 conjugation with the in vitro assay system. Therefore, detecting FAT10 conjugations in cells may facilitate the understanding of the role of E1-L2 in FAT10 modification. HEK293 cells were transfected with an expression plasmid encoding the wild-type FAT10 or its mutant harboring two alanine residues at the C-terminus (AA). Both wild-type and mutant FAT10 have an N-terminal tag consisted of His_6 followed by 3 tandem copies of FLAG peptide (His_6-3xFLAG-FAT10). Cells were lysed in TBS buffer containing 0.5% NP-40. FAT10 proteins were purified with nickel affinity chromatography under native condition and analyzed by SDS-

PAGE followed by immunoblotting with a FLAG specific antibody. As shown in Figure 30A, in addition to His_6-3xFLAG-FAT10 proteins, several additional bands above FAT10 proteins were observed in wild-type FAT10 overexpressing cells. These bands were not detected in mutant FAT10 overexpressing cells (compare lane 2 to lane 1), consistent with the prediction that the diglycine motif at the C-terminus of FAT10 is required for substrate modification. These results suggest that wild-type FAT10, but not the FAT10 mutant, forms covalent conjugates with unknown cellular targets.

To determine if E1-L2 is necessary for FAT10 conjugation in cells, I employed RNAi to silence the expression of E1-L2 in HEK293 cells as described in the Section II.B.2 (Figure 30B). Two different pairs of siRNA oligos against E1-L2 together with siRNA oligos against GFP or E1-L1 were transfected into HEK293 cells, which were subsequently transfected with an expression construct encoding the wild-type or mutant His_6-3xFLAG-FAT10. The cell lysates were prepared in TBS buffer containing 0.5% NP-40 and separated by SDS-PAGE under a reducing condition. The samples were then immunoblotted with an antibody against FLAG (to detect FAT10 conjugates), E1-L2 (to verify RNAi efficiency) or β-tubulin (as a loading control). The expression level of endogenous E1-L2 was reduced significantly upon RNAi treatment with siRNA oligos against E1-L2 but not with other control siRNA oligos, indicating the specificity of those siRNA oligos (Figure 30B, middle panel). The proteins formed covalent conjugations with His_6-3xFLAG-FAT10 were detected by a FLAG specific antibody. In control cells transfected with siRNA against GFP or E1-L1 or the ones without siRNA, wild-type FAT10, but not the FAT10 mutant, formed covalent conjugates with unknown cellular targets (Figure 30B, lanes 1 to 6 in upper panel). In contrast, both

pairs of siRNA oligos against E1-L2 prevented the formation of FAT10 conjugates in wild-type FAT10 overexpressed cells (Figure 30B, lanes 7 to 10 in upper panel). The lack of FAT10 conjugates was not due to the variations of sample loading or the different levels of FAT10 expression in cells since all the samples contained similar amounts of β-tubulin (a housekeeping protein) (Figure 30B, lower panel) and the expression level of wild-type or mutant His_6-3xFLAG-FAT10 in those cells were similar to each other (Figure 30B, upper panel). Taken together, these results indicate that E1-L2 is both necessary and sufficient to activate FAT10 in cells.

IV.E Identification of FAT10 substrates.

Although some non-covalent FAT10-binding proteins have been reported, no FAT10 substrates have been identified (Liu, Pan et al. 1999; Hipp, Raasi et al. 2004). Uncovering the substrates for FAT10 modification should help us to understand the implication and the function of FAT10 conjugation. Even with cytokine stimulation, endogenous FAT10 proteins are difficult to detect, which leads to low level of endogenous FAT10 conjugates in several cell lines. Although the endogenous FAT10 proteins can be detected in HEK293 cells after cytokine treatment, the FAT10 conjugates are undetectable by immunoblotting. However, as shown in the previous section, FAT10 conjugates were observed after overexpressing wild-type, but not mutant, His_6-3xFLAG-FAT10 in HEK293 cells. Overexpressing His_6-3xFLAG-FAT10 in HEK293 cells can be used as an approach to study FAT10 conjugation. The interaction between ubiquitin-like proteins and their substrates are covalent, hence the FAT10 conjugates are stable in the presence of high salt, reducing agents or the denaturing

reagents. Therefore, in order to identify FAT10 substrates, I performed tandem affinity purification under the denaturing condition with high stringency wash. After transfecetd with 3xFLAG-FAT10, HEK293 cells were lysed in TBS buffer containing 0.5% NP-40. FAT10 were immunoprecipitated with ANTI-FLAG M2 affinity gel at 4°C for 2hr. The beads were subsequently washed with lysis buffer, 1 M KCl buffer, RIPA buffer and then lysis buffer again. FAT10 and FAT10 conjugates were eluted with 8 M urea buffer (pH 4.5). The interaction between the FLAG tag and FLAG specific antibodies was disrupted by denaturing agent (urea), but not the buffers used for wash. The pH value was adjusted to pH 8.0 with 1 M Tris (pH 9.0) to facilitate the binding to the nickel affinity chromatography. The urea eluate was further incubated with nickel affinity chromatography at room temperature for 2 hours. After washing with 8 M urea buffer (pH 8.0), FAT10 and FAT10 conjugates were eluted with SDS sample buffer containing β-ME (Figure 31A). The unbound fraction and the final eluate from nickel affinity chromatography were analyzed with SDS-PAGE followed by immunoblotting (Figure 31B). The major bands in the unbound fractions were the heavy chain and light chain of FLAG specific antibodies. Several proteins were present in the final eluate but not in the unbound fraction (Figure 31B; compare lane 2 to lane 1). These proteins were potential substrates for FAT10 conjugation. The final eluate was then analyzed by SDS-PAGE and visualized by silver staining (Figure 31B; lane 3). The proteins in the final eluate, which were detected by both immunoblotting and silver staining, were cut and then identified by mass spectrometry (Figure 31B; compare lane 3 to lane 1).

FAT10 was identified in all the samples by mass spectrometry, indicating the proteins observed by immunoblotting and silver staining were indeed the FAT10 conjugates. One of

the peptides identified by mass spectrometry was corresponded to NUB1 or NUB1L (splice variants). NUB1/NUB1L was previously reported as a non-covalent FAT10-binding protein (Hipp, Raasi et al. 2004). To test whether NUB1/NUB1L was the FAT10 substrate under the condition I used, I cotransfected wild-type or mutant His_6-3xFLAG-FAT10 and HA-NUB1 or HA-NUB1L into HEK293 cells. FAT10 and FAT10 conjugates were purified with nickel affinity chromatography and immunoblotted with FLAG and HA antibodies under reducing conditions (Figure 32A). Although NUB1 and NUB1L were expressed at equal levels in transfected cells (Figure 32A; lane 4 to lane 9 in the third panel), NUB1 and NUB1L were copurified with wild-type, but not mutant FAT10 (Figure 32A; compare lane 6 and lane 7 to lane 8 and lane 9 in the top panel). The size of NUB1 and NUB1L remained the same and no additional bands were observed, indicating that both NUB1 and NUB1L were unmodified. Therefore, the interactions between NUB1/NUB1L and FAT10 are non-covalent but are resistant to high stringency wash.

Another peptides identified by mass spectrometry corresponded to RanGAP1. RanGAP1 was previously reported as a substrate for SUMO-1 (Schwartz and Hochstrasser 2003). In order to test whether RanGAP1 is a substrate for FAT10, I transfected wild-type or mutant His_6-3xFLAG-FAT10 together with HA-RanGAP1 into HEK293 cells. FAT10 and FAT10 conjugates were purified with nickel affinity chromatography, whereas RanGAP1 were immuoprecipitated with an HA specific antibody. The samples were resolved by SDS-PAGE and immunoblotted with FLAG or HA specific antibodies (Figure 32B). FAT10 and RanGAP1 proteins were well expressed at high levels in transfected cells and were efficiently purified with nickel affinity chromatography or an HA antibody (Figure 32B; the

second and the forth panels). However, neither FAT10 nor RanGAP1 were co-purified with each other (Figure 32B; the first and the third panels), indicating that FAT10 does not bind to RanGAP1 covalently or non-covalently. In other words, RanGAP1 is not a substrate for FAT10 conjugation. Since FAT10 proteins were identified by mass spectrometry in all the samples, the FAT10 conjugates observed in the gel were genuine. In order to improve the efficiency of substrate identification, FAT10 conjugates requires further purification. In addition, it is important to determine whether the FAT10 conjugates are composed of free poly-FAT10 chains without conjugating to the substrates.

IV.F Summary

Due to the similarity between E1-L1 and E1-L2, I further tested whether E1-L2 is the activating enzyme for FAT10. My findings show that in addition to ubiquitin, E1-L2 also forms a thioester with FAT10 both in vivo and in vitro in an ATP-dependent manner. E1-L2~FAT10 thioester intermediates were detected in the in vitro experiments using a FAT10 affinity column or by the thioester assay. E1-L2~FAT10 thioesters were formed as early as 1 minute after incubation and a small amount of E1-L2 formed a covalent bond with FAT10 after prolonged incubation. More importantly, the thioester bond was formed between Cys625 of E1-L2 and the C-terminal diglycine motif of FAT10. Among the activating enzymes I have tested, FAT10 only forms thioester with E1-L2, indicating the specificity between E1-L2 and FAT10. FAT10 mRNA is highly induced by TNFα and IFNγ. However, endogenous FAT10 proteins were only observed in HEK293 cells after cytokine treatment. Therefore, HEK293 cells were used for the in vivo thioester assay. E1-L2 formed a thioester

with FAT10 upon TNFα and IFNγ stimulation in the in vivo assay, whereas E1-L2~ubiquitin thioester intermediates were formed constitutively. This results indicate that despite the high concentration of ubiquitin and the higher affinity between E1-L2 and ubiquitin than E1-L2 and FAT10, E1-L2 is capable of forming a thioester with FAT10 in the presence of ubiquitin in HEK293 cells. Furthermore, the thioester formation between E1-L2 and FAT10 is also mediated by the C-terminal diglycine motif of FAT10. Moreover, knocking down the expression of endogenous E1-L2 by siRNA oligos against E1-L2 reduced FAT10 conjugation in wild-type FAT10 overexpressing cells. In addition to the formation of thioesters with FAT10, E1-L2 was both necessary and sufficient to promote FAT10 conjugation in HEK293 cells. These results show that E1-L2 is also an activating enzyme of FAT10 in addition to an activating enzyme for ubiquitin.

I have tested several known E2s, including Ubc3, Ubc5, Ubc13 and E2-25K for their ability to accept FAT10 from E1-L2, and found that none of these E2s could function as a FAT10 E2. Even though Ubc5 and Ubc13 can accept ubiquitin from E1-L2, E1-L2 cannot transfer FAT10 from E1-L2~FAT10 to these two E2 enzymes. This result suggests that both E1-L2 and FAT10 contribute to the selection of a cognate E2. E1-L2 is the first and the only enzyme identified in the FAT10 conjugation cascade. The conjugating enzymes and ligases for FAT10 conjugation remain unknown.

FAT10 conjugates could be detected in wild-type, but not mutant, His_6-3xFLAG-FAT10 overexpressing cells. The C-terminal diglycine motif of FAT10 is important not only for thioester formation but also for FAT10 conjugation. FAT10 conjugates were subsequently purified by tandem affinity purification with ANTI-FLAG M2 affinity gel and

nickel affinity chromatography. Two peptides identified from mass spectrometry represent ed NUB1/NUB1L or RanGAP1 proteins. After cotransfecting FAT10 and NUB1/NUB1L or RanGAP1 into cells, the interactions between FAT10 and these two proteins were verified by immunoprecipitation or affinity purification. Although NUB1 or NUB1L are copurified with FAT10, the interaction between these two proteins was non-covalent. RanGAP1 and FAT10 were not coimmunoprecipitated together. These findings indicate that neither of these proteins are the substrates for FAT10 conjugation. Further purification of the potential substrates is required for identification of bona fide substrates.

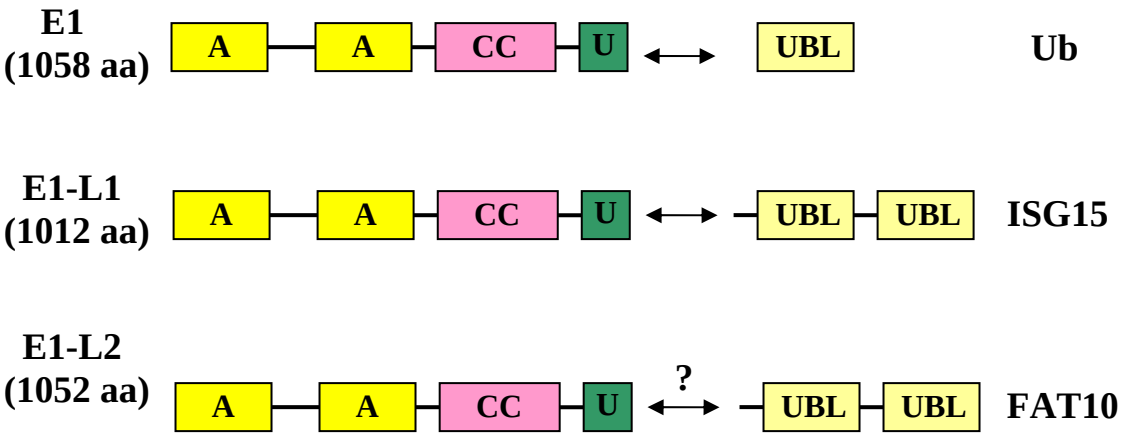


Figure 22. The relationship between E1, E1-L1, E1-L2 and their substrates.
The domain structure of E1-L2 is similar to E1 and E1-L1. Both FAT10 and ISG15 contain two ubiquitin-like domains. Since E1-L1 is the activating enzyme for ISG15, it is possible that E1-L2 is the activating enzyme for FAT10. A: adenylation domain; CC: catalytic cysteine domain; U: ubiquitin-fold domain; UBL: ubiquitin-like domain.

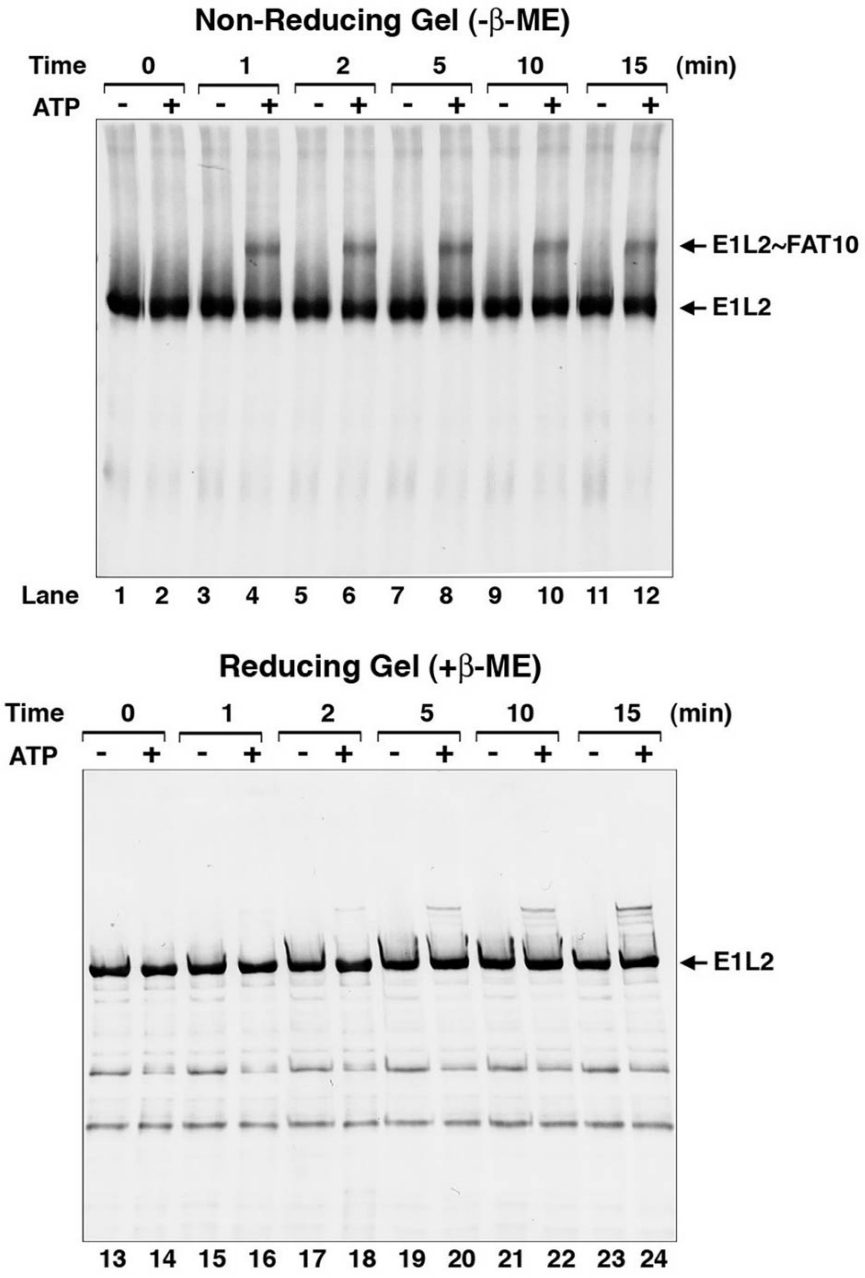


Figure 23. E1-L2 forms a thioester with FAT10 in vitro.
Purified E1-L2 was incubated with FAT10 in the presence or absence of ATP at 37^{o}C for the indicated lengths of time. An aliquot of the reaction mixtures was quenched in a SDS sample buffer in the absence or presence of β-mercaptoethanol (β-ME), and the samples were separated by electrophoresis followed by immunoblotting with a His_6 antibody to detect E1-L2.

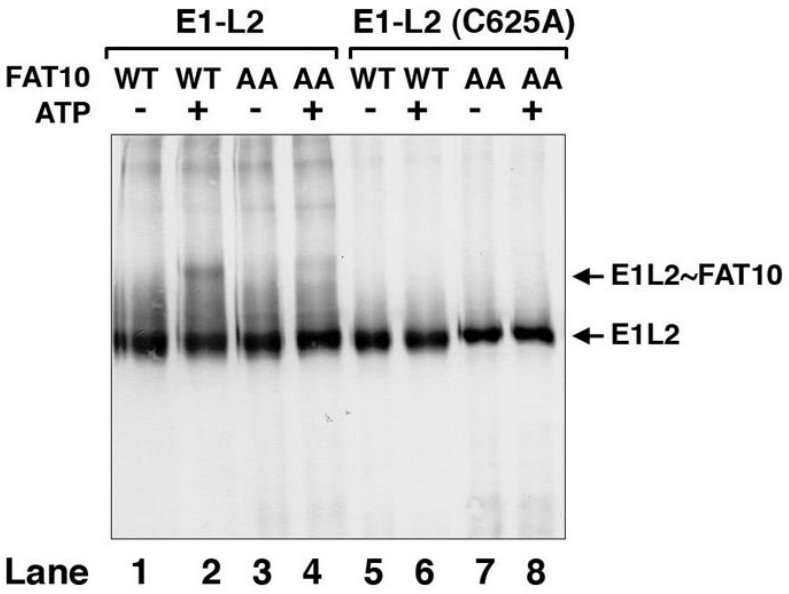


Figure 24. Thioester bond was formed between Cys625 of E1-L2 and the C-terminus of FAT10.

Purified E1-L2 or its C625A mutant was incubated with the wild-type or mutant FAT10 carrying two alanines (AA) at the C-terminus. After incubation in the presence or absence of ATP at 37^{o}C for 15 minutes, the thioester assay was performed as described in Figure 23.

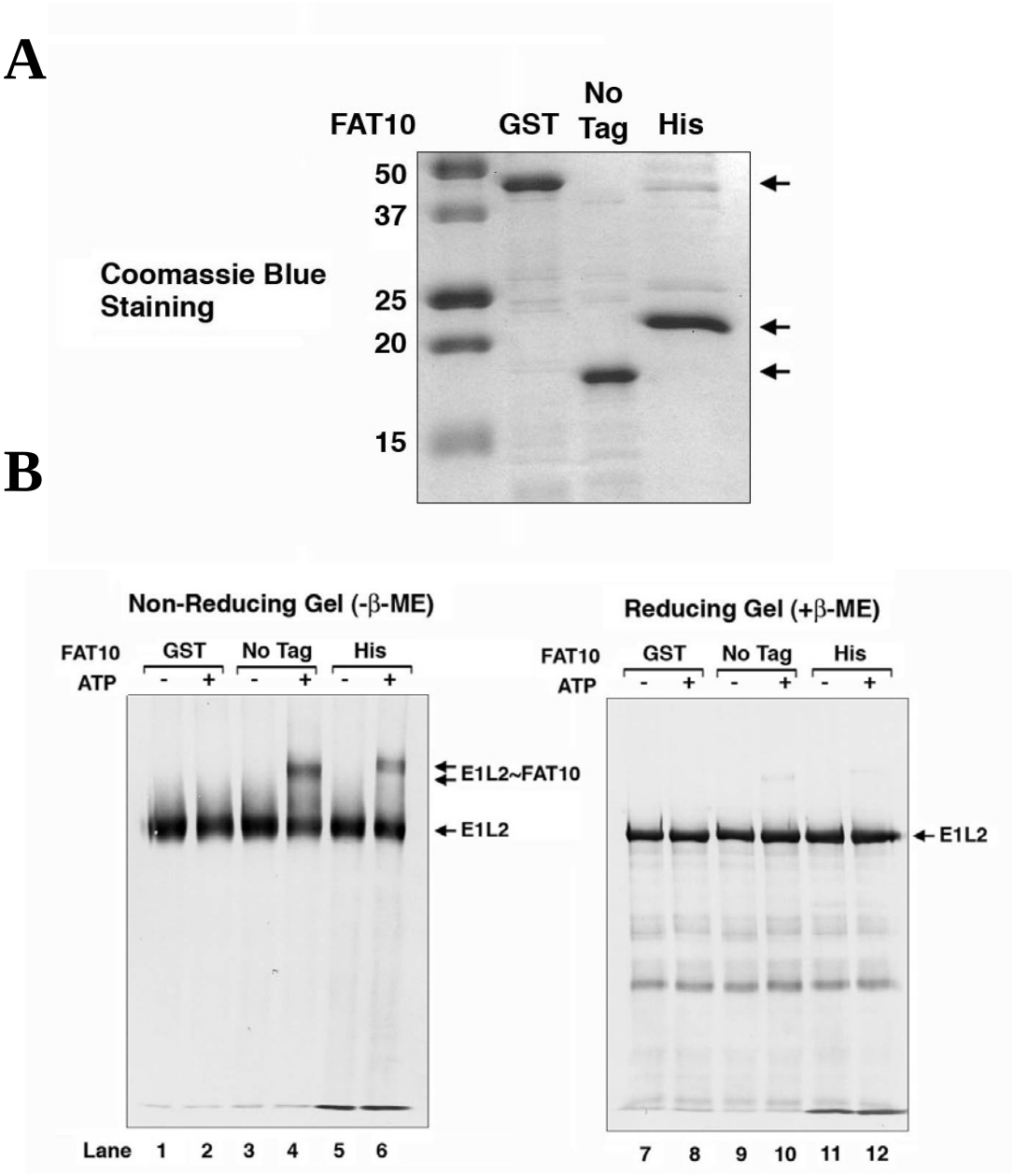


Figure 25. GST-FAT10 is not activated by E1-L2.
(A) Coomassie blue staining of recombinant FAT10 proteins. Lane 1: GST-FAT10; lane 2: GST was removed by Tev protease; lane 3: His_6-FAT10. (B) His_6-E1-L2 was incubated with the recombinant FAT10 proteins shown in (A) in the presence of ATP for 1 minute at 37°C, and then aliquots of the reaction mixtures were analyzed by non-reducing or reducing gel electrophoresis followed by immunoblotting with an antibody against His_6. β-ME: β-mercaptoethanol.

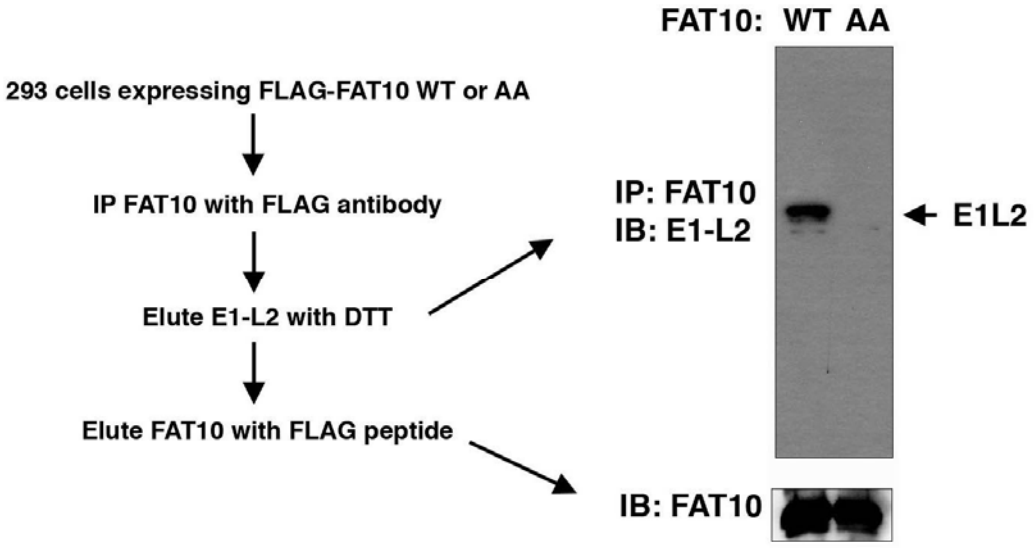
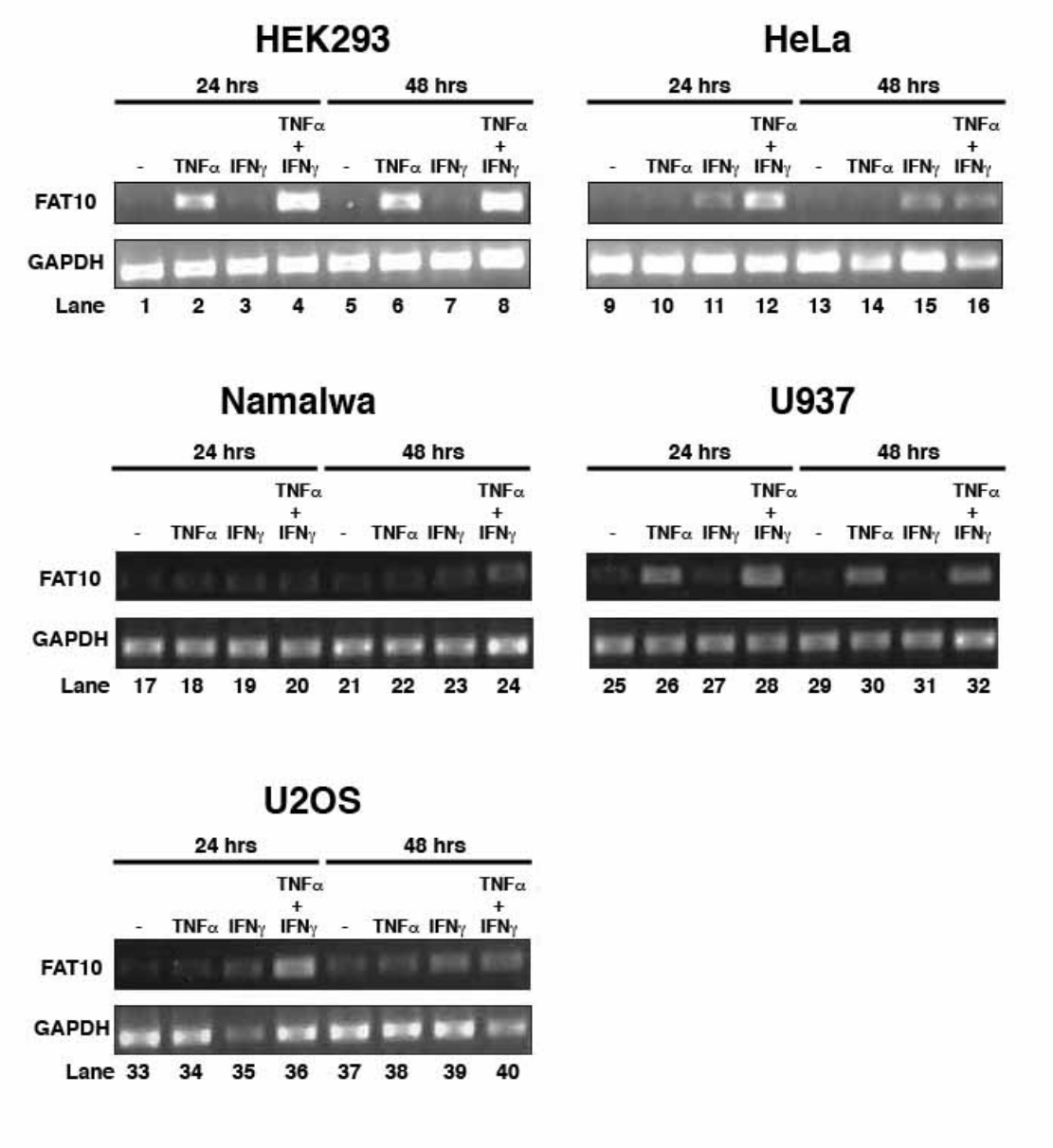


Figure 26. E1-L2 forms a thioester with exogenous FAT10 in cells.
An expression vector encoding FLAG-FAT10 was transfected into HEK293 cells, and then FAT10 and its associated proteins were immunoprecipitated with a FLAG antibody. Proteins that form a thioester bond with FAT10 were eluted with DTT and immunoblotted with an E1-L2 antibody. Subsequently FAT10 was eluted with a FLAG peptide and immunoblotted with a FAT10 antibody.

A


HEK293
HeLa
Namalwa
U937
U2OS
24 hrs
48 hrs
- TNFα IFNγ TNFα + IFNγ
FAT10
GAPDH
Lane 1 2 3 4 5 6 7 8
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Lane 25 26 27 28 29 30 31 32
Lane 33 34 35 36 37 38 39 40

B

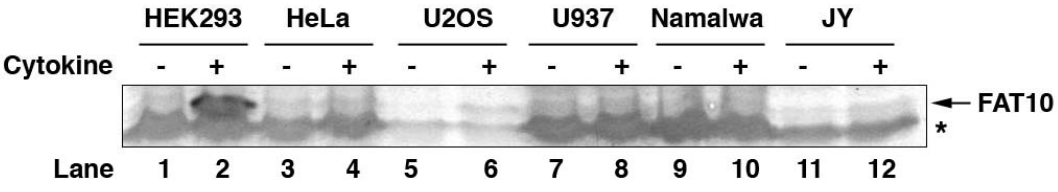

HEK293 HeLa U2OS U937 Namalwa JY
Cytokine - + - + - + - + - + - +
FAT10
*
Lane 1 2 3 4 5 6 7 8 9 10 11 12

Figure 27. Induction of FAT10 mRNA and protein in different cell lines.
(A) RT-PCR. Cells were stimulated with TNFα (0.2μg/ml), IFNγ (3000U/ml) or TNFα and IFNγ for 24 or 48 hours. Total RNAs were extracted from stimulated or unstimulated cells for RT-PCR with specific primer pairs against FAT10 or GAPDH. HEK293 cells: lane 1 to 8; HeLa cells: lane 9 to 16; Namalwa cells: lane 17 to 24; U937 cells: lane 25 to 32; U2OS: lane 33 to 40. (B) Immunoblotting. HEK293, HeLa, U2OS, U937, Namalwa, JY cells were stimulated with TNFα and IFNγ. Then cell lysates were immunoblotted with an antibody against FAT10. The nonspecific cross-reacting band was indicated by an asterisk.

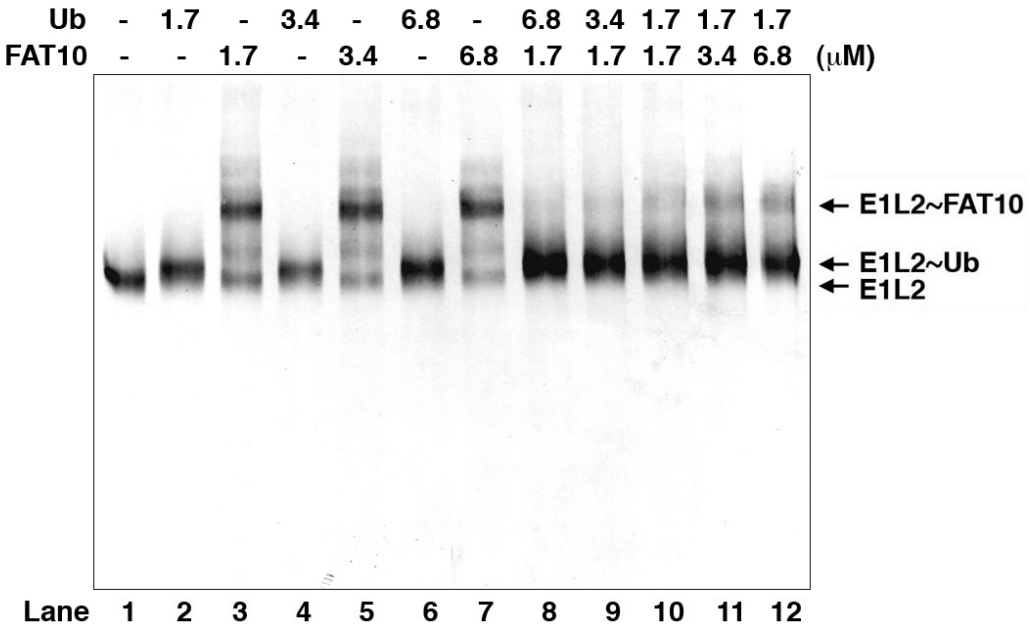


Figure 28. Competition of ubiquitin and FAT10 for E1-L2 in vitro.
His_6-E1-L2 (65 nM) was incubated with ubiquitin and FAT10 at the indicated concentrations for 15 minutes at 37°C. The resulting thioesters were analyzed by non-reducing gel electrophoresis followed by immunoblotting with an antibody against His_6.

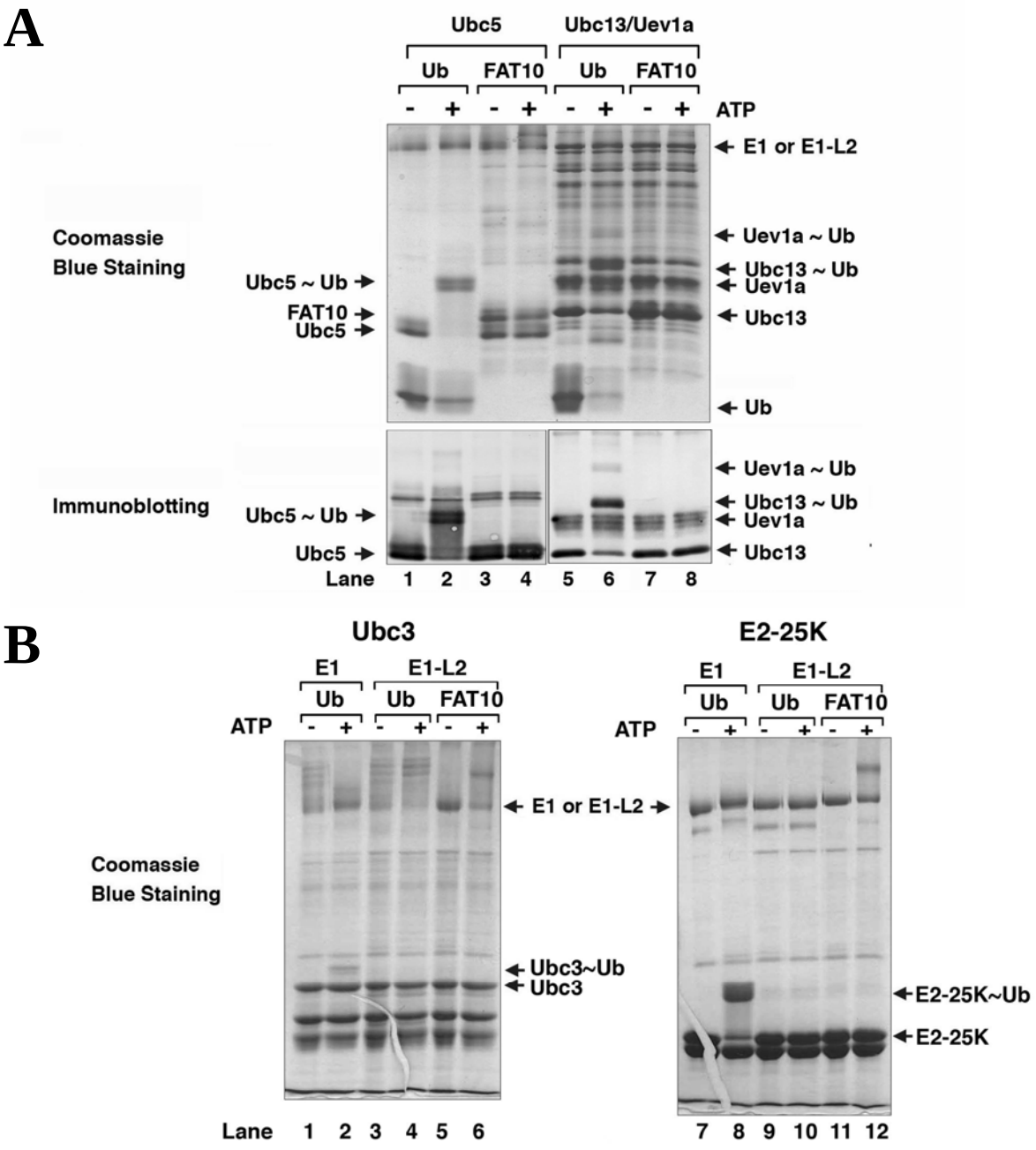


Figure 29. E1-L2 cannot transfer FAT10 to several ubiquitin E2 enzymes.
(A) Purified E1-L2 was incubated with Ubc5 or Ubc13 and ubiquitin or FAT10 in the presence of ATP. The thioester assays were carried out as described in the Section II.B.4. The proteins were visualized by Coomassie staining as well as by immunoblotting using antibodies against Ubc5 (lower left) or His_6 for Ubc13 (lower right). (B) Thioester assays were carried out as described in (A), except Ubc3 or E2-25K was used as the E2 enzymes. E1 was used as control for ubiquitin transfer.

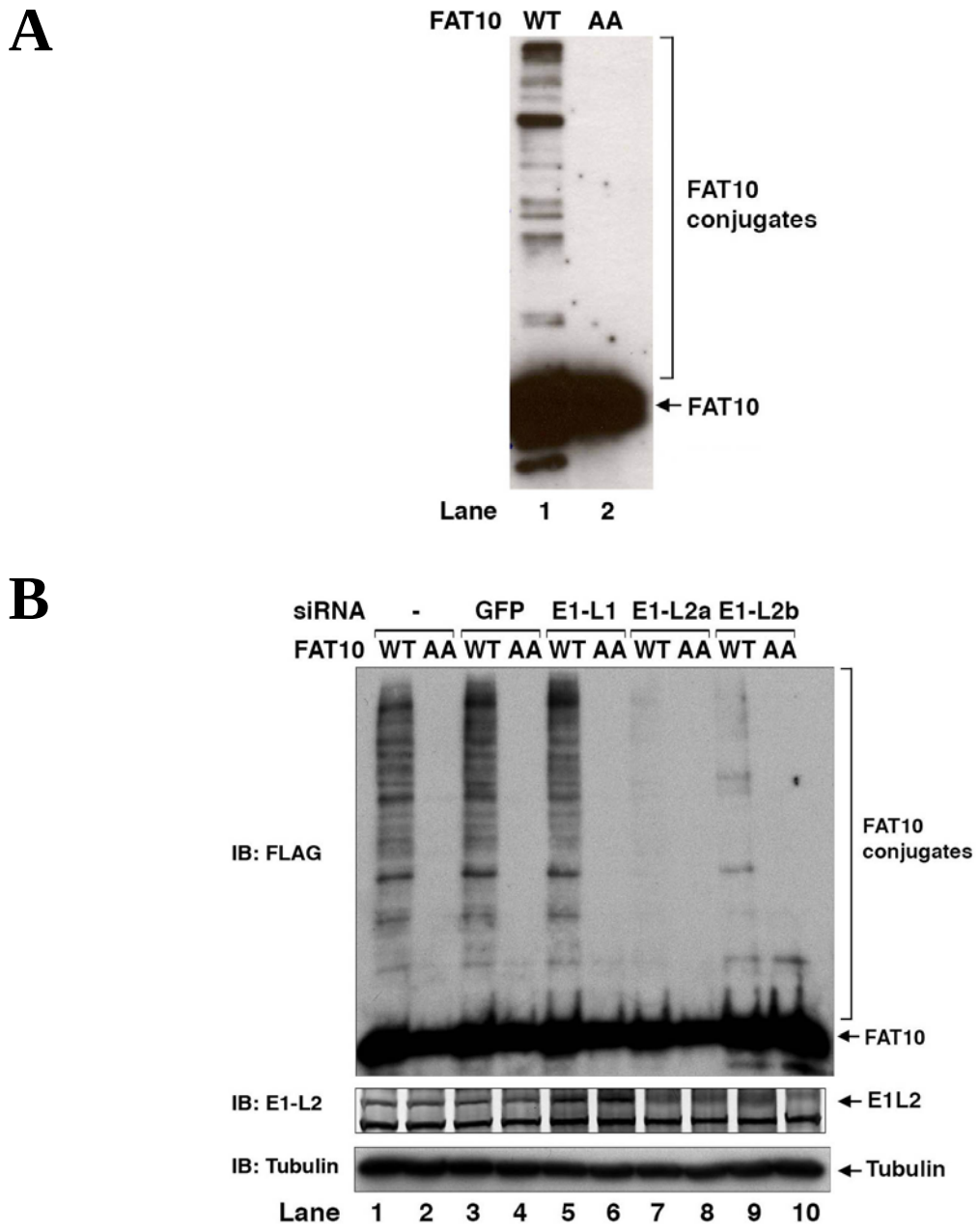


Figure 30. Knocking down E1-L2 reduces FAT10 conjugates in cells.
(A) HEK293 cells were transfected with wild-type or mutant His-3xFLAG-FAT10. FAT10 and its conjugates were purified with nickel affinity chromatography and immunoblotted with a FLAG specific antibody. (B) HEK293 cells were transfected with two different pairs of siRNA oligos (a & b) against E1-L2 (lanes 7-10) or control oligos (GFP: lanes 3 & 4; E1-L1: lanes 5 & 6). Subsequently, cells were transfected with expression plasmids encoding wild-type FLAG-FAT10 or its diglycine mutant (AA). Cell lysates were analyzed by immunoblotting with an antibody against FLAG (for FAT10 conjugates), E1-L2 (for RNAi efficiency) or β-tubulin (for loading control).

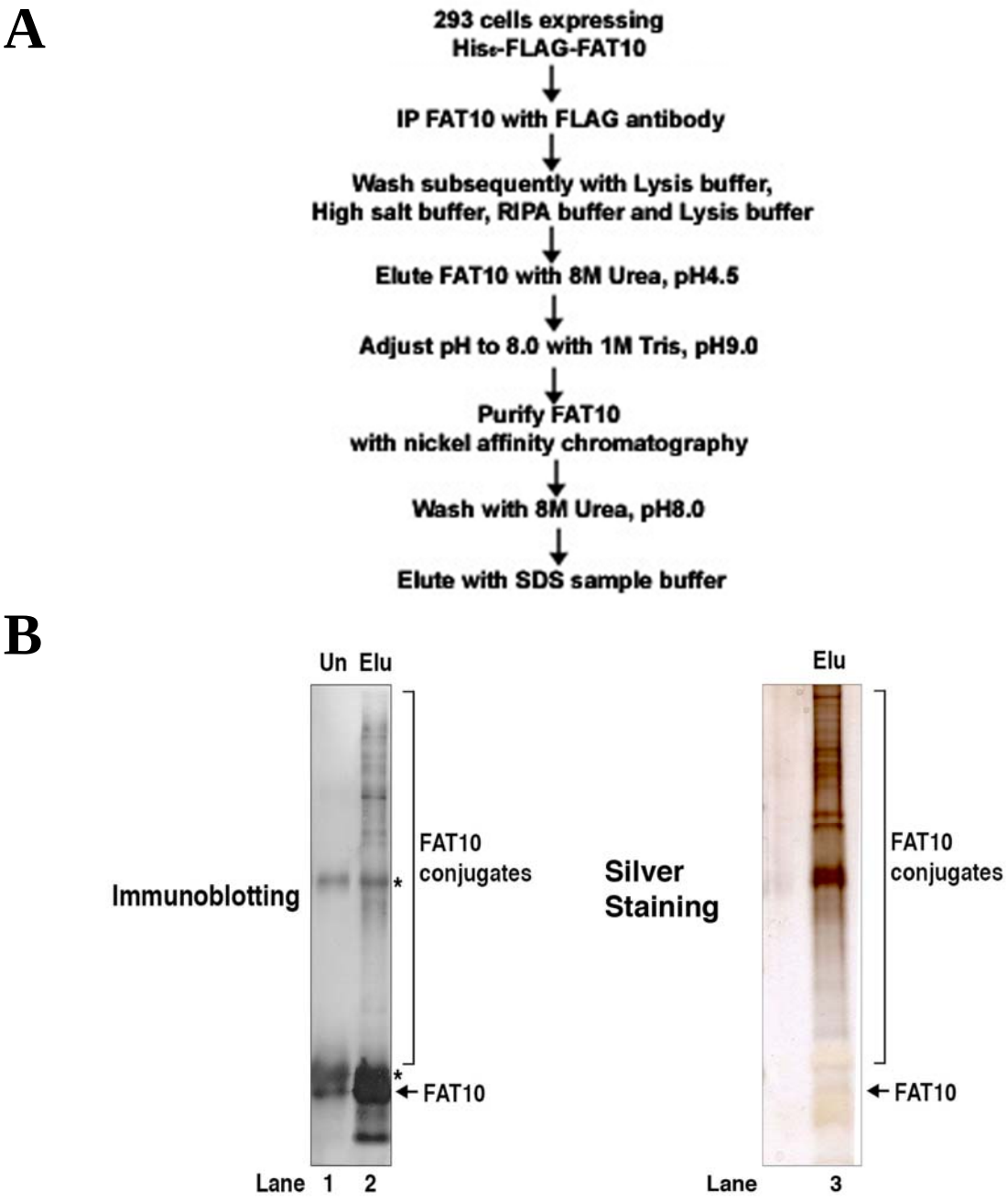
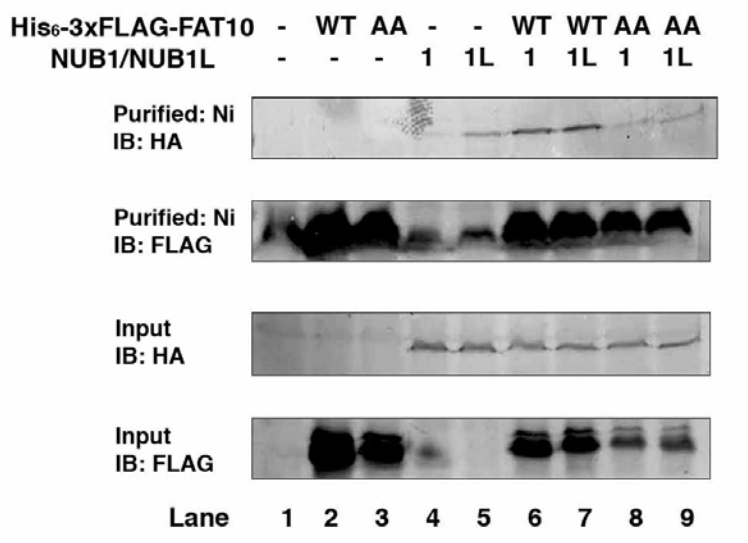


Figure 31. Purification of FAT10 substrates.
(A) Purification procedures. HEK293 cells were transfected with the wild-type His-3xFLAG-FAT10 construct, FAT10 and its conjugates were purified tandemly with ANTI-FLAG M2 affinity gel and nickel affinity chromatography. (B) The unbound fraction and the final eluate from nickel affinity chromatography were immunoblotted with a FLAG specific antibody (lane 1 and 2). The final eluate was visualized with silver staining (lane 3). Heavy chains and light chains of the FLAG antibodies were indicated by asterisks. Un: unbound fraction; Elu: the final eluate.

A



B

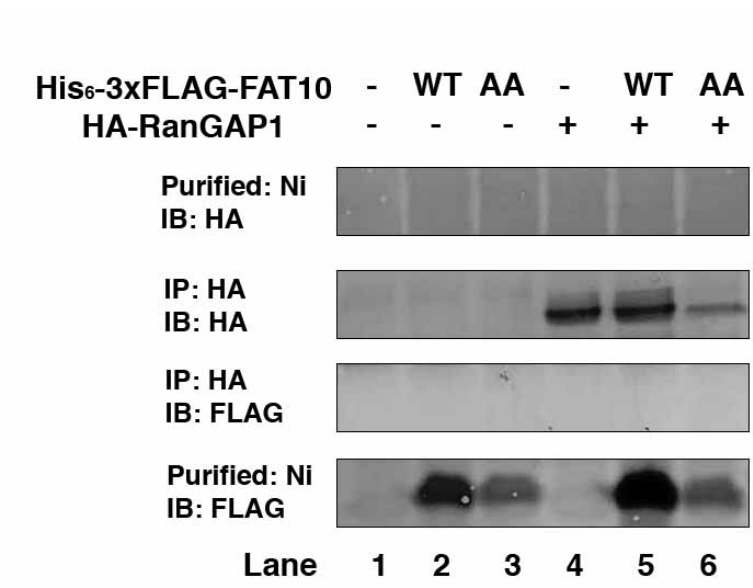


Figure 32. Verification of potential FAT10 substrates.
(A) HEK293 cells were cotransfected with His6-3xFLAG-FAT10 and HA-NUB1/NUB1L. FAT10 and FAT10 conjugates were purified with nickel affinity chromatography and immunoblotted with FLAG or HA specific antibodies. (B) HEK293 cells were cotransfected with His6-3xFLAG-FAT10 and HA-RanGAP1. FAT10 and FAT10 conjugates were purified with nickel affinity chromatography and immunoblotted with FLAG or HA specific antibodies. RanGAP1 was immunoprecipitated with an HA specific antibody and immunoblotted with FLAG or HA specific antibodies.

CHAPTER V: DISCUSSION AND FUTURE DIRECTIONS

In this dissertation, I have presented strong evidence that E1-L2 activates both ubiquitin and FAT10. First, I found that E1-L2 acts as an activating enzyme for ubiquitination, in which only one E1 had been identified before. E1-L2 formed a thioester with ubiquitin both in vitro and in vivo. This result suggests that E1-L2 is able to adenylate and activate ubiquitin. There are two important characteristics of activating enzymes: (1) transferring Ub-like proteins to E2s, and (2) promoting the conjugation of Ub-like proteins. I tested the ability of E1-L2 to transfer activated Ub to several different E2s. I found that, unlike ubiquitin E1, E1-L2 transfers ubiquitin to only a specific subset of E2s (Ubc5 and Ubc13) but not others (Ubc3 and E2-25K), suggesting that E1-L2 may only be involved in certain ubiquitination pathways. Furthermore, E1-L2 promoted polyubiquitination in the presence of E2 (Ubc13/Uev1A), E3 (TRAF6), ubiquitin and ATP. These findings strongly indicate that E1-L2 is another ubiquitin-activating enzyme. Second, I have shown that E1-L2 forms a thioester with FAT10 both in vitro and in vivo. I also detected FAT10 conjugates in HEK293 cells overexpressing wild-type, but not mutant, FAT10. Knocking down the expression of E1-L2 by RNA interference inhibits FAT10 conjugation. These findings show that E1-L2 is both necessary and sufficient to activate FAT10 as an activating enzyme. Although FAT10 conjugates have been detected in cells and an intact diglycine motif at the C-terminus of FAT10 is known to be important for its conjugation (Raasi, Schmidtke et al. 2001), the enzymes and substrates involved in FAT10 conjugation have remained unknown. E1-L2 is the first enzyme identified for the FAT10 conjugation cascade, a process I refer to as “fattenation” (in analogy to ubiquitination and other modifications such as sumoylation

and neddylation) (Figure 33). I have tested several known E2s, including Ubc3, Ubc5, Ubc13, and E2-25K for their ability to accept FAT10 from E1-L2. I found that none of these E2s could function as a FAT10 E2 even though Ubc5 and Ubc13 can accept ubiquitin from E1-L2. This result suggests that both E1-L2 and FAT10 contribute to the selection of a cognate E2. The FAT10-specific E2 enzymes are still unknown and need to be further studied. In the case of ISGylation, both ISG15 and its cognate E2 are induced by IFNβ. It is possible that some FAT10 specific E2 enzymes are induced by TNFα and IFNγ since FAT10 is highly upregulated in this condition. Identification of E1-L2 should facilitate the discovery of E2s and E3s for the fattenation cascade.

After most of the data presented in this dissertation were submitted for publication, Jin et al. and Pelzer et al. reported that E1-L2/Uba6/UbE1L2 could activate ubiquitin but not FAT10 , which is different from what I have found (Jin, Li et al. 2007; Pelzer, Kassner et al. 2007). I noticed that those authors used GST-FAT10 in their in vitro assays, which did not form thioester with E1-L2 in my hands (Figure 25B). GST is a 26 kDa protein which is bigger than FAT10 and might interfere with the activation of FAT10 by E1-L2. After I removed the GST tag from FAT10 with Tev protease or replaced GST with a smaller epitope such as a His_6, E1-L2~FAT10 thioester intermediate was observed in the assay. Therefore, I believe it is important to use smaller or no tag FAT10 proteins in the experiments either in vitro or in vivo in order to study its function. In addition, an E1-L2 specific ubiquitin E2, Use1 has been described in one of the papers (Jin, Li et al. 2007). Among the E2s they tested, Use1 was the only E2 that could uniquely accept ubiquitin from E1-L2 but not from ubiquitin E1. I further tested whether Use1 is the E2 enzyme for FAT10. Use1 proteins used in the

thioester assay were purified in *E. coli* expression system as GST tagged proteins and the GST tag was then removed by Tev protease. I found that FAT10 could not be transferred from E1-L2 to Use1, whereas Use1 formed a thioester intermediate with ubiquitin in the presence of E1-L2 as reported by the other group (Figure 34). Thus, the FAT10-conjugating enzyme (FAT10 E2) remains unknown. The biochemical approach with FAT10 affinity purification might be used for identification of FAT10 E2. Similar to ubiquitin affinity purification mentioned in the Section II.B.3, cell lysates will be incubated with FAT10-sepharose first in the presence of ATP. After washing with high salt and TE buffer, the column will be eluted with DTT buffer which breaks the thioester bond between FAT10 and other proteins. The DTT eluate should contain E1-L2 and potential FAT10 E2 proteins. Protein identification will be done and the thioester formation between FAT10 and those potential E2 enzymes should be further confirmed by thioester assay mentioned in the Section II.B.4. TNFα and IFNγ treated cell lysate should also be tested to investigate the existence of inducible FAT10 E2 enzymes.

FAT10 mRNA is highly upregulated by the TNFα and IFNγ treatment. However, endogenous FAT10 proteins were difficult to detect even after cytokine stimulation. Since FAT10 itself is a short-lived protein and fusion of FAT10 to a long-lived protein leads to reducing the half-life of that protein (Hipp, Kalveram et al. 2005), adding a proteasome inhibitor may stabilize the expression of endogenous FAT10 and further stabilize endogenous FAT10 conjugates. Furthermore, FAT10 conjugates should be observed by overexpressing wild-type, but not the diglycine mutant, FAT10. There are seventeen lysine residues in FAT10. Those FAT10 conjugates could be composed of several proteins

modified by one or more FAT10 molecules or a few proteins modified with various length of poly FAT10 chains. It is also possible that the FAT10 conjugates consist of free poly FAT10 chains without conjugation to the substrates. Expression of lysineless FAT10 will be able to distinguish these possibilities. Lysineless FAT10 can be generated by mutating all seventeen lysine residues of FAT10 into alanines or arginines, and this lysineless FAT10 should not be further conjugated by other FAT10 proteins or ubiquitin-like proteins. Therefore the formation of poly FAT10 chains should be blocked by using lysineless FAT10 in the assay. If only FAT10 monomers are present in those lysineless FAT10 overexpressing cells, it is likely that the FAT10 conjugates observed in my previous experiments consisted of free poly FAT10 chains. On the other hand, if FAT10 conjugates can still be detected, those conjugates might represent the modified substrates. Since the lysineless FAT10 cannot be further modified, one FAT10 conjugate should represent one kind of FAT10 substrate. In this case, using lysineless FAT10 in the assay should simplify the system and lower the background of the FAT10 conjugation reaction. These experiments should be able to facilitate the identification of FAT10 substrates.

Several functions of FAT10 have been suggested. It has been reported that FAT10 bound non-covalently to the mitotic spindle checkpoint protein MAD2, and overexpressing FAT10 in cells reduced the localization of MAD2 at the kinetochore during the prometaphase stage of the cell-cycle. The binding between FAT10 and MAD2 might cause chromosome instability in the cancer cells overexpressing FAT10 (Liu, Pan et al. 1999; Ren, Kan et al. 2006). In other studies, overexpression of the wild-type FAT10, but not its diglycine mutant, was found to cause apoptosis in a caspase-dependent manner, suggesting

that FAT10 conjugation of cellular targets may play a role in apoptosis (Raasi, Schmidtke et al. 2001). This is potentially important, as FAT10 is strongly induced by stimulation with TNFα and IFNγ, which synergistically promote apoptosis of many cell types including pancreatic beta cells and cancer cells (Lee 2002). RNA interference experiments should be able to provide insights into the role of FAT10 in TNFα and IFNγ induced apoptosis of those cells. Fusion of FAT10 to long-lived proteins such as GFP greatly facilitates the degradation of the fusion proteins by the 26S proteasome through a ubiquitin-independent mechanism (Hipp, Kalveram et al. 2005). Thus, fattenation may be another ubiquitin-like modification that targets protein degradation by the 26S proteasome. Despite these *ex vivo* studies, the physiological functions of FAT10 remain unclear, as FAT10-deficient mice displayed no obvious abnormality, except that these mice were more sensitive to endotoxin challenge than the wild-type mice and the lymphocytes of FAT10 knockout mice were slightly more prone to spontaneous apoptotic death (Canaan, Yu et al. 2006). As FAT10 is strongly induced by TNFα and IFNγ, phenotypic examination of FAT10 deficient cells following cytokine stimulation may help uncover the physiological function of FAT10.

In order to investigate the function of E1-L2 in vivo, Dr. Qinmiao Sun in our laboratory generated E1-L2 deficient mice by deleting the first two exons, including the ATG codon, of E1-L2 gene in the mouse germline by homologous recombination (Figure 35A and B). Around 140 viable F2 offspring were born and genotyped by PCR analysis with genomic DNAs extracted from tails. Surprisingly, all of the viable F2 offsprings were wild-type or heterozygous for E1-L2 at a ratio of 1 to 2 (Figure 35C), suggesting that deletion of E1-L2 caused embryonic lethality. The heterozygous E1-L2 mice were fertile and developed

normally. Dr. Sun and I dissected embryos at day 10.5 and 13.5 after fertilization and found that none of the live embryos carried homozygous deletion of E1-L2 alleles (data not shown), indicating that E1-L2 is essential for early embryogenesis in mice. The embryonic lethality at the early developmental stage in E1-L2 deficient mice is unlikely due to a defect in the fattenation pathway, because the FAT10-deficient mice are reported to be viable and fertile and display no abnormal developmental phenotypes (Canaan, Yu et al. 2006). In addition, E1-L2 is conserved in evolution and its orthologs can be found in the invertebrate animals including sea urchin (Fiure 12A), whereas FAT10 orthologs can only be found in mammalian species. The diverse phenotypes of E1-L2 and FAT10 knockout mice together with the difference in evolution indicate that E1-L2 has other functions in addition to activating FAT10. Given the fact that ubiquitination is conserved during evolution and is ubiquitously involved in many cellular regulatory mechanisms, it is likely that the embryonic phenotypes of the E1-L2 mice are due to some defects in certain ubiquitination pathways that cannot be compensated by ubiquitin E1 in early embryogenesis.

E1-L2 can promote polyubiquitination at least with Ubc13/Uev1A and TRAF6. However, the efficiency of E1-L2 in polyubiquitin chain synthesis is less than ubiquitin E1. Furthermore, E1-L2 is 75% less abundant than ubiquitin E1 in HEK293 cells. It remains a mystery that how E1-L2 competes with ubiquitin E1 for promoting ubiquitination and what is the function of E1-L2 in ubiquitination. Even though E1-L2 can activate ubiquitin, which is similar to ubiquitin E1, E1-L2 selectively transfers ubiquitin to the known ubiquitin E2 enzymes and might work with some E1-L2 specific E2s. In addition, unlike ubiquitin E1, E1-L2 cannot be found in yeast, *Drosophila* and *C. elegans*. These results suggest that E1-L2 is

not just a redundant activating enzyme for ubiquitin. E1-L2 together with its cognate E2 enzymes might play some unique roles in certain cellular regulatory mechanisms mediated by ubiquitination. Further research is required to determine the molecular mechanisms underlying the essential role of E1-L2.

E1-L2 activates both ubiquitin and FAT10. Implications of the dual functions of E1-L2 are still unknown. E1-L2 might play two distinct roles in regulating two different sets of cellular regulatory mechanisms mediated by ubiquitin and FAT10, respectively. However, it is also possible that E1-L2 is involved in certain pathways that required both ubiquitination and fattenation for transducing signals and executing certain cellular responses. Further dissection of the specific protein modification pathways activated by E1-L2, including identification of E2 and E3 enzymes for FAT10 and the substrates of ubiquitination and fattenation, should provide important insights into the mechanisms and functions of this new E1.

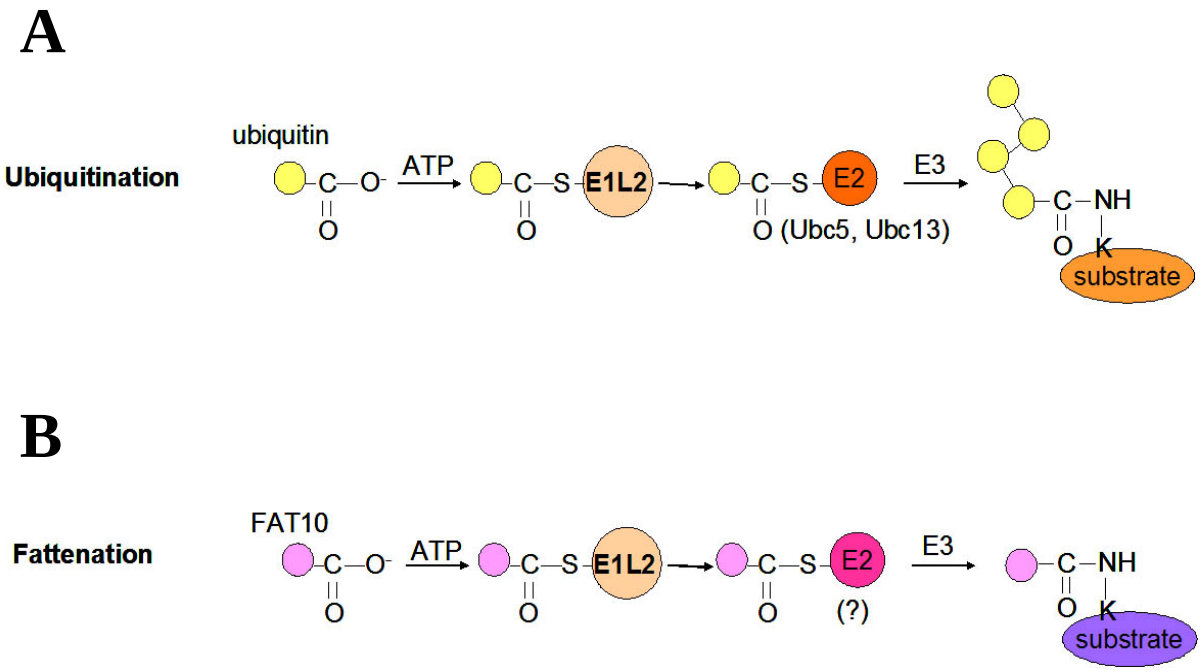


Figure 33. Schematic illustration of ubiquitination and fattenation.
(A) In ubiquitination, E1-L2 is involved in activating ubiquitin. Ubiquitin is then transferred to a subset of E2 enzymes. In the presence of E3, ubiquitins are conjugated to the substrate. (B) Similar to ubiquitination, E1-L2 activates FAT10 and transfers FAT10 to unidentified E2s. FAT10 is then conjugated to substrates and might require E3 enzymes for the conjugation. This conjugation pathway is referred as "Fattenation".

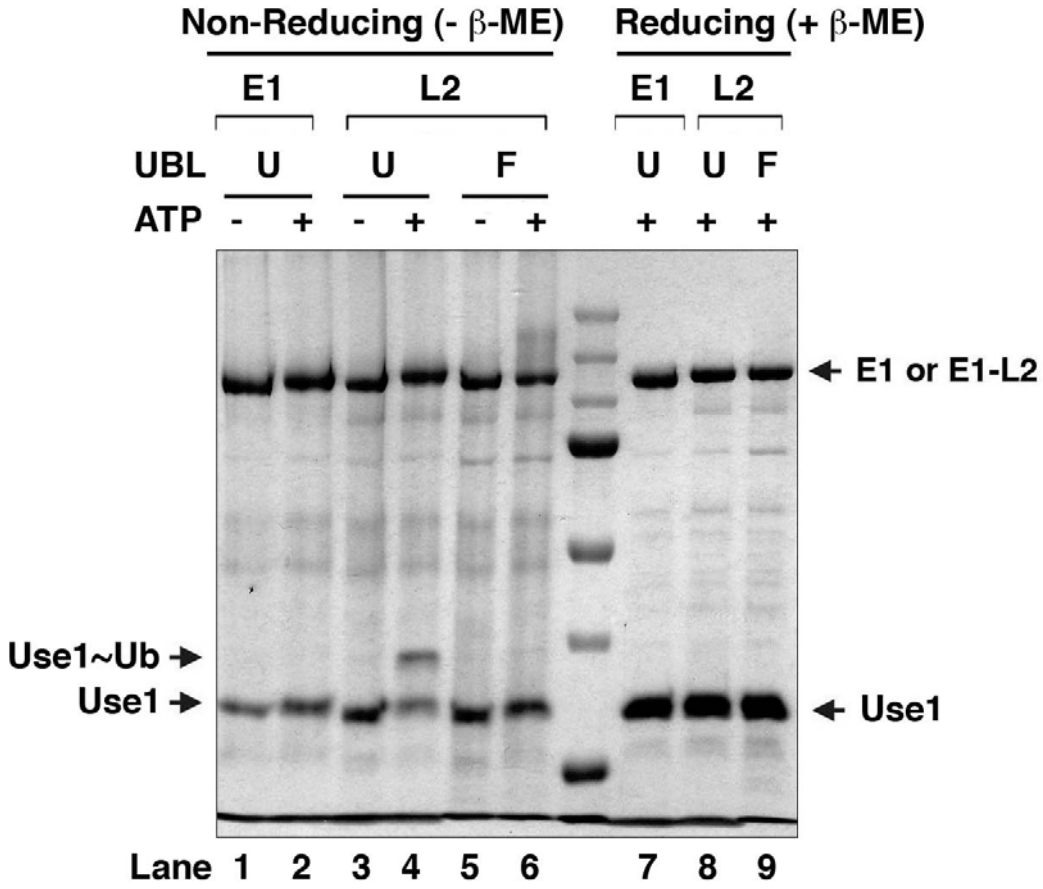
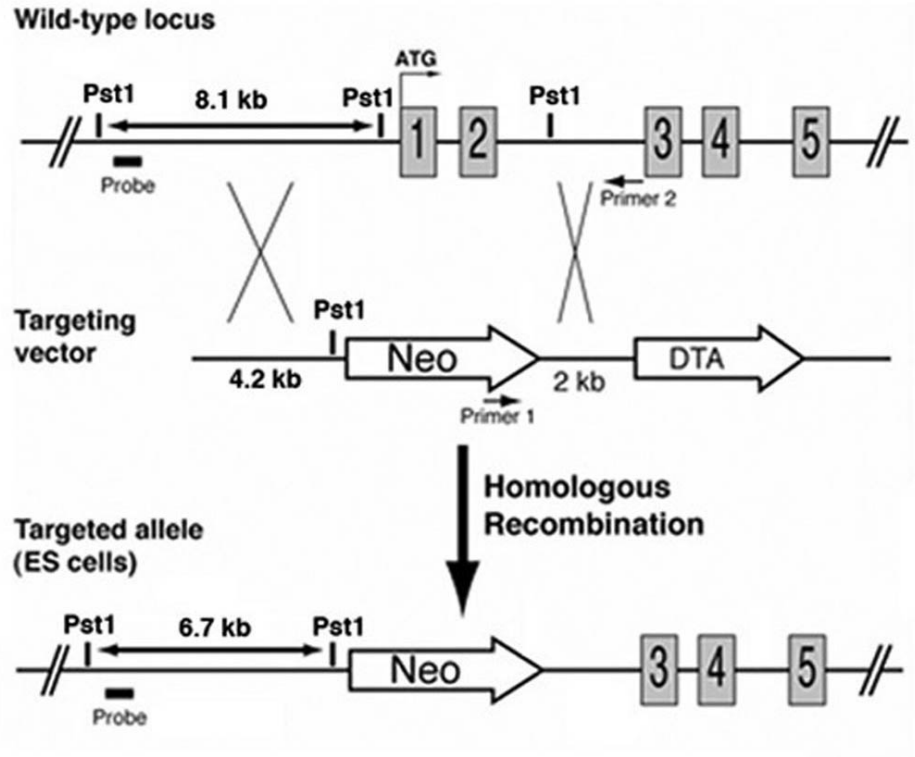


Figure 34. FAT10 cannot be transferred to Use1.
Purified E1 or E1-L2 was incubated with Use1 and ubiquitin or FAT10 in the presence of ATP. The thioester assays were carried out as described in the Section II.B.4. The proteins were analyzed under non-reducing and reducing conditions and visualized by Coomassie staining. L2: E1-L2, U: ubiquitin, F: FAT10.

A



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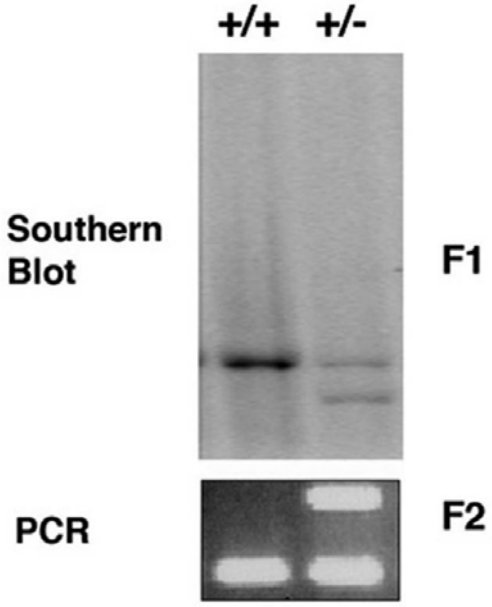

C

	Number	Percentage
+/+	46	33.3%
+/-	92	66.7%

Figure 35. E1-L2 deletion causes embryonic lethality in mice.
(A) Gene targeting strategy. Genomic DNA fragments flanking exon-1 and -2 of E1-L2 (4.2 and 2 kb, respectively) were cloned into a targeting vector to allow homologous recombination with the endogenous locus in mouse ES cells. (B) Southern blotting and PCR analyses of the genomic DNA from the tails of the F1 and F2 mice. The PCR primers and Southern blotting probe are indicated in (A). (C) Genotyping result of offspring from the breeding of heterozygous E1-L2 mice. Figure courtesy of Dr. Qinmiao Sun.

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VITAE

Yu-Hsin Chiu was born in Taipei, Taiwan, on October 2, 1977, the daughter of Chin-Hsin Chiou and Li-Lung Chen. After completing her study at Taipei First Girls High School in 1995, she entered the National Taiwan University in Taipei, Taiwan. She received the degree of Bachelor of Science with a major in zoology in 1999. In September 1999, she entered the immunology master program at the Graduate School of the National Taiwan University, and joined the laboratory of Dr. Ping-Ning Hsu in the Graduate Institute of Immunology. She was awarded the degree of Master of Science in June, 2001. During the following year she was employed as a research assistant at the Graduate Institute of Immunology in National Taiwan University. In August 2002, she entered the Division of Cellular and Molecular Biology (now the Division of Basic Science) in the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center at Dallas. The following year she joined the graduate program of Genetics and Development, and began research in the laboratory of Dr. Zhijian 'James' Chen in the Department of Molecular Biology. In 2005, she married Hon-Ren Huang.

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