國立臺灣大學生命科學院生化科學研究所

碩士論文

Graduate Institute of Biochemical Sciences
College of Life Science
National Taiwan University

Master Thesis

建立泛素化反應之高靈敏度螢光檢驗法

Development of a highly sensitive, fluorescence-based ubiquitination assay

張晏溥

Yen-Pu Chang

指導教授：陳佩燁 博士

Advisor: Rita Pei-Yeh Chen, Ph.D.

中華民國 103 年 7 月

July, 2014

致謝

記得不久前，當我正在趕論文時，總覺得時間過得很慢，希望通過口試交出論文的日子快點到來。但是當這個時刻真的來臨時，又覺得兩年的時光過得好快，而有些依依不捨了。

這篇論文可以順利完成，幕後最大推手當然首推陳佩燁老師。感謝老師這兩年來的耐心指導，讓我從一個懵懵懂懂的碩零生，到今天覺得自己好像會了一些東西，有點自信能夠在研究的路上繼續向前走的準畢業生。從老師身上，我不只學到了科學的知識，更看到了一個科學家對於研究的熱情以及格物致知的態度。

同時，我也感謝 504 實驗室的同伴們在這一路上給予我的幫助：幽默風趣的陳信良博士，除了實驗上指導我許多，在日常生活上也很熱心助人。柏廷學長雖然平時與我較少聊天，但在我實驗有問題時都會很有耐心地教導我。外表木訥的忠諭學長其實是個有趣的人，而且總是不厭其煩地教導我生化實驗的知識。認真踏實的桑傑學長，總會主動指出我實驗上的缺失，讓我不會重蹈覆轍，也是我在做實驗時常尋求的解惑對象。子騰學長熱心且知識豐富，時常在我實驗遭遇困難時主動給我建議，使我不致於走很多冤枉路。而剛加入實驗室的冠宇學長，以及宗諭、志豪兩位學弟，都讓實驗室增添了許多溫馨歡樂的氣氛。此外，雖然他們已經離開實驗室，但是陸逸麟博士、心彥學長、苔言學姐和惠玲學姐在實驗方面都給我許多的指導，我由衷地感謝他們。

在碩班的兩年中，我也要謝謝我的同學們，陪伴我一起度過這兩年的酸甜苦辣，並建立起珍貴的友誼。感謝辛苦的班代明五、立院的好夥伴邯宇、為人有趣總是逗人發笑的越荷、準備口試時熱心幫忙的示涵，以及支持我三不五時的揪團，最後還一起去了花東旅行的家嘉、軒芸、耿維、瑤瑜、維鈞等人。感謝蘊容，在我揪團時總是最熱情響應，也時常在生活上交換想法，雖然往後人生的路上少了

你的陪伴，但你會永遠活在我的心中。最後感謝珮雲，從立院的並肩作戰，分享彼此的心情點滴，後來在我修改論文時也時常督促我、幫助我，有你真好！

感謝我的家人，一路上一直無條件地支持我，讓我沒有後顧之憂。感謝我的父母，完全尊重我的想法，願意放手讓我在去追尋自己想要的夢。感謝我的阿姨與表弟，他們就像我的母親與親弟弟一般，總是給我許多的照顧與關心。

得之於人者太多，要謝的人永遠謝不完。謹將此論文獻給所有關心我、幫助我的人：有你們的存在，我的生命因而精彩。謝謝你們！

中文摘要

泛素可經由 C 端甘胺酸（glycine）與目標蛋白的賴胺酸（lysine）側鏈形成共價鍵結—異胜肽鍵（isopeptide bond），這個過程稱為泛素化。此外，因為泛素分子上具有七個賴胺酸，因此其本身也可以進行泛素化，形成聚泛素鏈（polyubiquitin chain）。不同的聚泛素鏈可將目標蛋白引導至細胞內不同的位置以及反應途徑，因此在做泛素化的研究時，辨識不同的聚泛素鏈是一個很重要但困難的工作。在本篇研究中，我們想要建立一個利用螢光標定的方法區分不同種類泛素化的辨識系統。我們先建立三種在不同位點的點突變泛素，每一種都有一個半胱胺酸（cysteine）的取代或插入，於是我們便可以使用易與硫醇基反應的（thiol-reactive）螢光分子標定突變的泛素。接著我們以酵母菌的 RNA 聚合酶 II（Pol II）做為泛素化的目標蛋白，並以螢光標定的泛素進行泛素化反應。實驗結果顯示，在三種我們所建立的螢光標定泛素中，有兩種可以結合到 Pol II 上，並為螢光成像（fluorescence imaging）所偵測。此外，我們也發現了一個在 Pol II 上未曾報導過的 E3 獨立泛素化反應。由以上結果得知，我們的方法可以用來偵測受泛素化修飾的蛋白，並且可以避免非專一反應所產生的不確定性；而螢光偵測的高敏感度，也提供我們一個發掘未知泛素化途徑的工具。

關鍵字：泛素化（ubiquitination）、泛素（ubiquitin）、RNA 聚合酶 II（RNA polymerase II）、螢光成像（fluorescence imaging）

Abstract

Ubiquitin can be covalently conjugated to other proteins through an isopeptide bond formed between the Lys side chain of the target protein and the C-terminal Gly of ubiquitin, and the process is called ubiquitination. This ubiquitin molecule could be further ubiquitinated to form polyubiquitin chain. Ubiquitin has totally seven Lys which could be used in polyubiquitination. The polyubiquitin chains linked by different Lys lead the ubiquitinated target proteins to different cell locations and pathways, hence polyubiquitin chain identification is an important but difficult work for investigating ubiquitination. In this study, we aimed to construct a fluorescence detection system for identifying ubiquitination. We constructed three ubiquitin mutants by introducing cysteine residue in three different sites, and then thiol-reactive fluorescent dye were labeled to the cysteine residue of these mutants. Using RNA polymerase II (Pol II) as ubiquitination target and these dye-labeled ubiquitin mutants to proceed ubiquitination, we have shown that 2 out 3 dye-labeled ubiquitin mutants could be conjugated to Pol II and detected by fluorescence imaging. Furthermore, we found an unreported E3 independent ubiquitination on Pol II. In conclusion, our method allows us to detect ubiquitinated proteins without the uncertainties from non-specific interactions, and the high sensitivity of this detection system could provideus a new way to discover unknown ubiquitination pathways.

Keywords: ubiquitination, ubiquitin, RNA polymerase II, fluorescence imaging

Abbreviations

Alexa-488/A488	Alexa Fluor 488® C5 maleimide dye
ATP	adenosine triphosphate
Bis-Tris	Bis(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane
CV	column volume
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ESI-TOF	electrospray ionization - time of flight
HPLC	high performance liquid chromatography
IEC	Ion exchange chromatography
IMAC	Immobilized metal-ion affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	lysogeny broth
MES	2-(N-morpholino)ethanesulfonic acid
MS	mass spectrometry
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride

Pol II	RNA polymerase II
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
TAE buffer	Tris-acetate-EDTA buffer
TB	terrific broth
TCEP	*tris*(2-carboxyethyl)phosphine
TEMED	tetramethylethylenediamine
TFA	trifluoroacetic acid
Tris	*tris*(hydroxymethyl)aminomethane
Ub	ubiquitin

C (Cys)	cysteine
D (Asp)	aspartate / aspartic acid
K (Lys)	lysine
M (Met)	methionine
R (Arg)	arginine

Contents

Figure Contents

Chapter 1: Introduction

1-1 Introduction to ubiquitin and ubiquitination

Today, nearly everyone in biology fields knows ubiquitin for its involvement in proteasomal degradation, yet the discovery and the function identification of this protein is a dramatic story. Protein degradation was identified long before ubiquitin was discovered; on the other hand, ubiquitin was discovered to be ubiquitious in all eukaryotic organisms and was first proposed to be a thymopoietic hormone [1, 2]. Several years later, proteasome and proteasomal degradation were identified, and a protein named APF1 (ATP-dependent proteolysis factor 1) was described to be covalently conjugated to substrate protein, serving as a degradation signal [3, 4]. Further studies eventually showed that ubiquitin is APF1 [5].

Ubiquitin is a small protein with 76 residues and a molecular mass of 8.5 kDa with 7 lysine residues (figure 1.1). The sequence of ubiquitin among different eukaryotic species is highly conserved. For example, human and yeast ubiquitin only differ by 3 residues. The covalent conjugating process of ubiquitin to the substrate protein, which is called ubiquitination (or ubiquitylation), is an isopeptide bond forming between the ε-amino group of one lysine in the substrate protein and the carboxyl group of the C-terminal glycine of ubiquitin [6]. This reaction is sequentially catalyzed by three

enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3) [7, 8]. For proteasomal degradation, not a single ubiquitin, but a polyubiquitin chain instead, is added onto the substrate protein. When this chain is synthesized, the C-terminal glycine of a free ubiquitin is conjugated to a lysine side chain of the previously conjugated ubiquitin moiety, forming a new isopeptide bond. Because there are seven lysine residues in ubiquitin, the possible isopeptide bond linkages between ubiquitin moieties within a polyubiquitin chain could be numerous. For proteins to be degraded in proteasomal degradation, all ubiquitin moieties except the first one (which is conjugated to the substrate lysine) in a polyubiquitin chain are conjugated to Lys48 (K48) of another ubiquitin. This chain is called K48-polyubiquitin chain and serves as a recognition signal for proteolysis [9].

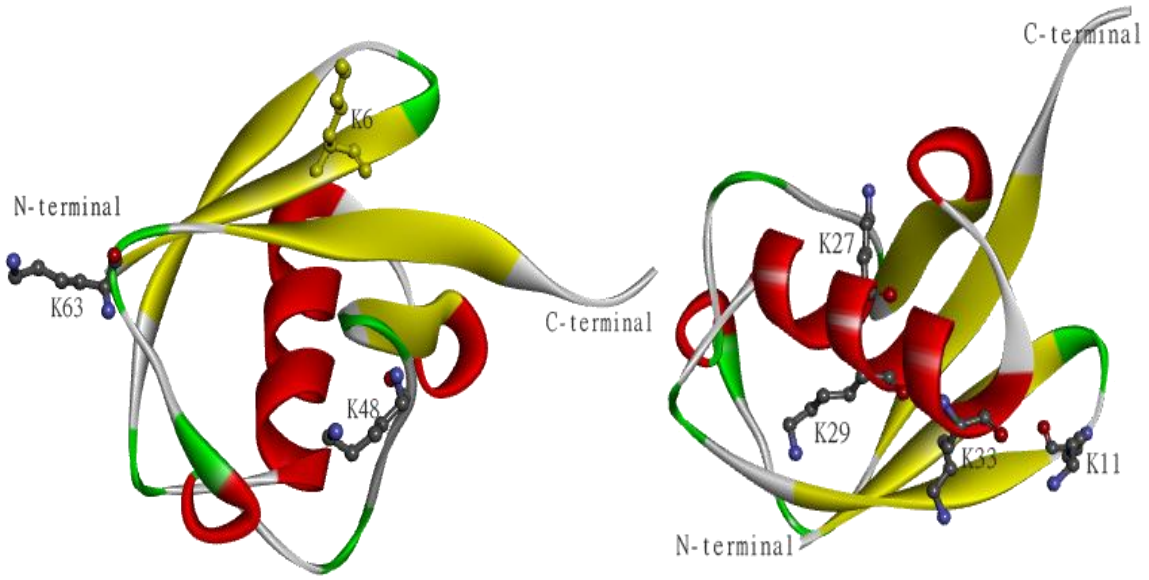


Figure 1.1 The structure of ubiquitin

Alpha-helix is shown in red and beta-sheet structure is shown in yellow. The two figures show the structure of ubiquitin with different orientations, and the position of lysine residues are all marked.

Besides proteasomal degradation, protein ubiquitinaion also plays roles in many other pathways. For example, monoubiquitination of histone H2B regulates the histone methylation and therefore regulates gene expression indirectly [10]; K63-polyubiquitin chain might be involved in DNA repair [11], ribosomal function [12], endocytosis [13], and NF-κB signaling [14]. In recent years, more and more functions of other polyubiquitin chains were also unearthed [15, 16]. However, many chains and their functions remain elusive. Therefore, ubiquitination is still an important topic that deserves deeper investigation.

1-2 Yeast RNA polymerase II and its ubiquitination

RNA polymerase II (abbreviated as Pol II or RNAPII) is a eukaryotic enzyme that catalyzes the transcription of DNA to synthesize mRNA (and also shRNA and microRNA) [17, 18]. Combination of Pol II, transcription factors, and regulatory proteins forms RNA polymerase II holoenzyme, which then binds to the promoters of protein-coding genes [19, 20]. For yeast, RNA polymerase II is a 550 kDa complex, which consists of 12 subunits. Among these subunits, Rpb1 is the largest one and contains a carboxy terminal domain (CTD) with 26 heptapeptide repeats (YSPTSPS), and the CTD repeats are essential for polymerase activity [21]. The serine residues in CTD undergo extensive phosphorylation and dephosphorylation during transcription

cycles. The phosphorylation and dephosphorylation of serine 5 in heptapeptide repeats (Ser5) regulates transcription initiation, whereas phosphorylation state of serine 2 (Ser2) regulates the elongation [18, 22].

Besides being phosphorylated, Pol II Rpb1 could also be ubiquitinated [23]. When DNA transcription is stalled, cells will proceed with Rad26-mediated transcription-coupled nucleotide excision repair (TC-NER) first [24], then undergo ubiquitination and even Def1-mediated degradation if previous attempts fails [25, 26]. Earlier researches showed that yeast Pol II Rpb1 can be ubiquitinated by E1 Uba1, E2 Ubc5 and HECT E3 Rsp5 [27, 28], forming K63-polyubiquitin chains at K330 and K695 [28-30]. There is also a cross-talk between phosphorylation and ubiquitination: only CTD Ser2 phosphorylated Pol II, but not Ser5 phosphorylated ones, efficiently ubiquitinated by Rsp5, agrees with the fact that Ser2 phosphorylation appears in elongation stage, which Pol II ubiquitination should occur [28]. However, since K63 polyubiquitin chains basically don't mediate proteasomal degradation [31], there must be other pathway, say, K48-polyubiquitination take place in following degradation process [30]. Later, more and more evidence showed that Rsp5 can actually do mono- or poly-ubiquitination on Pol II *in vivo*, and a deubiquitinating enzyme called Ubp2 will trim the K63-polyubiquitin chains to monoubiquitin modifications if there's any in the cell [30, 32]. Then an Elc1–Cul3 complex and Def1 recognizes the monoubiquitylated

Pol II and creates K48-polyubiquitin chains. At last, if it is needed, the ATPase Cdc48–Ubx5 and 26S proteasome are recruited to K48 chain modified Pol II, and then Rpb1 will be dissociated from Pol II and undergo proteasomal degradation [26].

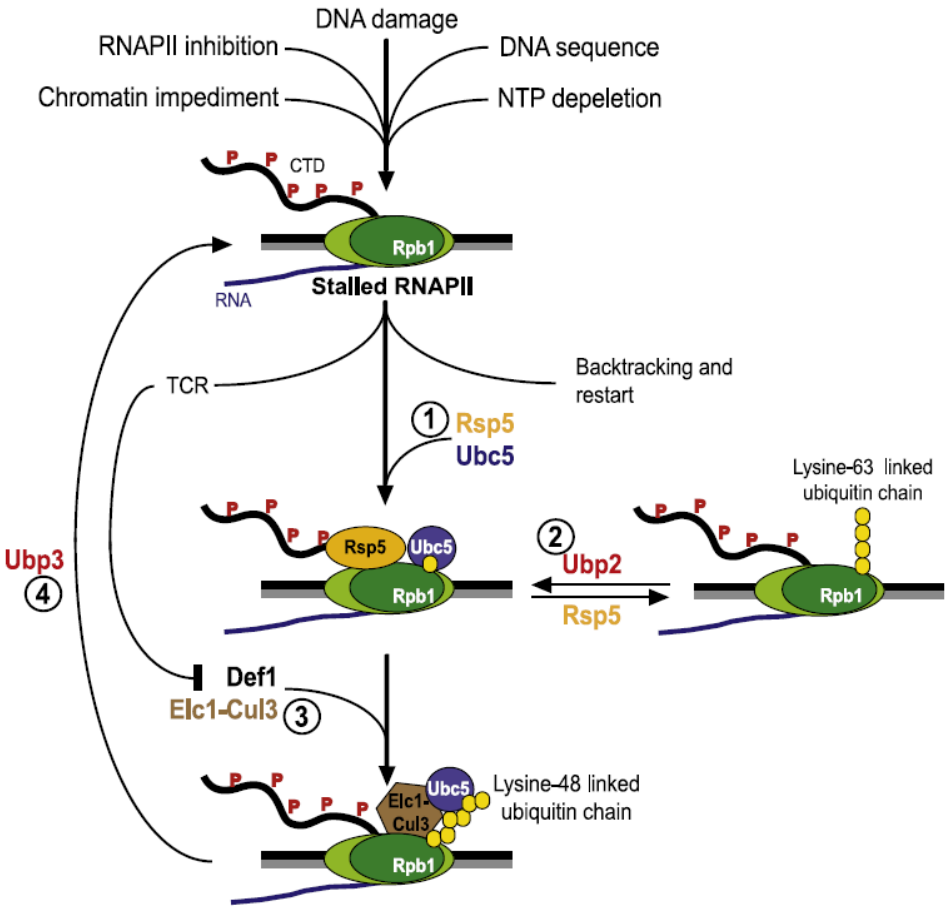


Figure 1.2 Model for Pol II polyubiquitination and degradation

(1) Rsp5–Ubc5 mono-ubiquitinates stalled Pol II.

(2) This can be extended to lysine-63 linked poly-ubiquitin chains, and it can be reversed by the associated de-ubiquitylating enzyme Ubp2.

(3) An Elc1–Cul3 complex and Def1 recognizes the mono-ubiquitylated form of Pol II and creates lysine-48 linked poly-ubiquitin chains.

(4) This can be removed by Ubp3 to prevent unnecessary degradation.

(5) The ATPase Cdc48–Ubx5 and 26S proteasome is recruited and The Pol II complex is disassembled. Poly-ubiquitylated Rpb1 is fed into the proteasome.

(adopted from Wilson et al. [26])

1-3 Ubiquitination assay

In many previous researches, *in vitro* ubiquitination assay was performed to investigate protein ubiquitination. Substrate protein, ubiquitin, and all enzymes needed (E1, E2 and E3), in addition to ATP, are mixed together in a suitable buffer condition [28]. Though not being identical to reactions *in vivo*, it indeed provides researchers an easier and simpler way to investigate ubiquitination, which is important for proposing new models [30]. In our experiments, ubiquitination assay was used to assess the quality of our detecting system.

1-4 Fluorophore

A fluorophore is part of a molecule responsible for creating a fluorescent emission upon light excitation. Fluorophore molecules usually contain alternating single and double bonds, with a planar molecular shape and even with several combined aromatic rings, thus are able to delocalize the π-electrons and form so called conjugated systems that can absorb light of the right wavelengths. When a fluorophore absorbs a photon, the π-electron of the molecule is excited from ground state to a higher energy state, excited state. After that, the excited electron will release the absorbed energy and go back to the ground state. Besides fast relaxation via non-radiative thermal dissipation to the surrounding environment, a fluorophore also release the energy via emitting a photon of

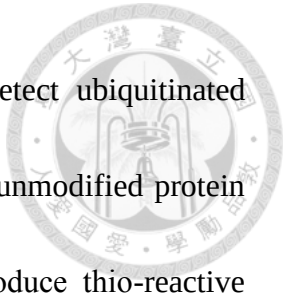
specific energy, and it is where the fluorescence comes from.

Fluorophores have several applications on researches. They can be used as dye for staining certain structures (e.g., DAPI), or covalently bonded to target molecules as markers (e.g., Alexa Fluor and ATTO dyes), therefore can further be applied on fluorescent imaging and spectroscopy. Fluorophores can also be used to quench the fluorescence of other fluorescent dyes, or to relay their fluorescence at even longer wavelength, thus enable some single molecule applications like Förster resonance energy transfer (FRET) [33, 34].

1-5 The aim of this thesis

From other referral studies in ubiquitination, we found that Western blot is the most widely used method for detecting protein ubiquitination. With various antibodies and K to R mutated ubiquitin, researchers are able to tell: (1) Is there any ubiquitination; (2) Is that mono- or polyubiquitination, and (3) Which lysine-linked polyubiquitin chain appears. However, in these studies, antibody specificity remains an issue hard to solve. That is non-specific binding. Therefore we think of developing another method to overcome them.

In this thesis, we aimed to construct a fluorescence detection system for ubiquitination, in which ubiquitin proteins are labeled with fluorescent dye. The usage



of dye-labeled ubiquitin in ubiquitination assays allows us to detect ubiquitinated proteins without uncertainties from non-specific interactions, since unmodified protein doesn't emit fluorescence. To achieve this goal, we need to introduce thio-reactive fluorescent dyes by labeling them to ubiquitin. Because ubiquitin doesn't contain cysteine residue, we need to substitute a residue on ubiquitin to cysteine, or insert a cysteine residue into ubiquitin sequence. Considering sizes and properties of amino acids, side-chain orientations in ubiquitin, in addition to some kinetic and dynamic study in our previous work, we chose three point (Met1, Asp39 and Arg72) for point mutation. We found the stability of dye-labeled M[C]Q would be slightly lowered [35], while stabilities of labeled D39C and R72C remained unchanged (unpublished result).

Dye-labeled ubiquitin was used to perform ubiquitination assay as normal, and the substrate was yeast RNA polymerase II mentioned above. By reference articles, Pol II would be polyubiquitinated and form a K63-linked polyubiquitin chain *in vitro* [30]. We expect our system can distinguish different polyubiquitination chain by introducing K-to-R mutants to dye-labeled ubiquitin, similar to K-to-R ubiquitin mutant used in traditional methods such as Western blot and mass spectrometry [30]. If it works, since we can get the picture right after SDS-PAGE and no following staining is method needed, this system can be used as a convenient and high-specific tool for detecting and identify ubiquitination and polyubiquitin chain.

Chapter 2: Materials and Methods

2-1 Materials

2-1.1 Water

Water was distilled and deionized by Milli-RO PLUS 60 and Milli-QSP reagent water system (EMD Millipore, USA).

2-1.2 Chemicals

40% acrylamide/bis (29:1) solution	Bio-Rad
95% industrial ethanol	Uni-Onward (友和)
Absolute ethanol	Merck KGaA
Acetic acid	Sigma-Aldrich
Acetic acid, glacial	J.T. Baker
Acetonitrile	J.T. Baker
Agar	AMRESCO
Agarose	AMRESCO
Alexa Fluor 488® C5 maleimide dye	Life Technologies
Ammonium sulfate	AMRESCO
Ammonium persulfate	Sigma-Aldrich

Ampicillin sodium salt	AMRESCO
Anti-ubiquitin antibody (rabbit)	Sigma-Aldrich
ATP magnesium salt	Sigma-Aldrich
BME (2-mercaptoethanol / β-mercaptoethanol)	Sigma-Aldrich
Bis-Tris	Sigma-Aldrich
Bromophenol blue	AMRESCO
CelLytic B 10X reagent	Sigma-Aldrich
Coomassie brilliant blue R-250	Sigma-Aldrich
DMSO (dimethyl sulfoxide)	Sigma-Aldrich
DNase I	Roche Applied Science
DpnI restriction enzyme	New England Biolabs
DTT (dithiothreitol)	AMRESCO
EDTA (ethylenediaminetetraacetic acid)	J.T. Baker
Formalin (formaldehyde solution 36.5 - 38%)	Sigma-Aldrich
Glucose	Sigma-Aldrich
Glycerol	AMRESCO
Glycine	AMRESCO
Goat anti-rabbit-IgG, HRP peroxidase conjugated	EMD Millipore
HEPES	J.T. Baker

Hydrochloric acid 36.5 - 38%	J.T. Baker
Imidazole	Sigma-Aldrich
IPTG (isopropyl β-D-1-thiogalactopyranoside)	AMRESCO
Isopropanol (2-propanol)	J.T. Baker
LB medium	Aurora Biotech
Lysozyme	Sigma-Aldrich
Magnesium chloride hexadydrate	Merck KGaA
MES (2-(N-morpholino)ethanesulfonic acid) hydrate	Sigma-Aldrich
Methanol alcohol anhydrous	Macron Fine Chemicals
Nickel sulfate hexadydrate	Riedel-de Haën
Potassium chloride	J.T. Baker
Potassium phosphate monobasic	Macron Fine Chemicals
Peptone from casein	Merck KGaA / Bionovas
PfuUltra II fusion HS DNA polymerase	Stratagene
PMSF (phenylmethylsulfonyl fluoride)	Sigma-Aldrich
SDS (sodium dodecyl sulfate)	AMRESCO
Silver nitrate	Sigma-Aldrich
Sodium acetate	Sigma-Aldrich
Sodium carbonate	Sigma-Aldrich

Sodium chloride	Sigma-Aldrich
Sodium hydroxide	Sigma-Aldrich
Sodium phosphate monobasic	Sigma-Aldrich
Sodium phosphate dibasic	Sigma-Aldrich
Sodium thiosulfate	Shimakyu's Pure Chemicals
SUMO protease Ulp1	Invitrogen
T4 DNA ligase	Roche Applied Science
T4 PNK (T4 polynucleotide kinase)	New England Biolabs
TAE buffer (50X)	AMRESCO
TCEP (*tris*(2-carboxyethyl)phosphine)	Sigma-Aldrich
TEMED (tetramethylethylenediamine)	AMRESCO
TFA (trifluoroacetic acid)	Alfa Aesar
Tris (*tris*(hydroxymethyl)aminomethane)	AMRESCO
Tween-20 (Polyoxyethylene (20) sorbitan monolaurate)	J.T. Baker
Ubiquitin	Sigma-Aldrich
Ubiquitin activating enzyme (E1) human, recombinant	Sigma-Aldrich
Western chemiluminescent HRP substrate	EMD Millipore
Yeast extract	Merck KGaA / Bionovas
Zinc chloride	Fluka

2-2 Methods

2-2.1 Expression constructs and site-directed mutagenesis

We had plasmid containing ubiquitin gene in pET-11b vector. Because this construct had a 6-His tag on C-terminus of ubiquitin and could not be used in the ubiquitination assay, construct without 6-His tag were made from the tagged ones by PCR-based mutagenesis. Primers that changed the first histidine codon (CAT) on 6-His tag to a stop codon (TGA) were used. The primer sequences were listed below, with mutation sites underlined:

UbqDHis forward: 5'-CATTG AGGAT CCGGC TGCTA ACAAA-3'

UbqDHis reversed: 5'-ATGAT GATGA TGTCA ACCAC CTCTT AG-3'

Three Cys mutations M[C]Q (cysteine insertion between M1 and Q2), D39C (D39 is replaced by cysteine), and R72C (R72 is replaced by cysteine) were constructed by site-directed mutagenesis (D39C was constructed previously in our lab). In later experiments, seven lysine-to-arginine mutants of ubiquitin M[C]Q, namely, M[C]Q/K6R, M[C]Q/K11R, M[C]Q/K27R, M[C]Q/K29R, M[C]Q/K33R, M[C]Q/K48R, and M[C]Q/K63R, were also constructed by PCR-based method from the template, ubiquitin M[C]Q construct. The primer sequences used were listed below: (mutation sites were underlined)

M[C]Q/K6R forward	5’- AGGAC GTTAA CCGGT AAAAC CAT-3’
M[C]Q/K6Rreversed	5’-GACGA AGATC TGGCA CATAT GTAT-3’
K11R forward	5’-CGAAC CATAA CTCTA GAAGT TG-3’
K11R reversed	5’-ACCGG TTAAC GTCTT GACGA AG-3’
K27R forward	5’-AGGGC TAAAA TTCAA GACAA GGAAG G-3’
K27R reversed	5’-AACGT TTTCG ATGGT ATCGG ATGG-3’
K29R forward	5’-CGAAT TCAAG ACAAG GAAGG CATTC C-3’
K29R reversed	5’-AGCCT TAACG TTTTC GATGG TATCG G-3’
K33R forward	5’-AGGGA AGGCA TTCCA CCTGA TC-3’
K33R reversed	5’-GTCTT GAATT TTAGC CTTAA CC-3’
K48R forward	5’-AGGCA GCTCG AGGAC GGTAG AACG-3’
K48R reversed	5’-ACCGG CAAAG ATCAA TCTTT G-3’
K63R forward	5’-AGGGA GTCGA CCTTA CATCT TGTC-3’
K63R reversed	5’-CTGAA TGTTG TAATC AGACA GCGTT C-3’

After PCR amplification, the products were checked by electrophoresis in 1% agarose gel and 1x TAE buffer (50x stock from AMRESCO, USA) under 100 volts for 30 minutes. The products were then treated with Dpn1 (New England Biolabs, USA) to digest parental plasmids, and purified by QIAquick PCR Purification Kit (QIAGEN, Germany). The purified plasmids were 5’-phosphorylated and ligated by T4

polynucleotide kinase (New England Biolabs, USA) and T4 ligase (Roche Applied Science, Germany), respectively. Ligated plasmids were first transformed into ECOS™ DH5α competent cells (Yeastern Biotech, Taiwan), cultured overnight on LB agar plate containing 100 μg/ml ampicillin. A few colonies on the plate were picked and subcultured in LB medium. The amplified plasmids were extracted and purified by QIAprep Spin Miniprep Kit (QIAGEN, Germany). The clones with desired sequences were confirmed by DNA sequencing conducted in ABI PRISM® 96-capillary 3730 xl DNA Analyzer (Applied Biosystems, Life Technologies, USA) in DNA Sequencing Core Facility, Academia Sinica.

2-2.2 Small scale expression testing

Small scale expression tests were performed by transforming desired plasmid into *E. coli* BL21 Star™ (DE3) competent cells (Invitrogen, Life Technologies, USA) on agar plate. Transformed cells were inoculated into LB medium with 100 μg/mL ampicillin and incubated overnight at 37℃, 250 rpm. 200 μL of overnight culture was subcultured into 5 ml LB/ampicillin medium and incubated for 3 hr. Then 5μL of 1M IPTG were added (with the final concentration of 1mM) and induced for 5 hr at 37℃, 250 rpm. The cell culture was centrifuged, then the cell pellet was collected and resuspeneded by lysis

buffer (40 mM Tris, 150 mM NaCl, pH 8.0). SDS-PAGE sample buffer was added and the expression efficiency was checked by SDS-PAGE and Coomassie Blue R250 staining.

2-2.3 Large scale protein expression and purification

2-2.3.1 Glycerol cell stock preparation

In previous test, the cell culture with good expression efficiency was prepared as glycerol stock for stable yield and convenient storage. When OD_{600} of the cell culture exceeded 0.6, 600 μL of 50% sterilized glycerol was added to 600 μL of culture, resulting a 1.2 mL glycerol stock. The glycerol stock was kept at -30℃ overnight and moved to -80℃ refrigerator for long term storage.

2-2.3.2 Expression of recombinant ubiquitin protein

100 μL of glycerol stock was added to 100 mL TB medium (17.6 mM Na_2HPO_4, 7.4 mM KH_2PO_4, 137 mM NaCl, 2% peptone, 1.5% yeast extract, pH 7.4) with 10 mM glucose and 100 μg/mL ampicillin, and incubated overnight at 37℃, 250 rpm. The culture was inoculated equally to four 2-liter flasks with 500 ml TB medium (also with 10 mM glucose and 100 μg/mL ampicillin), and the resulting 2L culture was further

incubated at the same condition for 3 hours. Protein expression was induced by 1 mM IPTG for 4 hours at 37℃, 250 rpm. After induction, the culture was centrifuged (8,950 g for 30 min) and the cell pellet was collected. Then we resuspended the cell pellet with lysis buffer (40 mM Tris, 150 mM NaCl, pH 8.0). Cell lysis was done by adding 0.4 X CellLytic B (Sigma-Aldrich, USA), 150 μg/mL lysozyme, 10 μg/mL DNaseI, 5 mM $MgCl_2$ and 1 mM PMSF and stirring for 30 minutes at room temperature. Then the cell lysate was centrifuged at 30,000 g for 30 minutes at 4℃ and the supernatant was collected for further purification.

2-2.3.3 Ammonium sulfate precipitation

Next step, ammonium sulfate precipitation was applied to give a preliminary separation of different proteins in the supernatant collected before. For every 100 mL of cell lysate supernatant, 24.3 g of ammonium sulfate was added to give the solution of 40% ammonium sulfate saturation at 25℃. The solution was centrifuged at 10,000 g for 30 minutes at 4℃. Since ubiquitin remained soluble in this step, we collected the supernatant and keep adding ammonium sulfate. This time we added 28.5 g of ammonium sulfate for every 100 mL of supernatant, to give the solution of 80% saturation. Ubiquitin would be precipitated at this step, therefore the solution was centrifuged again at 10,000 g for 30 minutes at 4℃, and the pellet was collected and

dialysis (Cellu-Sep T1 3500 MWCO dialysis membrane; Membrane Filtration Products, USA) overnight in IEC A buffer (50 mM acetic acid, 50 mM sodium acetate, pH 4.7). Some proteins became insoluble in this step and appeared as precipitant (whereas ubiquitin could be dissolved in the buffer), so the sample was centrifuged at 30,000 g for 30 minutes at 4℃, and the supernatant was collected.

2-2.3.4 Ion exchange chromatography and size exclusion chromatography

In ion exchange chromatography (IEC) and size exclusion chromatography (SEC), ÄKTA Protein Purification Systems (GE Healthcare, UK) were applied. We concentrated the supernatant from last step to less than 50 mL and then filtered it by 0.22 μm filter (33mm; EMD Millipore, USA) for IEC. HiPrep SP Sepharose XL 16/10 cation-exchange column (GE Healthcare, UK) was used to purify mutated ubiquitin. The buffer gradient was from 100% A buffer (50 mM acetic acid, 50 mM sodium acetate, pH 4.7) to 100% B buffer (50 mM acetic acid, 50 mM sodium acetate, 1M sodium chloride pH 4.7). Ubiquitin bound to negative-charged cation exchange columns and eluted at around 30% to 40% B buffer. Then we concentrated the fractions with ubiquitin to less than 5 mL and performed SEC. 1.6/60 cm Superdex-75 column (GE Healthcare, UK) and SEC buffer (150 mM sodium phosphate buffer, 1 mM DTT, pH 7.0) were used to remove the contaminations in the IEC flow through. 2 column volume

(CV) of SEC buffer was pumped into the column to elute ubiquitin. The protein was eluted between 1.4 and 1.7 CV. Then the eluate was collected and dialyzed in 0.1% TFA. Finally, the sample was lyophilized and the resulting ubiquitin powder was kept at -30℃.

2-2.4 Fluorescence dye labeling and purification

One vial of Alexa Fluor 488® C5 maleimide dye (1 mg; Life Technologies, USA) was dissolved in 100 μL DMSO to make a 13.9 mM stock. Ubiquitin powder prepared previouly was dissolved in 30 mM sodium phosphate buffer (pH 7.0) to form a 30 μM ubiquitin solution and then filtered by 0.22 μm filter (33mm; EMD Millipore, USA). 1 mL of filtered solution was taken and 1 μL of 100 mM TCEP was added to it. The sample was degassed, then 5 μL of Alexa-488 stock was added and mixed gently. The conjugation reaction was kept away from light at 25℃ for 1.5 hours. Since Alexa-488 free dye eluted at approximately the same time as ubiquitin, dialysis was performed to remove free dye. About 1 mL of dye-labeled ubiquitin sample was kept away from light and dialysis in 500 mL of distilled water overnight by using Cellu-Sep T1 3500 MWCO dialysis membrane (Membrane Filtration Products, USA).

Next, reversed-phase HPLC was applied to separate the products. It was performed

by using C18 column (DiscoveryBIO Wide Pore C18, 25 cm × 10 μm, Supelco, Sigma-Aldrich, USA) with Agilent HPLC system, and buffer A (94.9% water, 5% acetonitrile, 0.1% TFA) and buffer B (99.9% acetonitrile, 0.1% TFA) were used. The purification condition was set with a gradient from 25% to 55% of B buffer within 30 minutes. Dye-labeled ubiquitin would be eluted at about 30% B buffer and could be separated from unlabeled protein. Purified products were collected and lyophilized, then stored at -30℃. The identification of dye-labeled ubiquitin was done with ESI-TOF mass spectrometry by Waters LCT Premier XE time-of-flight benchtop mass spectrometer (Waters, UK) in Mass Spectrometry Service Center, Academia Sinica.

2-2.5 Enzyme expression and purification

2-2.5.1 Expression of yeast E2 and E3 protein

Plasmid constructs of yeast E2 ubiquitin conjugating enzyme, Ubc5, and E3 ubiquitin ligase, Rsp5, were kindly provided by Dr. Hung-Ta Chen, Institute of Molecular Biology, Academia Sinica. Both these two constructs has an N-terminal hexahistidine tag following a SUMO-1 tag for purification purposes (figure 2.1). Plasmids were transformed into *E. coli.* BL21 Star^{TM} (DE3) to make glycerol stock (The methods are the same as 2-2.2 and 2-2.3.1). For each enzyme (both E2 and E3), 100 μL

of glycerol stock was inoculated into 100 mL of sterile LB/ampicillin medium, and the culture was incubated overnight at 37℃, 250 rpm. On the next day, 20 mL of the overnight culture was subcultured into 500 mL sterile LB/ampicillin medium and incubated at the same conditions. After the absorbance OD_{600} of the culture exceeded 0.6 (about 3 hours later), 500 μL of 1M IPTG was added to give a final concentration of 1 mM for induced expression. 4 hours later, the culture was centrifuged (8,950 g for 30 min) and the supernatant was discarded. The cell pellet was kept in -80℃ refrigerator until the next step was performed.

Figure 2.1 Constructs of yeast Ubc5 and Rsp5
Hexahistidine (6 His) tag is used for IMAC purification (described in chapter 2-2.5.2), and SUMO tag can undergo SUMO cleavage (chapter 2-2.5.3) to get pure products.

2-2.5.2 Immobilized metal-ion affinity chromatography (IMAC)

The cell pellet was resuspended with 30 mL of lysis buffer for IMAC (20 mM HEPES, 500 mM NaCl, 10 μM $ZnCl_2$, 2.5 mM imidazole, 0.5 mM TCEP, 2 mM $MgCl_2$, 0.2 mM PMSF, 0.3 mg/mL lysozyme, 2 μg/mL2 DNaseI, pH 8.0) and then lysed with three cycles of freezing and thawing (freezing with liquid nitrogen and thawing with 37℃ water bath). Cell lysate was centrifuged at 30,000 g for 30 minutes at 4℃, and the

supernatant was collected. Pre-packed column with Ni^{2+}-charged Chelating Sepharose Fast Flow (GE healthcare, UK) was used and balanced with IMAC A buffer (20 mM HEPES, 500 mM NaCl, 10 μM $ZnCl_2$, 2.5 mM imidazole , 0.5 mM TCEP, pH 8.0) beforehand. Filtered supernatant was applied to the column and the column was shaken for 30 minutes at 4℃ to enhance the specific binding. The flow through was discarded, and 5 CV of IMAC D buffer (20 mM HEPES, 500 mM NaCl, 10 μM $ZnCl_2$, 30 mM imidazole , 0.5 mM TCEP, pH 8.0) was applied twice to wash out nonspecific, loosely binding proteins. Finally, hexahistidine-tagged proteins were eluted by 5 CV of IMAC E buffer (20 mM HEPES, 500 mM NaCl, 10 μM $ZnCl_2$, 500 mM imidazole , 0.5 mM TCEP, pH 8.0).

2-2.5.3 SUMO-tag cleavage and enzyme purification

Two samples, IMAC eluate with Ubc5 and eluate with Rsp5 from last step were dialyzed in IMAC A buffer to reduce imidazole concentration. Then the samples were concentrated 10 folds or more. To remove N-terminal hexahistidine tag and SUMO tag, 150 μL of concentrated sample was added with 290 μL of distilled water, 50 μL of 10× SUMO protease buffer (500 mM Tris-HCl, pH 8.0, 2% Igepal (NP-40), 10 mM DTT) and 10 μL SUMO protease Ulp1 (1U/μL; Invitrogen, Life Technologies, USA). The final buffer condition was 150 mM NaCl (from IMAC A buffer), 50 mM Tris-HCl, 0.2%

Igepal (NP-40), 1 mM DTT, pH 8.0, and the reaction was held at 4℃ overnight.

After overnight reaction, a second round of IMAC was performed to separate the cleavage product and other proteins in the sample. The 500 μL reacted sample was mixed with 15 mL of IMAC B buffer (20 mM HEPES, 500 mM NaCl, 10 μM $ZnCl_2$, 10 mM imidazole , 0.5 mM TCEP, pH 8.0) and applied to B buffer pre-balanced Ni^{2+}-charged column. After shaken for 30 minutes at 4℃, the flow through was collected, and the sample was then eluted by IMAC C buffer (20 mM HEPES, 500 mM NaCl, 10 μM $ZnCl_2$, 20 mM imidazole , 0.5 mM TCEP, pH 8.0) and IMAC D buffer (the same as D buffer in 2-2.5.2) sequentially. In E2 purification, Ubc5 didn't bind to the column and would appear in flow through; whereas in E3 purification, most Rsp5 would be eluted by IMAC C buffer. The collected flow through and eluate with high purity of E2 or E3 were dialyzed in 1× ubiquitination buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 50 μM $ZnCl_2$, 1 mM DTT, pH 7.5), concentrated, and then store at -30℃ until use.

2-2.6 Ubiquitination assay with dye-labeled ubiquitin

When dye-labeled ubiquitin mutants and E2, E3 enzymes were ready to use, we performed *in vitro* ubiquitination. Our substrate was yeast RNA polymerase II (Pol II),

which was kindly provided by Dr. Wei-Hau Chang, Institute of Chemistry, Academia Sinica. 300 ng Pol II, 0.5 μg human E1 activating enzyme UBE1 (Sigma-Aldrich, USA), 1 μg yeast E2 Ubc5, 300 ng yeast E3 Rsp5, 2 μg ubiquitin (wild-type or dye-labeled) were mixed in ubiquitination buffer (50 mM Tris, 10 mM $MgCl_2$, 50 μm $ZnCl_2$, 1 mM DTT, pH 7.5) and 2 mM ATP, with a total volume of 30 μL. Ubiquitin would conjugate to Pol II subunit Rpb1 in this reaction. After 30℃ incubation with mild shaking for overnight, silver staining, Western blotting and fluorescent imaging were used to investigate the efficacy of ubiquitination.

2-2.7 Gel electrophoresis and imaging

20 μL of each sample was mixed with 5 μL of 5× SDS loading dye (250 mM Tris-HCl, 10% SDS, 50% glycerol, 10 mM EDTA, 0.25 mg/mL bromophenol blue, 25% BME in distilled water), heated on dry bath at 95℃ for 10 minutes. Then 10 μL of the sample was loaded into a well in gel. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 8% Bis-Tris polyacrylamide gel 1.0 mm (The protocol of this gel is listed below) under 150V for 35 minutes, or 4 – 12% gradient gel (NuPAGE® Novex 4-12% Bis-Tris Gel 1.0 mm, 10 Well; Life Technologies, USA) under 200V for 35 minutes.

2-2.7.1 Fluorescence imaging

When electrophoresis was done, the proteins on gel were fixed by fixing buffer (30% ethanol, 10% acetic acid in distilled water) for 30 minutes. The gel was then rinsed twice in 20% ethanol, for 10 minutes for each wash, and then twice in water, for 10 minutes for each wash. After that, the gel was scanned by fluorescent scanner Amersham Typhoon^{TM} 9200 Imager (GE Healthcare, UK). The laser and emission filter were set to 488 nm and 520 nm, respectively. The sensitivity was set to normal, and the pixel size was set to 100 microns or 200 microns.

2-2.7.2 Silver stain

After fluorescence imagine, the gel could be used directly to do silver stain. The gel was sensitized by 0.8 mM sodium thiosulfate for 1 minute and then rinsed twice in water for 1 minute for each wash. Next, the gel was soaked in 0.2% silver nitrate (12 mM) for 20 minutes. After silver impregnation, the gel was dipped in water for 10 seconds and then moved to developer (3% sodium carbonate, 250 μl formalin and 125 μl 10% sodium thiosulfate per liter) for 5 minutes. Last step, the gel was immersed in stop solution (4% Tris and 2% acetic acid in water). The detailed protocol of silver stain was published by Chevallet et al. [36].

2-2.7.3 Western blot

Another gel just finished electrophoresis was used to do Western blot. First, the proteins on gel were transferred to Whatman® Protran® nitrocellulose membrane BA85, pore size 0.45 μm (PerkinElmer, USA), in Western blot transfer buffer (5.8 g glycine, 2.9 g Tris, 10% methanol per liter) under 300 mA for 80 minutes. Meanwhile, 1 liter of wash buffer (8.7 g sodium chloride, 6 g Tris, 1 mL Tween-20 in 1 liter buffer, pH 7.5) was also prepared. Later, 10 mL of blocking buffer (wash buffer plus 5% milk powder) was used to avoid nonspecific bindings on the membrane. After 30 minutes of blocking, 1^{st} antibody (rabbit anti-ubiquitin antibody; Sigma-Aldrich, USA) was added directly, and binding overnight at 4℃ with shaking. On the next day, the membrane was washed 3 times in wash buffer, 5 minutes each time. Then 10 mL of blocking buffer was added, in addition with 2 μL of 2^{nd} antibody (goat anti-rabbit-IgG antibody, HRP peroxidase conjugated; EMD Millipore, USA), binding for 1 hour with shaking. After additional 3 times of washing (5 minutes each), Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore, USA) was used for chemiluminescent detection. Then FUJI SuperRX developing film (Fujifilm, Japan) was used, and the developing time was set from 20 seceonds to 3 minutes. KODAK RP X-OMAT Developer and Replenisher (Eastman Kodak, USA) was used to develop the film.

Chapter 3: Result

3-1 Design and expression of mutant ubiquitin constructs

To make thio-reactive fluorescence dye Alexa-488 conjugated ubiquitin, we need ubiquitin mutants with cysteine residue for dye labeling. We already had wild-type ubiquitin in pET-11b expression vector in our lab, and ubiquitin D39C, D39C/K63R M[C]Q-6His, and R72C-6His were constructed later based on the wild-type plasmid. D39C and D39C/K63R mutant could be used directly, while M[C]Q-6His and R72C-6His could only be used after hexahistidine tag deletion. For this purpose, we mutated the first histidine codon (CAT) to stop codon (TGA) (figure 3.1). The resulting M[C]Q and R72C constructs were used to express cysteine-containing ubiquitins. Furthermore, for better understandings of ubiquitination, we mutated lysine residues on ubiquitin M[C]Q to arginines. Seven mutants, M[C]Q/K6R, M[C]Q/K11R, M[C]Q/K27R, M[C]Q/K29R, M[C]Q/K33R, M[C]Q/K48R, and M[C]Q/K63R were therefore constructed and used in K-to-R ubiquitins expression later (figure 3.2). During making some constructs, the first DH5α colony picked on the plate didn't contain the desired sequences in plasmids, and we had to pick and analyze several colonies to get the right ones.

(A)

10x reaction buffer	5 µL
dNTP mix (2.5 mM)	8 µL
Template plasmid	150 ng
Forward primer (10 µM)	2 µL
Reverse primer (10 µM)	2 µL
Polymerase	1 µL
DMSO	2.5 µL
Add distilled H_2O to 50 µL	

(B)

Segment 1		95°C	2 min
1 cycle			
Segment 2	Denaturation:	95°C	20 sec
30 cycles	Annealing:	T*	20 sec
	Elongation:	72°C	3 min
Segment 3		72°C	3min
1 cycle			

Table 3.1 PCR protocol of site-directed mutagenesis

(A) The reagents added in PCR reactions. The polymerase used is *PfuUltra* II Fusion HS DNA Polymerase (Stratagene, Agilent, USA).

(B) PCR cycle condition. Determination of annealing temperature T* is according to the melting temperature (T_m) of the primer used.

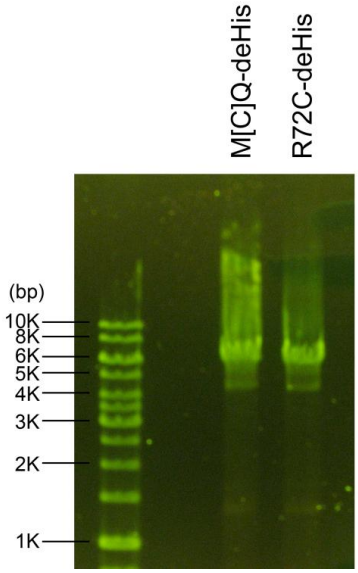


Figure 3.1 PCR result of ubiquitin M[C]Q and R72C 6-His tag deletion

The protocol of PCR reaction is listed in Table 3.1 and the annealing temperature (T*) was set at 55℃. The PCR products were checked directly by DNA electrophoresis under 100 volts for 30 minutes. The gel was composed of 1% agarose in 1x TAE buffer with 1x SYBR® Safe DNA Gel Stain (Invitrogen, Life Technologies, USA). The result shows that the sizes of products were correct (about 6k bp). After plasmid 5'-phosphorylation and ligation, the samples were transformed to DH5α competent cells. The sequences of plasmids were confirmed to be the expected ones by sequencing as chapter 2-2.1 described.

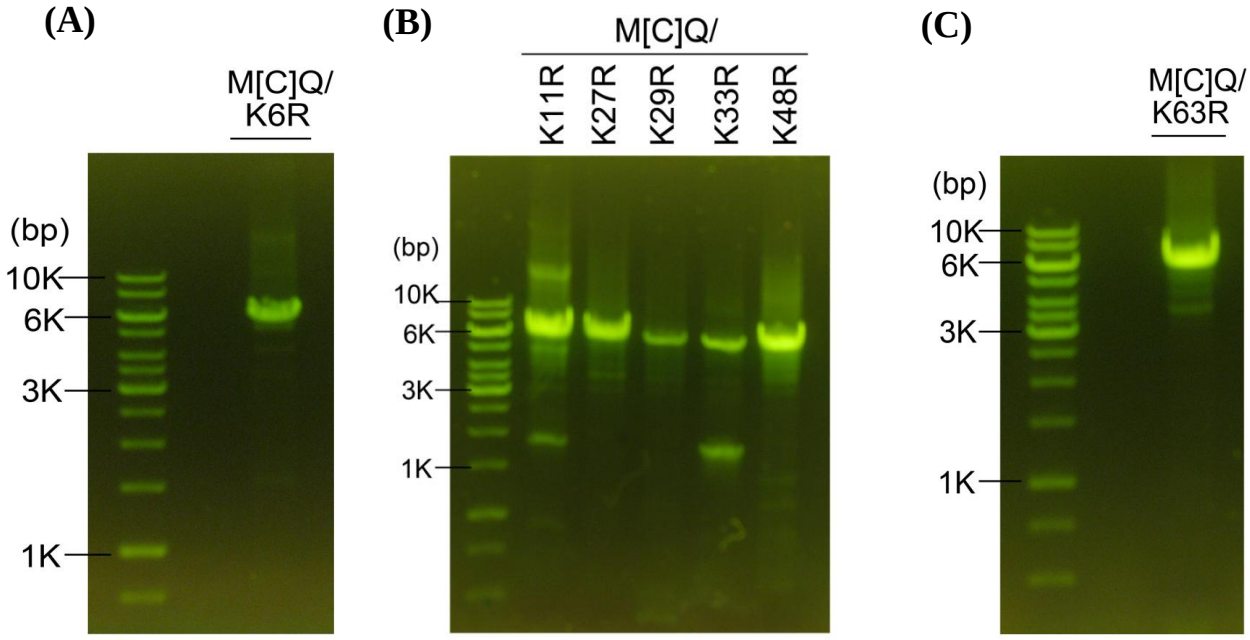


Figure 3.2 PCR result of ubiquitin M[C]Q/K to R mutants.

The protocol of PCR reaction is listed in Table 3.1

(A) PCR result of ubiquitin M[C]Q/K6R. The annealing temperature was set at 52℃.

(B) PCR result of ubiquitin M[C]Q/K11R, K27R, K29R, K33R and K48R, respectively. The annealing temperatures of all these 5 reactions were all set at 51℃.

(C) PCR result of ubiquitin M[C]Q/K63R. The annealing temperature was set at 55℃.

The DNA electrophoresis was done in gel composed of 1% agarose in 1x TAE buffer with 1x SYBR® Safe DNA Gel Stain (Invitrogen, Life Technologies, USA) under 100 volts for 30 minutes. The sizes of PCR products were all correct (about 6k bp). After plasmid 5'-phosphorylation and ligation, the samples were transformed to DH5α competent cells and the plasmids were amplified and extracted afterward. The sequences of plasmids were confirmed to be the expected ones by sequencing as chapter 2-2.1 described.

3-2 Small scale expression analysis

After transforming plasmid into *E. coli* BL21 Star™ (DE3) competent cells, we cultured the cells overnight on agar plates. The colonies on the plates were inoculated into LB/ampicillin medium. After overnight culture and the following subculture, IPTG was added for induction test. As shown in figure 3.3, the expression levels of ubiquitin mutants were all highly elevated. Those high-expression strains were prepared as glycerol stocks for later expression. 2 tubes of 1.2 mL glycerol stock were made for every mutant.

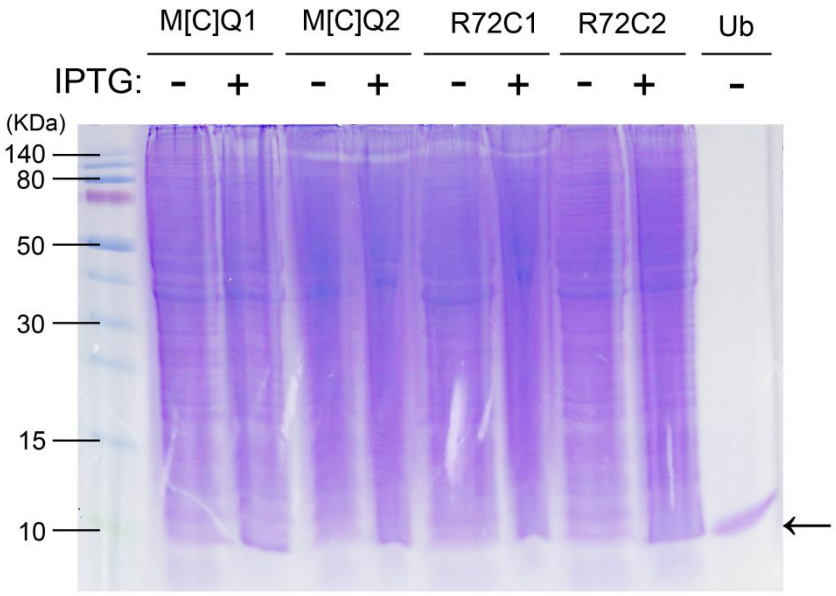


Figure 3.3 Small scale expression of ubiquitin M[C]Q and R72C (without 6-His tag). Two colonies with ubiquitin M[C]Q plasmid (M[C]Q1 and M[C]Q2) and two colonies with ubiquitin R72C plasmid (R72C1 and R72C2) were picked and subcultured for induction test. The full procedures are described in chapter 2-2.2. The result shows that all cultures with IPTG induction overexpressed ubiquitin (arrow on the bottom). ("Ub" stands for commercial wild-type ubiquitin as a positive control.)

3-3 Large scale expression and purification

3-3.1 Large scale protein expression

100 μL of ubiquitin M[C]Q, D39C, R72C and D39C/K63R glycerol stock was added to 100 mL TB medium respectively and incubated overnight. Then the cultures were subcultured and ubiquitin expression was induced by IPTG. 4 hours later, the cultures were centrifuged and about 20 g of the cell pellet was collected for each culture. Later the pellets underwent cell lysis, and about 200 mL of cell lysate was produced. Then ammonium precipitation was performed. In every step, 20 uL of sample was collect and analyzed by electrophoresis (figure 3.4).

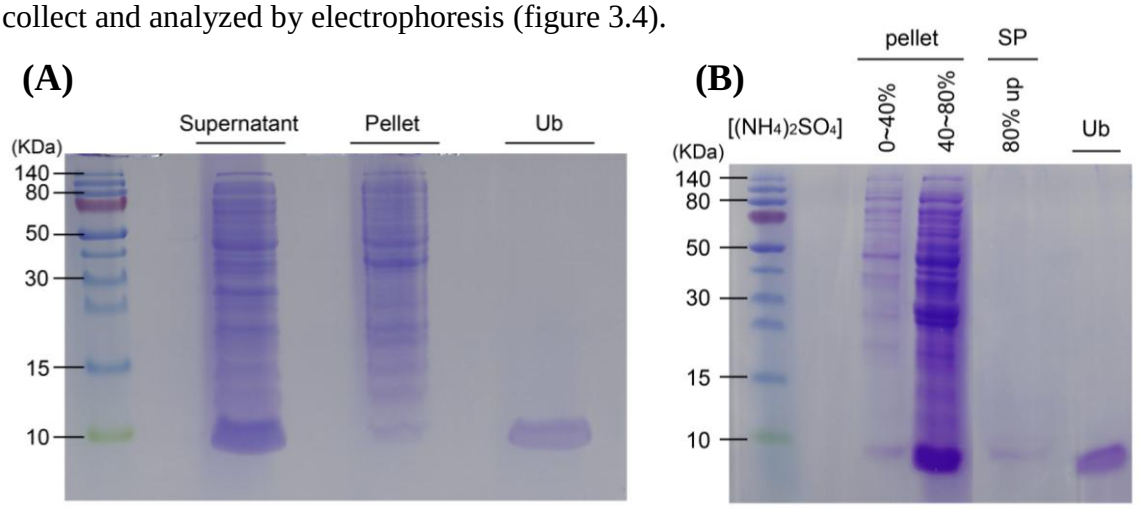


Figure 3.4 Examples of ubiquitin purification.
The purification method is listed in chapter 2-2.3.2 and 2-2.3.3. Ubiquitin D39C/K63R (D39 is replaced by cysteine, and K63 is replaced by arginine) construct was used to optimize purification procedures.

(A) Cell lysis and centrifugation after ubiquitin overexpression. As shown in the pictures, expressed protein appeared in supernatant, not pellet.

(B) Ammonium sulfate precipitation of collected supernatant. Most ubiquitin was precipitated within 40 - 80% saturation. (“SP” = supernatant; “Ub” = WT ubiquitin)

3-3.2 Ion exchange chromatography & size exclusion chromatography

The supernatant was condensed to less than 50 mL and then filtered by 0.22 μm filter for IEC. Ubiquitin was eluted at 30% to 40% B buffer, as it shown in figure 3.5. The fractions which contained ubiquitin were pooled and concentrated for SEC. As shown in figure 3.6, ubiquitin appeared between fraction 23 and 32 (from 82 mL to 102 mL). However, since ubiquitin was not finely separated from the contaminants in this step, the collected fractions were concentrated and underwent a second round of SEC (figure 3.7). Figure 3.5 to 3.7 show the results of ubiquitin M[C]Q purification, and the purification of ubiquitin D39C and R72C had similar results. The eluate with purified ubiquitin was dialyzed in 0.1% TFA and then lyophilized. Finally, about 5 μg of ubiquitin dry powder was acquired.

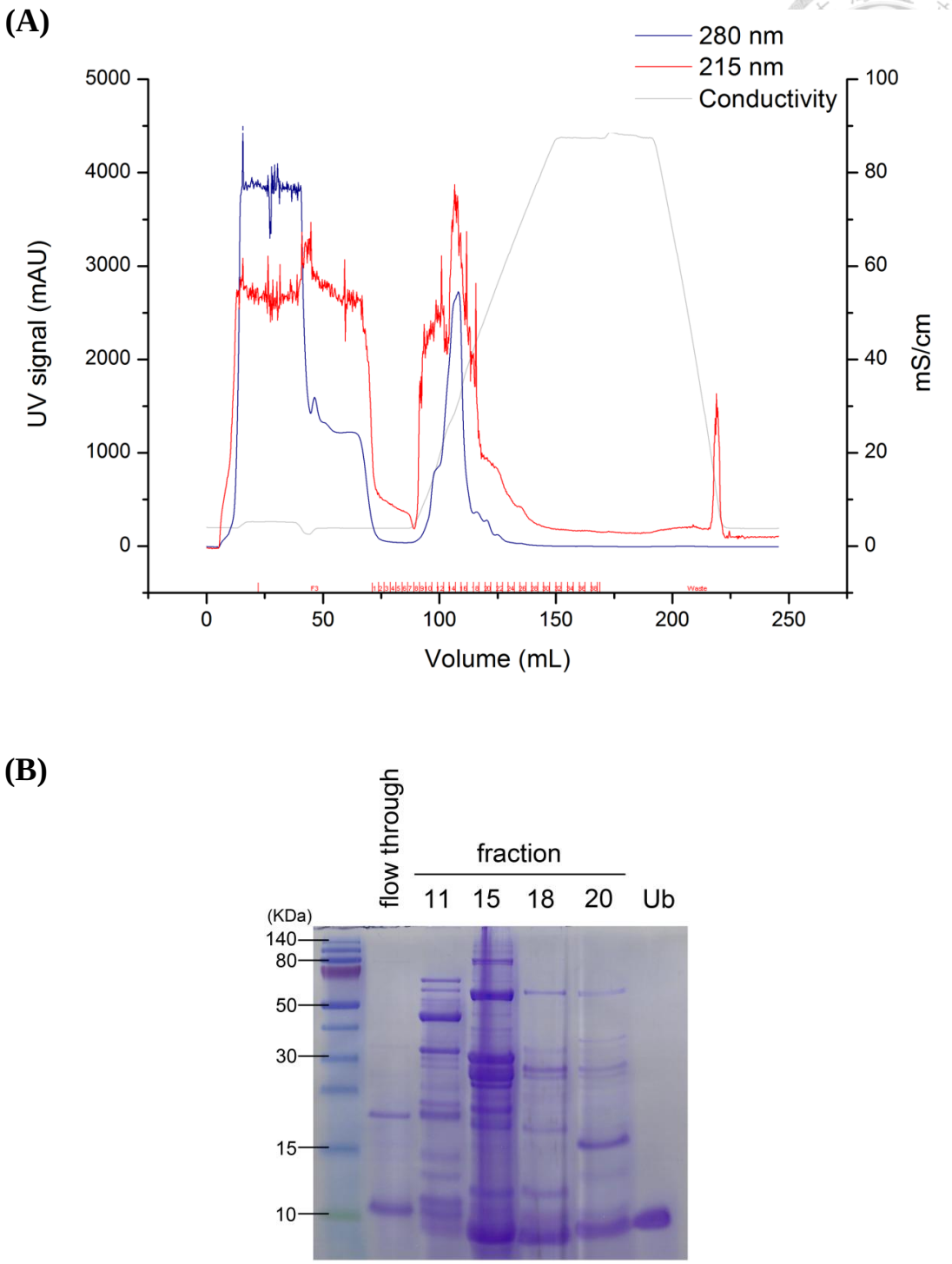


Figure 3.5 IEC purification of Ub M[C]Q.

Ub M[C]Q mostly appeared in fraction 14 to 18, and these fractions were collected for SEC. ("Ub" represents "wild-type ubiquitin", which was used as positive control.)

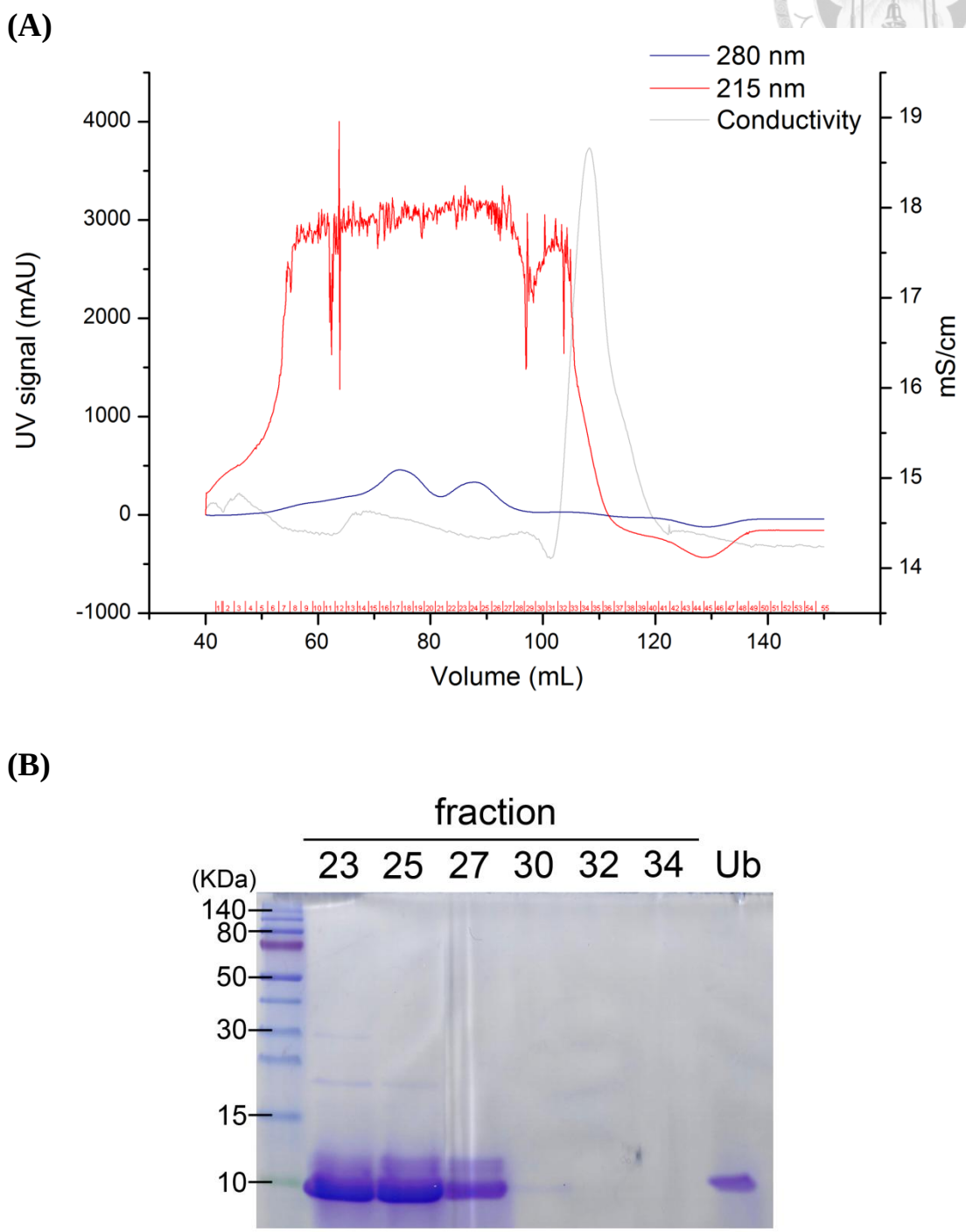


Figure 3.6 SEC purification of Ub M[C]Q

Ub M[C]Q appeared in fraction 22 to 28, and these fractions were pooled for second SEC. ("Ub" = WT Ub)

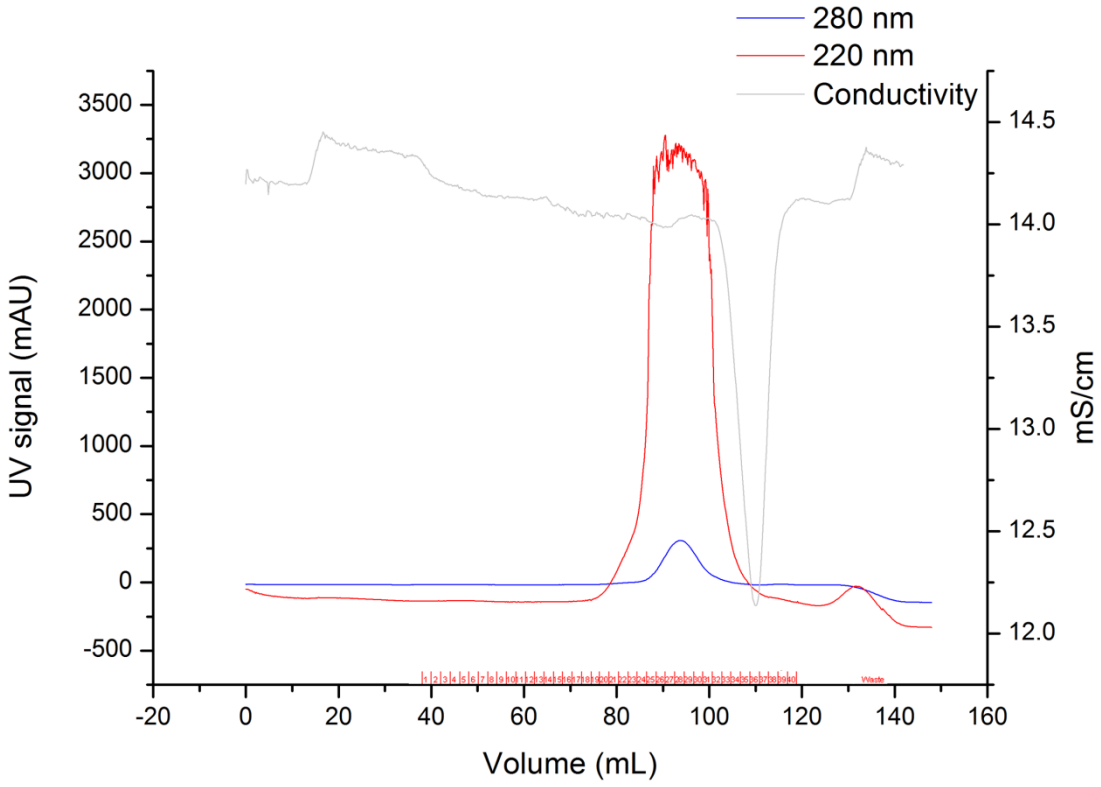


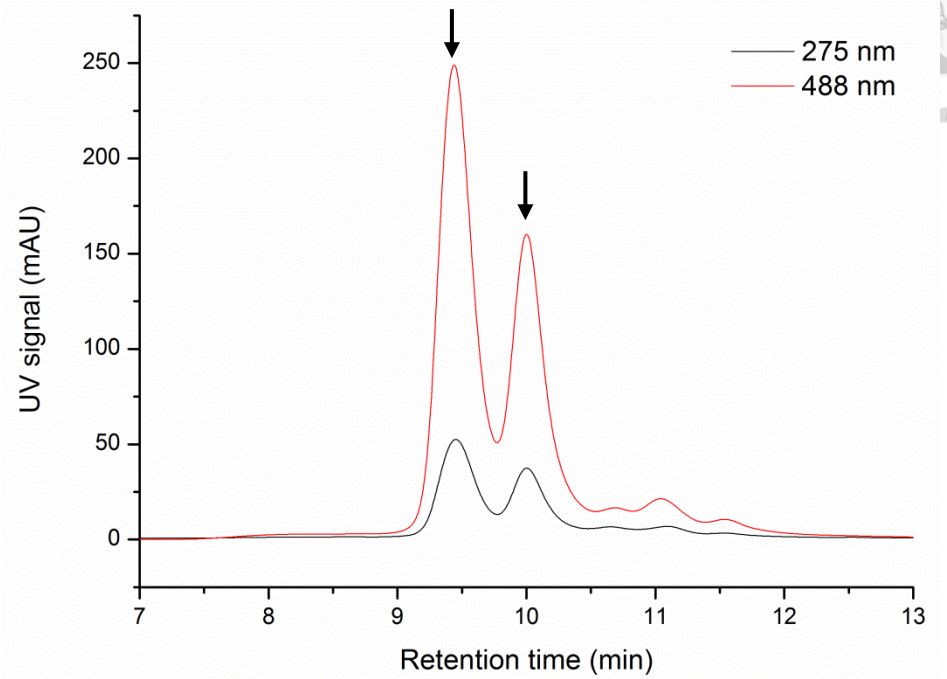
Figure 3.7 Second SEC of Ub M[C]Q

A single peak appeared in FPLC plot, and fraction 23 to 34 were collected, dialysis in 0.1% TFA, and then lyophilized.

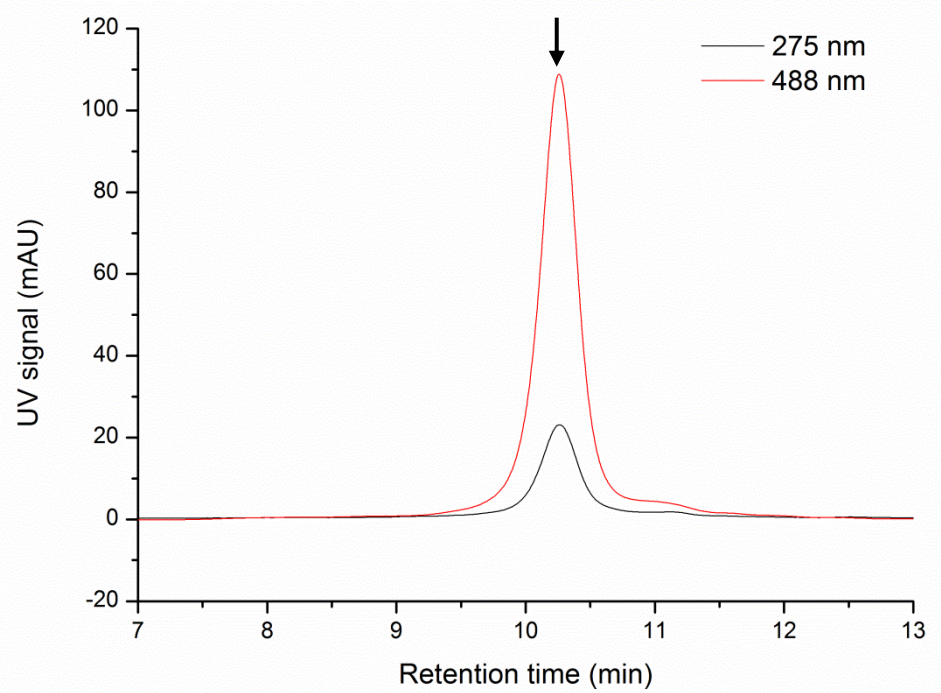
3-4 Fluorescence dye labeling and purification

Ubiquitin M[C]Q, D39C, R72C and D39C/K63R were used to make Alexa-488 labeled ubiquitin, which called M[C]Q-A488, D39C-A488, R72C-A488 and D39C/K63R-A488. After reaction and dialysis, HPLC was applied to separate labeled ubiquitin from free ubiquitin. All samples eluted at 30% to 32% B buffer (figure 3.8 B-E). It should be noted that two peaks in M[C]Q-A488 sample with 488 nm absorption appeared, therefore we collected both peaks for further analysis. Purified and lyophilized samples were then sent to do ESI-TOF mass spectrometry analysis (figure 3.9 and 3.10). We confirmed that R72C-A488 was the correct product (figure 3.10), but it seemed that the Met1 of both M[C]Q-A488 samples were lost (figure 3.9), which agreed with previous result in our lab and researches from other groups [37, 38].

(A) Sample volume = 220 μL



(B) Sample volume = 50 μL



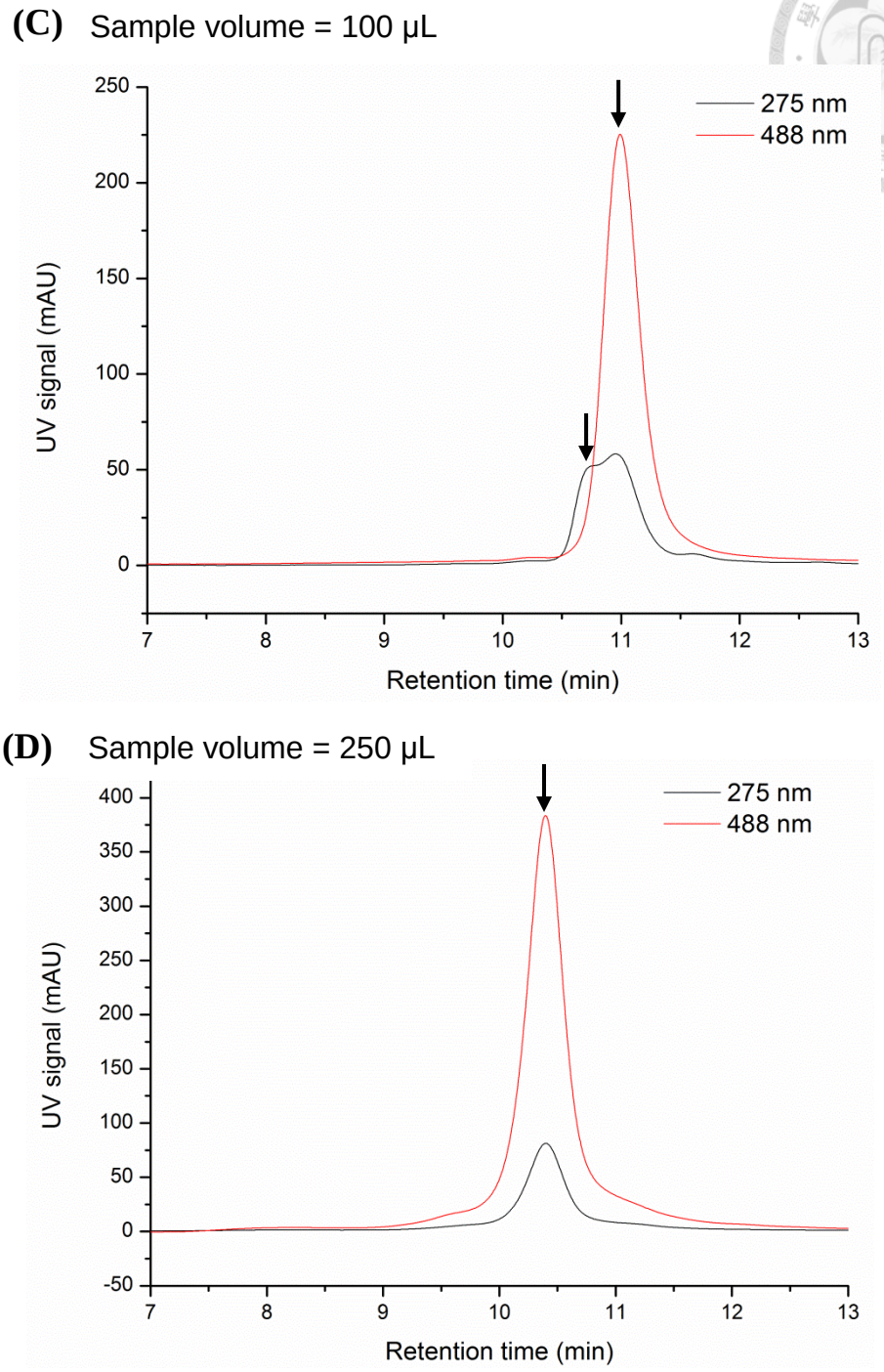


Figure 3.8 HPLC purification of Alexa-488 labeled ubiquitin

(A) HPLC purification of Ub M[C]Q-A488. Two peaks were eluted in M[C]Q-A488 sample, and both of them were collected.

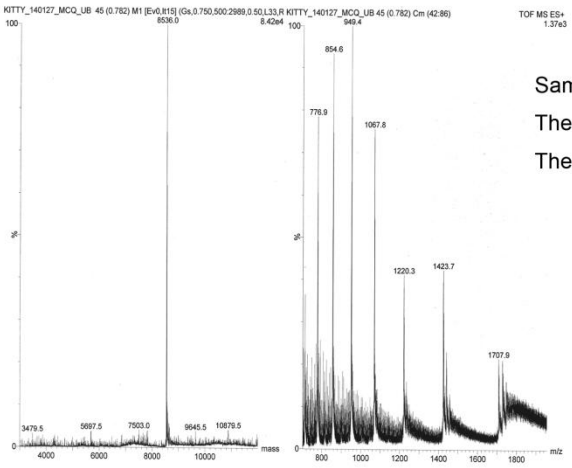
(B) HPLC purification of Ub D39C-A488. A single peak was shown.

(C) HPLC purification of Ub R72C-A488. The right peak is dye-labeled R72C-A488, and the left peak is R72C free protein.

(D) HPLC purification of Ub D39C/K63R-A488. A single peak was shown.

Free Alexa-488 eluted at 11 - 13 minutes, at similar position as Ub-A488 samples.

(A)

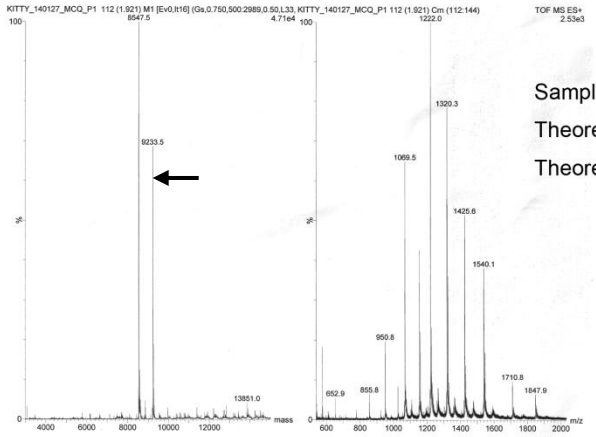


Sample: Ub M[C]Q

Theoretic mass: 8668.0Da for Ub M[C]Q

Theoretic mass: 8536.8Da for M1 excised Ub M[C]Q

(B)

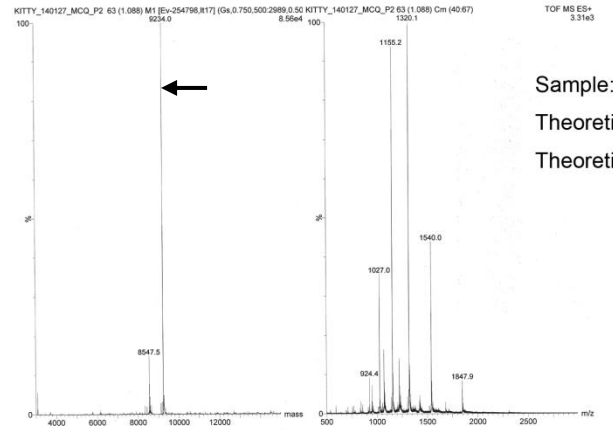


Sample: Ub M[C]Q-A488 peak1

Theoretic mass: 9365.7Da for Ub M[C]Q-A488

Theoretic mass: 9234.5Da for M1 excised Ub M[C]Q-A488

(C)



Sample: Ub M[C]Q-A488 peak2

Theoretic mass: 9365.7Da for Ub M[C]Q-A488

Theoretic mass: 9234.5Da for M1 excised Ub M[C]Q-A488

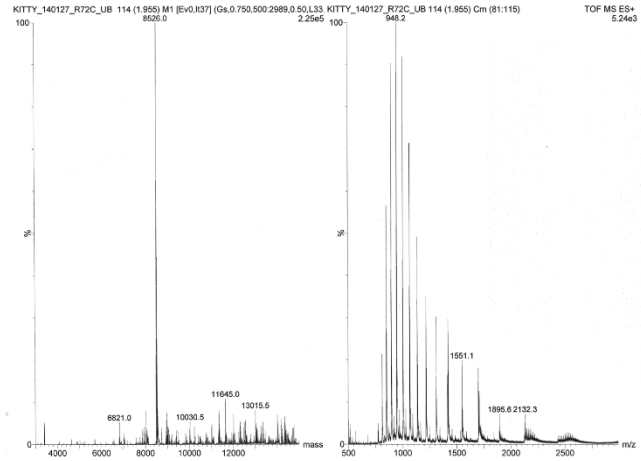
Figure 3.9 ESI-TOF mass spectra of Ub M[C]Q and Ub M[C]Q-A488

(A) Theoretic mass of Met1 excised Ub M[C]Q is shown in the spectra.

(B) Theoretic mass of Met1 excised Ub M[C]Q-A488 (black arrow) is shown in the spectra of Ub M[C]Q sample peak 1.

(C) Theoretic mass of Met1 excised Ub M[C]Q-A488 (black arrow) is also shown in the spectra of Ub M[C]Q sample peak 2.

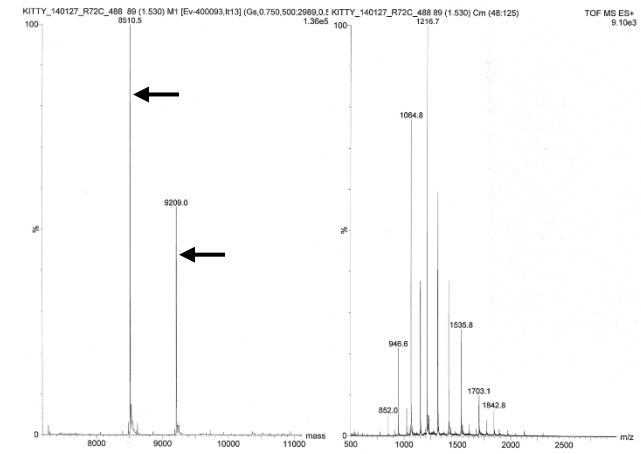
(A)



Sample: Ub R72C

Theoretic mass: 8511.8Da

(B)



Sample: Ub R72C-A488

Theoretic mass: 9209.5Da

Figure 3.10 ESI-TOF mass spectra of Ub R72C and Ub R72C-A488

(A) No theoretic mass of Ub R72C (8511.8 Da) was obtained in this figure.

(B) However, two peaks with molecular weight of 8510.5 Da and 9209.0 Da (black arrows), which are related to Ub R72C (predicted mass = 8511.8 Da) and R72C-A488 (predicted mass = 9209.5 Da), appeared here.

3-5 Enzyme expression and purification

After ubiquitin-A488 samples are ready, Ubc5 (E2) and Rsp5 (E3) enzymes are expressed. Prepared glycerol stocks were used for large scale expression, and IMAC was performed to purify His-tagged enzymes. Our enzymes appeared in eluate (1st IMAC in figure 3.11A & B). In following SUMO-tag cleavage, Ubc5 and Rsp5 eluted at low imidazole concentration (2nd IMAC in figure 3.11 A & B).

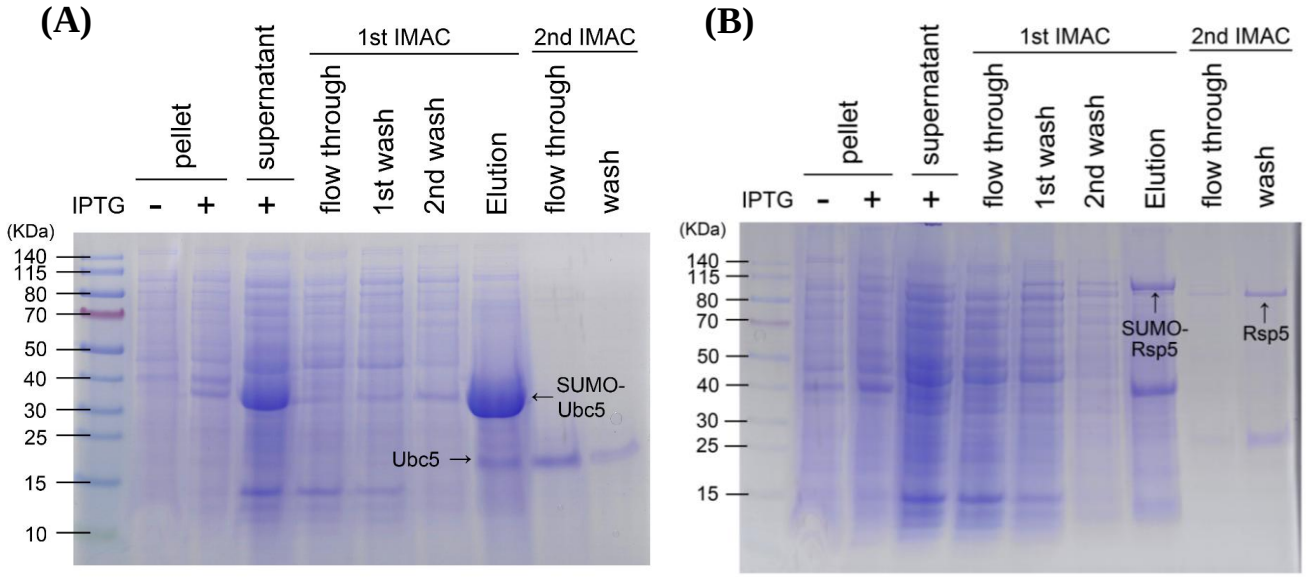


Figure 3.11 Ubc5 (E2) purification and Rsp5 (E3) purification

The method of IMAC purification is described in chapter 2-2.5.2, and SUMO cleavage method is described in 2-2.5.3.

(A) Ubc5 purification. After cell lysis and centrifugation, Ubc5 appeared in supernatant. In the first IMAC, Ubc5 was eluted when elution buffer with high imidazole was applied. After SUMO cleavage, the sample underwent the second IMAC. Ubc5 protein didn't bind to IMAC column and appeared in flow through (imidazole concentration = 10 mM).

(B) Rsp5 purification. After cell lysis and centrifugation, Rsp5 appeared in supernatant. In the first IMAC, Rsp5 was eluted when elution buffer with high imidazole was applied. After SUMO cleavage, Rsp5 protein bind loosely to IMAC column and was eluted when imidazole concentration > 20 mM

3-6 Ubiquitination assay and its results

We performed *in vitro* ubiquitination assay after dye-labeled ubiquitin mutants and E2, E3 enzymes were ready to use. Every component was checked by silver staining prior to ubiquitination assay to confirm its position on gel (figure 3.12). We designed a series of ubiquitination assay, which specific reagents were added or not (table 3.2). With this design, we showed that all reagents we added were required in ubiquitination (figure 3.13-3.16).

The fluorescent imaging, silver staining and Western blotting result of different dye-labeled ubiquitin (M[C]Q-A488, D39C-A488 and R72C-A488) are shown below. We observed that ubiquitin M[C]Q-A488 and D39C-A488 conjugated to Pol II (figure 3.13-3.16), while R72C-A488 didn't conjugate to substrate in the same condition (figure 3.17). To our surprise, no polyubiquitin signal was detected on fluorescent imaging, and it maybe indicates that dye-labeled ubiquitin is unable to form polyubiquitin chain. On the other hand, besides expected singnals, Western blot result revealed that non-specific bindings almost appeared in every plot (figure 3.13B, 3.15B and 3.17B).

	1	2	3	4	5	6	7	8
pol II:	+	-	+	+	+	+	+	+
E1:	+	+	-	+	+	+	+	+
E2:	+	+	+	-	+	+	+	+
E3:	+	+	+	+	-	+	+	+
ATP:	+	+	+	+	+	-	+	+
Ub:	F	F	F	F	F	F	W	-

Table 3.2 Experimental design of ubiquitination assay

A plus sign (+) means the reagent was added in this reaction, and a minus sign (-) means the reagent was absent in this reaction. An "F" means fluorescent-dye-labeled ubiquitin was added, while a "W" means wild-type ubiquitin was used instead. We expected that only reaction 1 and 7 would show Pol II ubiquitination.

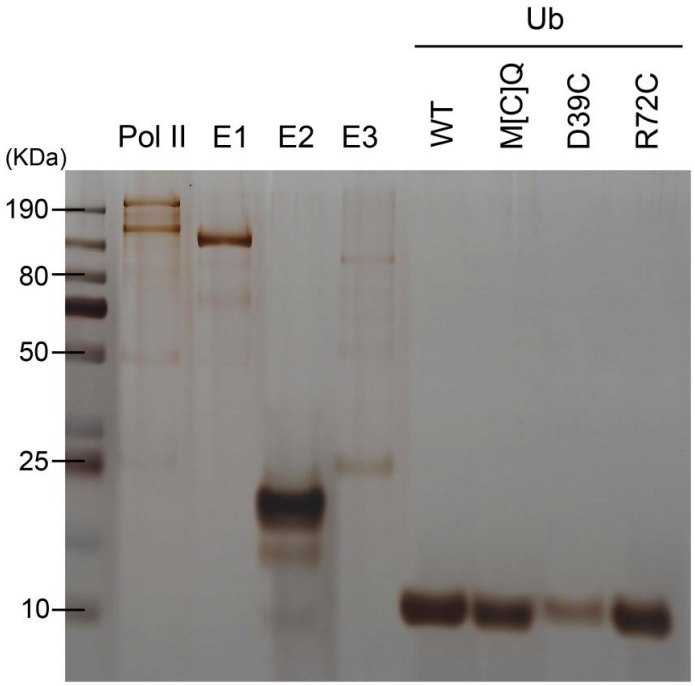


Figure 3.12 Silver stain result of each reagent in ubiquitination

Pol II = yeast RNA polymerase II, E1 = human UBE1 (Sigma-Aldrich, USA), E2 = yeast Ubc5, E3 = yeast Rsp5, WT Ub = human wild-type ubiquitin (Sigma-Aldrich, USA). Ub M[C]Q, D39C and R72C were expressed and purified previously.

Two major bands in Pol II sample were Pol II subunit Rpb1 (upper) and Rpb2 (lower).

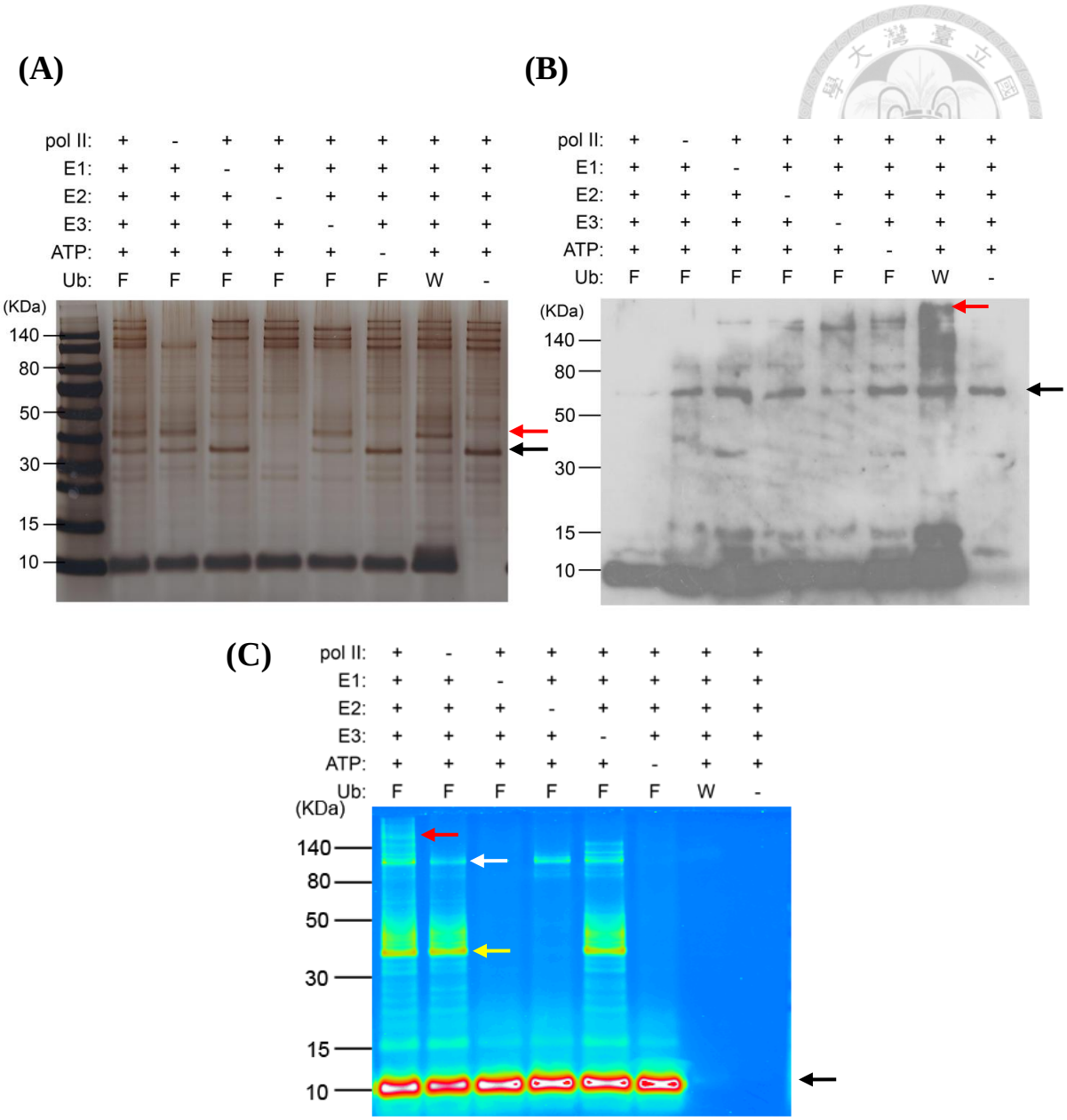


Figure 3.13 Ubiquitination assay using Ub M[C]Q-A488 (E2, E3 with SUMO tag)
Which reagents were added is listed above. "+" means this reagent was added, "-" means this reagent was not added. At the row of Ub (ubiquitin), an "F" means fluorescent-dye-labeled Ub M[C]Q-A488, while a "W" means wild-type ubiquitin.
(A) Silver stain: in this figure, band shift after ubiquitin conjugation (especially E2-Ub) could be observed. The unmodified and Ub-conjugated E2 are marked by black arrow and red arrow, respectively.
(B) Western blot: polyubiquitination signal could be seen in ubiquitination assay using wild-type Ub (red arrow). Non-specific binding band is marked by black arrow.
(C) Fluorescent image: in this figure, we can distinguish ubiquitin monomer (marked by black arrwo), Ub conjugated E1 (E1-Ub, white arrow), E2-Ub (yellow arrow) and Rpb1-Ub (red arrow)

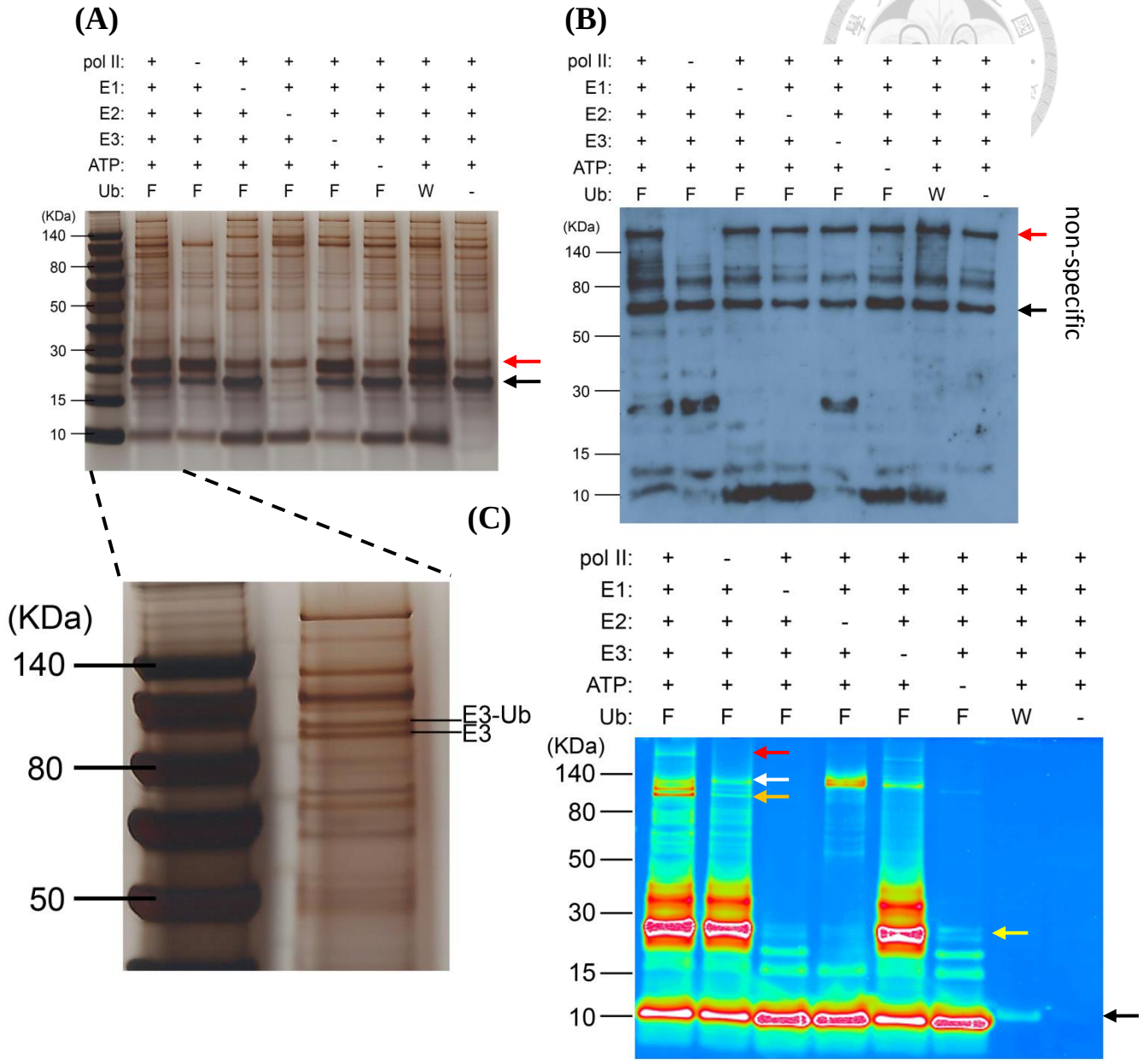


Figure 3.14 Ubiquitination assay using Ub M[C]Q-A488 (E2, E3 without SUMO tag)

Which reagents were added is listed above. The meanings of "+" and "-" are the same as figure 3.12. At the last row, an "F" means fluorescent-dye-labeled ubiquitin M[C]Q-A488, and a "W" means wild-type ubiquitin.

(A) Silver stain: in this figure, band shift after ubiquitin conjugation could be observed. The unmodified and Ub-conjugated E2 are marked by black arrow and red arrow, respectively. Ub-conjugated E3 could also be seen in this figure (blue arrow).

(B) Western blot (C) Fluorescent image: in this figure, we can distinguish ubiquitin monomer (marked by black arrwo), Ub conjugated E1 (E1-Ub, white arrow), E2-Ub (yellow arrow), E3-Ub (orange arrow) and Rpb1-Ub (red arrow)

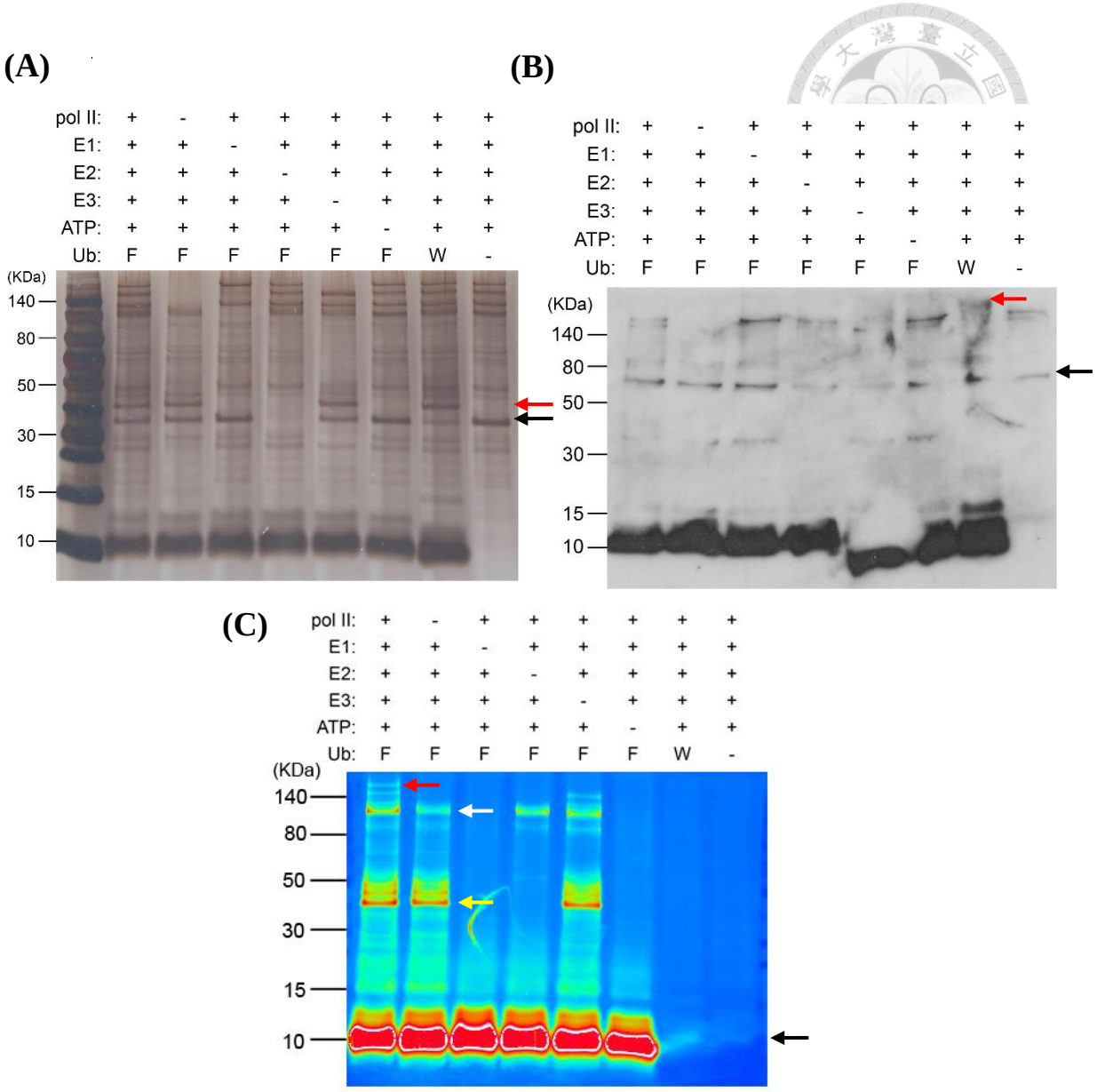


Figure 3.15 Ubiquitination assay using Ub D39C-A488 (E2, E3 with SUMO tag)
Which reagents were added is listed above. The meanings of "+" and "-" are the same as figure 3.12. At the last row, an "F" means fluorescent-dye-labeled ubiquitin D39C-A488, and a "W" means wild-type ubiquitin.
(A) Silver stain: in this figure, band shift after ubiquitin conjugation could be observed. The unmodified and Ub-conjugated E2 are marked by black arrow and red arrow, respectively.
(B) Western blot: polyubiquitination signal could be seen in ubiquitination assay using wild-type Ub (red arrow). Non-specific binding band is marked by black arrow.
(C) Fluorescent image: in this figure, we can distinguish ubiquitin monomer (marked by black arrwo), Ub conjugated E1 (E1-Ub, white arrow), E2-Ub (yellow arrow) and Rpb1-Ub (red arrow)

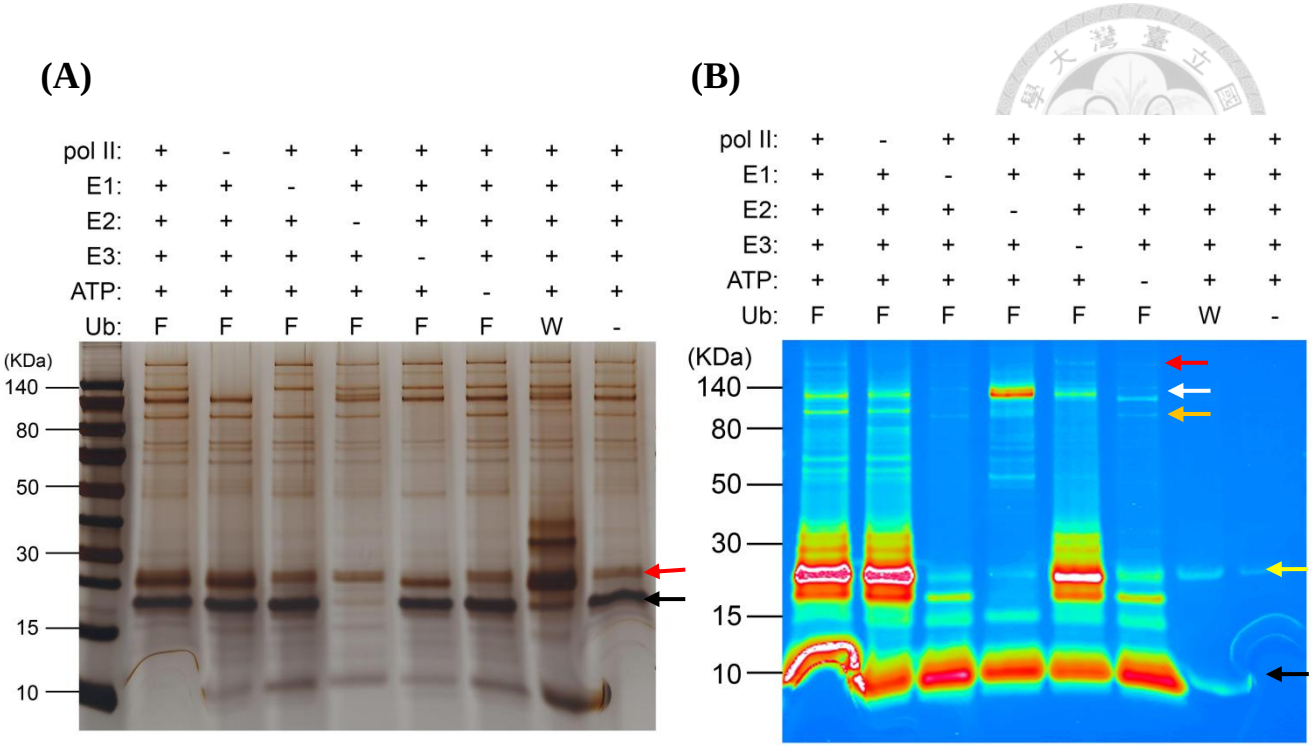


Figure 3.16 Ubiquitination assay using Ub D39C-A488 (E2, E3 without SUMO tag)
Which reagents were added is listed above. The meanings of "+" and "-" are the same as figure 3.12. At the last row, an "F" means fluorescent-dye-labeled ubiquitin D39C-A488, and a "W" means wild-type ubiquitin.
(A) Silver stain: in this figure, band shift after ubiquitin conjugation (especially E2-Ub) could be observed. The unmodified and Ub-conjugated E2 are marked by black arrow and red arrow, respectively.
(B) Fluorescent image: in this figure, we can distinguish ubiquitin monomer (marked by black arrwo), Ub conjugated E1 (E1-Ub, white arrow), E2-Ub (yellow arrow), E3-Ub (orange arrow) and Rpb1-Ub (red arrow)

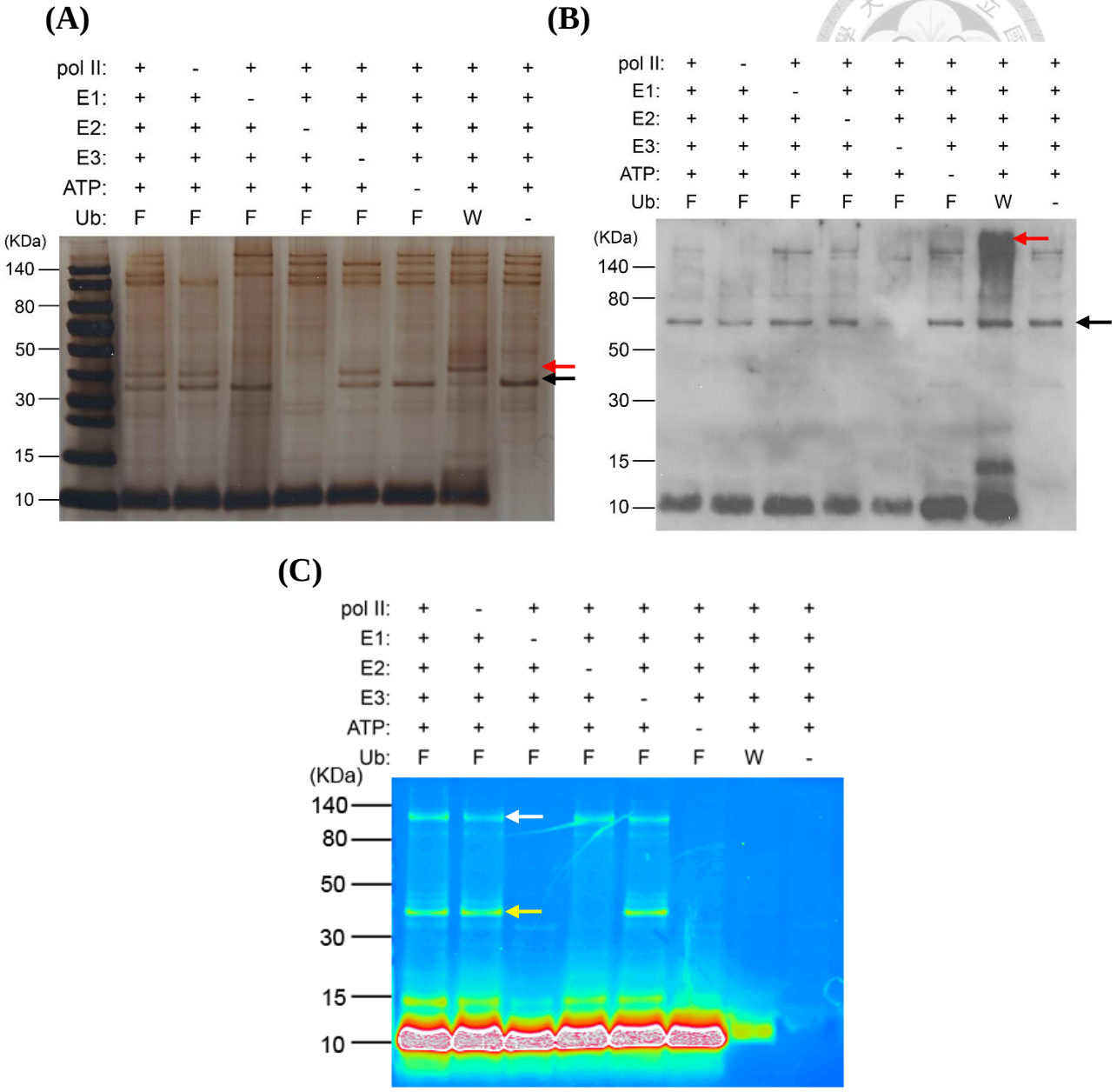


Figure 3.17 Ubiquitination assay using Ub R72C-A488 (E2, E3 with SUMO tag)
Which reagents were added is listed above. The meanings of "+" and "-" are the same as figure 3.12. At the last row, an "F" means fluorescent-dye-labeled ubiquitin R72C-A488, and a "W" means wild-type ubiquitin.
(A) Silver stain: in this figure, band shift after ubiquitin conjugation could be observed. The unmodified and Ub-conjugated E2 are marked by black arrow and red arrow, respectively.
(B) Western blot: polyubiquitination signal could be seen in ubiquitination assay using wild-type Ub (red arrow). Non-specific binding band is marked by black arrow.
(C) Fluorescent image: only E1 (white arrow) and small amount of E2 (yellow arrow) are conjugated by R72C-A488. No fluorescent signal of Pol II could be seen on gel.

Chapter 4: Discussion

In this thesis, we tried to construct a fluorescence based method that can give us a brief and clear result without ambiguity. From previous studies, researchers took advantage of Western blot to detect Pol II ubiquitination on Rpb1, and they could identify mono- or poly-ubiquitination [28-30]. However, when we looked at our Western blot results, we found that there are many non-specific binding bands. Anti-ubiquitin antibody bound not only to impurities in E2 and E3 enzyme but also to Pol II itself (figure 3.14B). This is a serious problem, since it would be difficult for us to distinguish monoubiquitinated Rpb1 from unmodified ones. Clearly, this was the drawback that pushed us to seek for alternative methods in ubiquitination research.

Fluorescent dye Alexa-488, which has a high quantum yield and higher stability [39], was used in ubiquitin conjugation. Our result shows that ubiquitin reacted almost completely in excess amoun of Alexa-488 dye (figure 3.8 B&D, no free ubiquitin peak shown in the results). In following ESI-TOF analysis, we confirmed that samples of R72C and R72C-A488 were indeed the expected products (figure 3.10 A&B), whereas samples of M[C]Q underwent N-terminal methionine excision (NME) [37, 38] and the N-terminal methionine of M[C]Q was excised (figure 3.9 A-C).

Since ubiquitination reaction involves several enzymes and reagents, many of them

should be freshly prepared to ensure a good experiment quality. In our experience, purifying E2 and E3 without an ideal procedure led to inactivity of these enzymes and unsuccessful ubiquitination (figure A1). In ubiquitination assay, we showed that Ubc5 (E2) and Rsp5 (E3) with SUMO tag could also catalyze ubiquitination (figure 3.13 and 3.15) as the tag-removed enzymes did (figure 3.14 and 3.16), though using the enzymes without SUMO tags are considered closer to the conditions *in vivo*.

In our works, we used fluorescent-dye-labeled ubiquitin as a probe to identify ubiquitinated proteins. When M[C]Q-A488 and D39C-A488 were used, Rpb1 band could be detected under fluorescent scanner (figure 3.13C, 3.14C, 3.15C and 3.16B), indicating that these two dye-labeled ubiquitin mutant were able to work similarly to wild-type ubiquitin in Pol II ubiquitination, and maybe also in ubiquitination of other substrates. On the other hand, when using R72C-A488, there was no fluorescent signal on Rpb1 band (figure 3.17C), indicating that R72C-A488 could not conjugate normally to the substrate. It might be attributed to the bulky molecule Alexa-488, which is conjugated at Cys72 of Ub R72C and is near C-terminal. Since Gly76 would be conjugated to the substrate directly in ubiquitination, C-terminal tail plays an important role in ubiquitination, and the appearance of a bulky group near C-terminal might hinder the normal function of ubiquitin. R72C-A488 could still be labeled onto E1 and E2, though the intensity is lower than M[C]Q-A488 and D39C-A488. In conclusion,

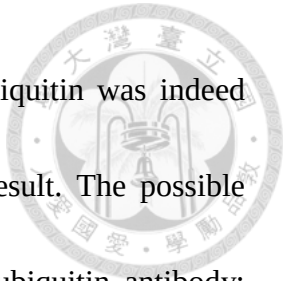
two out of three dye-labeled ubiquitin mutants could work, representing a positive result at the first step.

Besides, another thing we should also take note of is the signs of E3-independent ubiquitination. Our fluorescent images indicates that Pol II could also be monoubiquitinated in the absence of E3 (figure 3.13C, 3.14C, 3.15C and 3.16C), as long as E2 is in presence. The phenomenon of E3 independent ubiquitination has been stated previously [40, 41], but no studies before has reported this occurs on Pol II. The accidental discovery further marks the value of our method.

Nevertheless, considering the type of ubiquitination (mono- or poly-), we didn't observe clear polyubiquitination signal (marks by a smeared band over 250 kDa) in our work. From our result, it seems that only one D39C-A488 molecule was conjugated to Rpb1 of Pol II (figure 3.15C); M[C]Q-A488 probably forms polyubiquitin chain on Pol II, but the evidence is not clear enough (figure 3.13C). Using a K63R mutant ubiquitin (e.g. M[C]Q/K63R-A488 or D39C/K63R-A488) in the assay, or reducing the amount of enzymes and ubiquitin to lower the interference from other proteins, might be feasible ways to clarify it. However, in our Western blot result, we could hardly find a smeared band when M[C]Q-A488 or D39C-A488 was used in ubiquitination assay (the leftmost lanes of figure 3.13B and 3.15B). In contrast, those lanes which used wild-type ubiquitin to carry out ubiquitination assay show bands at high molecular weight (over

200 kDa), and it possibly marks polyubiquitinated Pol II.

Therefore, there are two questions remained unanswered: (1) Was mono- or poly-ubiquitination (from previous studies, it should be a K63 chain) really formed in our system? (2) Why didn't our Western blot show a similar result as wild-type protein, when dye-labeled ubiquitin was used in the assay? Trying to answer those questions, we looked into our previous trials, in which ubiquitin D39C and D39C/K63R were used and labeled by another fluorescent dye, Cy3 (GE Healthcare, UK). In those trials, Ub D39C-Cy3 and D39C/K63R-Cy3 were used in ubiquitination assay, and fluorescent image was also taken after that (figure A2C). In this figure, we can see D39C/K63R-Cy3 fluorescent signal only appeared as a single band at the top, indicating that only monoubiquitinated Pol II was formed; while D39C-Cy3 signal formed a smeared band at the same place, indicating that polyubiquitination might exist. When comparing D39C-Cy3 and D39C-A488, the only difference between them is the conjugated dye at position 39. It should be noted that when wild-type ubiquitin, Ub K29R and Ub K48R were used, smeared black lanes appeared on Western blot result, and Ub K63R didn't show this feature (figure A2B). This result is in agreement with our previous study and previous research from other group [30]. However, in figure A3B, few antibodies were bound to dye-labeled ubiquitin, and no signal was seen at the position of Pol II Rpb1 (over 200 kDa) on lane 5 and lane 6 in Western blot. It is



contradictory, since fluorescent imaging shows that dye-labeled ubiquitin was indeed conjugated to Pol II, but Western blot does not show the same result. The possible explanation is the antibody we used in Western blot (rabbit anti-ubiquitin antibody; Sigma-Aldrich, USA) is not that specific to dye-labeled ubiquitin as it is to wild-type and K-to-R-mutant ubiquitin. It probably points out another drawback of Western blot: besides non-specific interactions, lacking affinity to desired targets might also be a problematic issue (even though we used polyclonal antibody in our study). Actually, this problem could already be seen in ubiquitin conjugated E2: in ubiquitination assay, large amount of wild-type ubiquitin was conjugated to E2 enzyme, which could be confirmed by silver staining (figure 3.13A, 3.14A, 3.15A and 3,17A), but few of them could be seen on Western blot (none could be seen on figure 3.13B, 3.15B and 3.17B, and only some could be ssen on figure 3.14B). Maybe it is because the epitopes of ubiquitin were covered by E2 and could not be identified and bound by antibody. If we also take non-specific binding, which we have mentioned above, into account, it might further reduce the credibility of Western blot results.

Above all, though dye-labeled protein is required, fluorescent imaging gives a more reliable result (e.g. figure 3.13C and 3.14C, where E2-Ub signals are clearly shown). The availability of a commercial kit for detecting ubiquitination using fluorescent imaging and FRET (LanthaScreen™ Ubiquitin and SUMO Assay Reagents, Invitrogen,

Life Technologies, USA) also supports our hypothesis that fluorescent-dye-labeled ubiquitin could be used as a probe for high sensitivity and accuracy.

Chapter 5: Future work

Since we have constructed seven K-to-R mutant of M[C]Q ubiquitin (i.e. M[C]Q/K6R, M[C]Q/K11R, and so on), in the future, we will add fluorophore to those proteins to make dye-labeled K-to-R ubiquitin. Then they could be used in ubiquitination assay similar to method Harreman et al. used before [30]. The only difference is we will use fluorescent imaging instead of Western blotting. If we can get the similar result (for example, all mutant except K63R show fluorescent signals of polyubiquitination, and K63R mutant can only form monoubiquitinated product), then this system can be used as a tool to detect and identify ubiquitin chain when investigating ubiquitination system.

Another feasible application of our method is FRET. If Alexa-488 labeled ubiquitin is confirmed to perform monoubiquitination on Pol II, or if K63R mutated ubiquitin is used in our system instead, we can get monoubiquitinated Pol II. Since the monoubiquitinated Pol II can work normally [26], therefore enables us to use it on FRET experiments. A similar method that using FRET to investigate the mechanistic behavior of Pol II has been developed by Chang et al. [42], however, it might be much easier to perform if we can put a donor or acceptor molecule on Pol II just by ubiquitination assay.

Appendix

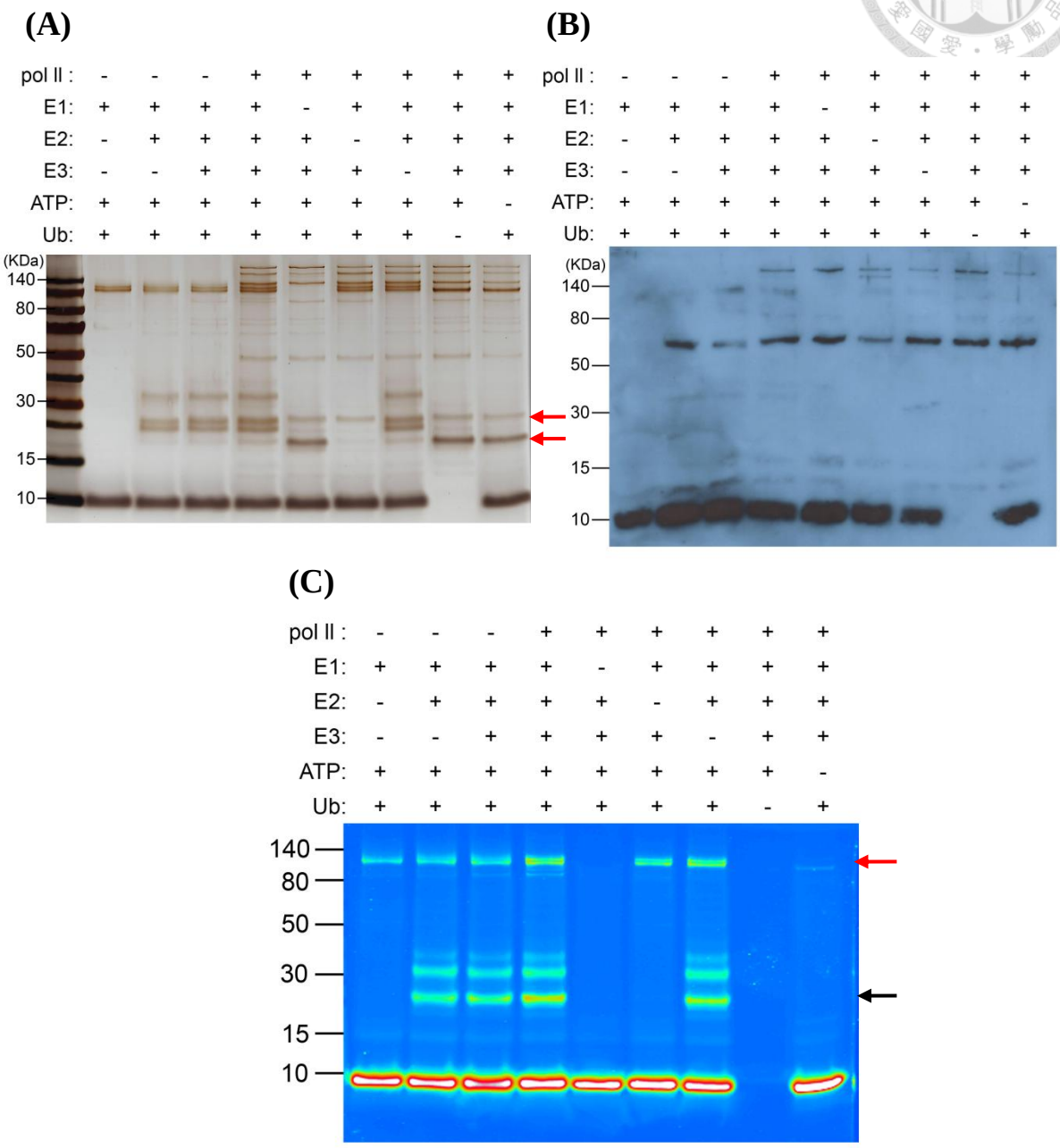


Figure A1 Ubiquitination assay in which enzymes failed to catalyze reactions
Which reagents were added is listed above. "+" means this reagent was added, "-" means this reagent was not added. At the row of Ub (ubiquitin), an "F" means fluorescent-dye-labeled ubiquitin M[C]Q-A488, and a "W" means wild-type ubiquitin. (A) Silver stain: in this figure, E2-Ub could be observed, indicating E1 had catalytic ability. (B) Western blot. (C) Fluorescent image: in this figure, we can see Ub conjugated E1 (E1-Ub, red arrow) and E2-Ub (black arrow), but no E3-Ub and Rpb-Ub, indicating the catalytic ability of E2 had lost.

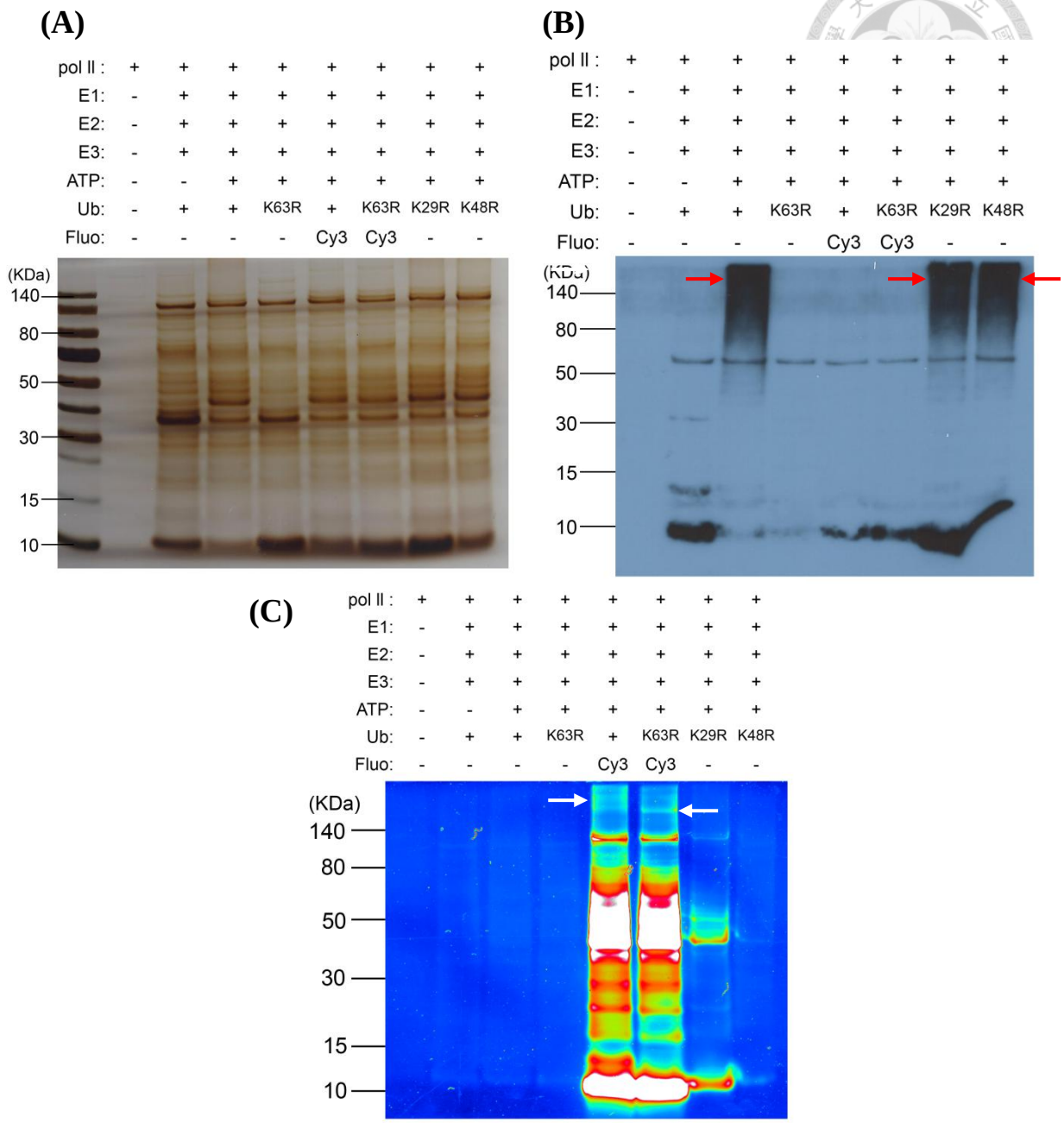


Figure A2 Ubiquitination assay using Ub D39C-Cy3 (E2, E3 with SUMO tag)

Which reagents were added is listed above. "+" means this reagent was added, and "-" means it was not added. From lane 2 to lane 8, wild-type Ub, wild-type Ub, Ub K63R, Ub D39C-Cy3, Ub D39C/K63R-Cy3, Ub K29R and Ub K48R were used, respectively.

(A) Silver stain

(B) Western blot: polyubiquitination signal could be seen in ubiquitination assay using wild-type Ub, Ub K29R and Ub K48R (arrows).

(C) Fluorescent image: this figure shows that Ub D39C-Cy3 (which has Lys63) could form a smeared band (polyubiquitination) on the top of gel, but Ub D39C/K63R-Cy3 (which does not have Lys63) could only form a single band (monoubiquitination).

References

1. Goldstein, G., *Isolation of bovine thymin: a polypeptide hormone of the thymus.* Nature, 1974. **247**(5435): p. 11-4.

2. Goldstein, G., et al., *Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells.* Proc Natl Acad Sci U S A, 1975. **72**(1): p. 11-5.

3. Ciechanover, A., et al., *ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation.* Proc Natl Acad Sci U S A, 1980. **77**(3): p. 1365-8.

4. Hershko, A., et al., *Proposed role of ATP in protein breakdown: conjugation of protein with multiple chains of the polypeptide of ATP-dependent proteolysis.* Proc Natl Acad Sci U S A, 1980. **77**(4): p. 1783-6.

5. Wilkinson, K.D., M.K. Urban, and A.L. Haas, *Ubiquitin is the ATP-dependent proteolysis factor I of rabbit reticulocytes.* J Biol Chem, 1980. **255**(16): p. 7529-32.

6. Ciechanover, A., et al., *Activation of the heat-stable polypeptide of the ATP-dependent proteolytic system.* Proc Natl Acad Sci U S A, 1981. **78**(2): p. 761-5.

7. Ciechanover, A., et al., *"Covalent affinity" purification of ubiquitin-activating enzyme.* J Biol Chem, 1982. **257**(5): p. 2537-42.

8. Hershko, A., et al., *Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown.* J Biol Chem, 1983. **258**(13): p. 8206-14.

9. Chau, V., et al., *A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein.* Science, 1989. **243**(4898): p. 1576-83.

10. Osley, M.A., *H2B ubiquitylation: the end is in sight.* Biochim Biophys Acta,

2004. **1677**(1-3): p. 74-8.

11. Metcalf, J.L., et al., *K63-ubiquitylation of VHL by SOCS1 mediates DNA double-strand break repair.* Oncogene, 2014. **33**(8): p. 1055-65.

12. Spence, J., et al., *Cell cycle-regulated modification of the ribosome by a variant multiubiquitin chain.* Cell, 2000. **102**(1): p. 67-76.

13. Duncan, L.M., et al., *Lysine-63-linked ubiquitination is required for endolysosomal degradation of class I molecules.* Embo j, 2006. **25**(8): p. 1635-45.

14. Deng, L., et al., *Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain.* Cell, 2000. **103**(2): p. 351-61.

15. Xu, P., et al., *Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation.* Cell, 2009. **137**(1): p. 133-45.

16. Yuan, W.C., et al., *K33-Linked Polyubiquitination of Coronin 7 by Cul3-KLHL20 Ubiquitin E3 Ligase Regulates Protein Trafficking.* Mol Cell, 2014. **54**(4): p. 586-600.

17. Kornberg, R.D., *Eukaryotic transcriptional control.* Trends Cell Biol, 1999. **9**(12): p. M46-9.

18. Sims, R.J., 3rd, S.S. Mandal, and D. Reinberg, *Recent highlights of RNA-polymerase-II-mediated transcription.* Curr Opin Cell Biol, 2004. **16**(3): p. 263-71.

19. Koleske, A.J. and R.A. Young, *An RNA polymerase II holoenzyme responsive to activators.* Nature, 1994. **368**(6470): p. 466-9.

20. Myer, V.E. and R.A. Young, *RNA Polymerase II Holoenzymes and Subcomplexes.* Journal of Biological Chemistry, 1998. **273**(43): p. 27757-27760.

21. Brickey, W.J. and A.L. Greenleaf, *Functional studies of the carboxy-terminal repeat domain of Drosophila RNA polymerase II in vivo.* Genetics, 1995. **140**(2):

p. 599-613.

22. Orphanides, G. and D. Reinberg, *A unified theory of gene expression.* Cell, 2002. **108**(4): p. 439-51.

23. Peng, J., et al., *A proteomics approach to understanding protein ubiquitination.* Nat Biotechnol, 2003. **21**(8): p. 921-6.

24. Woudstra, E.C., et al., *A Rad26-Def1 complex coordinates repair and RNA pol II proteolysis in response to DNA damage.* Nature, 2002. **415**(6874): p. 929-33.

25. Anindya, R., O. Aygun, and J.Q. Svejstrup, *Damage-induced ubiquitylation of human RNA polymerase II by the ubiquitin ligase Nedd4, but not Cockayne syndrome proteins or BRCA1.* Mol Cell, 2007. **28**(3): p. 386-97.

26. Wilson, M.D., M. Harreman, and J.Q. Svejstrup, *Ubiquitylation and degradation of elongating RNA polymerase II: the last resort.* Biochim Biophys Acta, 2013. **1829**(1): p. 151-7.

27. Huibregtse, J.M., J.C. Yang, and S.L. Beaudenon, *The large subunit of RNA polymerase II is a substrate of the Rsp5 ubiquitin-protein ligase.* Proc Natl Acad Sci U S A, 1997. **94**(8): p. 3656-61.

28. Somesh, B.P., et al., *Multiple mechanisms confining RNA polymerase II ubiquitylation to polymerases undergoing transcriptional arrest.* Cell, 2005. **121**(6): p. 913-23.

29. Somesh, B.P., et al., *Communication between distant sites in RNA polymerase II through ubiquitylation factors and the polymerase CTD.* Cell, 2007. **129**(1): p. 57-68.

30. Harreman, M., et al., *Distinct ubiquitin ligases act sequentially for RNA polymerase II polyubiquitylation.* Proc Natl Acad Sci U S A, 2009. **106**(49): p. 20705-10.

31. Ulrich, H.D. and H. Walden, *Ubiquitin signalling in DNA replication and repair.* Nat Rev Mol Cell Biol, 2010. **11**(7): p. 479-89.

32. Kvint, K., et al., *Reversal of RNA polymerase II ubiquitylation by the ubiquitin protease Ubp3.* Mol Cell, 2008. **30**(4): p. 498-506.

33. Ha, T., et al., *Probing the interaction between two single molecules: fluorescence resonance energy transfer between a single donor and a single acceptor.* Proc Natl Acad Sci U S A, 1996. **93**(13): p. 6264-8.

34. Deniz, A.A., et al., *Single-pair fluorescence resonance energy transfer on freely diffusing molecules: observation of Forster distance dependence and subpopulations.* Proc Natl Acad Sci U S A, 1999. **96**(7): p. 3670-5.

35. Kao, M.W., et al., *Strategy for efficient site-specific FRET-dye labeling of ubiquitin.* Bioconjug Chem, 2008. **19**(6): p. 1124-6.

36. Chevallet, M., S. Luche, and T. Rabilloud, *Silver staining of proteins in polyacrylamide gels.* Nat Protoc, 2006. **1**(4): p. 1852-8.

37. Hirel, P.H., et al., *Extent of N-terminal methionine excision from Escherichia coli proteins is governed by the side-chain length of the penultimate amino acid.* Proc Natl Acad Sci U S A, 1989. **86**(21): p. 8247-51.

38. Giglione, C., A. Boularot, and T. Meinnel, *Protein N-terminal methionine excision.* Cell Mol Life Sci, 2004. **61**(12): p. 1455-74.

39. Panchuk-Voloshina, N., et al., *Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates.* J Histochem Cytochem, 1999. **47**(9): p. 1179-88.

40. Hoeller, D., et al., *E3-independent monoubiquitination of ubiquitin-binding proteins.* Mol Cell, 2007. **26**(6): p. 891-8.

41. Kao, A.L., et al., *E3-independent ubiquitination of AtMAPR/MSBP1.* Phytochemistry, 2012. **78**: p. 7-19.

42. Chang, J.W., et al., *Hybrid electron microscopy-FRET imaging localizes the dynamical C-terminus of Tfg2 in RNA polymerase II-TFIIF with nanometer precision.* J Struct Biol, 2013. **184**(1): p. 52-62.