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# 泛素化在細胞自噬分解酵母菌胞內蛋白質中所扮演的角色

# Role of ubiquitylation in autophagic degradation of cytosolic soluble protein in *Saccharomyces cerevisiae*

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# 摘要

泛素是種小而保守的蛋白質，普遍的被用來標定在真核生物中將要被蛋白脢體分解的異常構型蛋白質。細胞自噬則是真核生物中另一條降解蛋白質的路徑；透過由雙層膜包裹的細胞質所形成的自噬小體與細胞中的液胞或溶小體進行融合，細胞能有效的分解胞器以及蛋白質，以獲得養分應付充滿壓力的環境。已知在哺乳動物細胞中，被泛素所標定的蛋白質除了透過蛋白脢體分解外，也會形成蛋白質聚集體，堆積在細胞質當中，而這些蛋白質聚集體，已被證實能選擇性的透過細胞自噬所清除。這次研究，我們發現在酵母菌中泛素修飾並不會促進蛋白質形成聚集體，且泛素化的修飾不但不會促進蛋白質透過細胞自噬分解，反而扮演著抑制性的角色。我們同時發現，泛素的一個已知的突變能破壞泛素與大部分泛素結合區的交互作用，而這種泛素突變也喪失抑制細胞自噬分解蛋白質的作用。我們認為，透過泛素與某種未知蛋白質的交互作用，阻礙了泛素化蛋白質被自噬小體包裹的過程。這個發現，是泛素抑制細胞自噬分解作用的首件案例。

## Abstract

Ubiquitin is a small, conserved molecule among eukaryotes that serves as a tag for the breakdown of misfolded-proteins by the 26s proteasome in eukaryotes. Autophagy is another protein turnover process, which sequesters cytoplasm into double membrane vesicles, called autophagosomes. Subsequent fusion with the vacuole/lysosome mediates breakdown of proteins or organelles in eukaryotes facing stressful environments. In mammalian cells, ubiquitylated protein aggregates in cytosol associated with neural degeneration diseases were shown to be specific substrates for autophagic degradation. However, in this study, we showed that the ubiquitin modification does not trigger the formation of protein aggregates in *saccharomyces cerevisiae*. Moreover, we found that ubiquitylation impedes, instead promotes, the degradation of cytosolic proteins by starvation-induced autophagy in yeast. We also identified an ubiquitin mutant, which was previously shown defective in interacting with most known ubiquitin-binding domains (UBD), lost the delay on autophagic degradation of cytosolic proteins. We propose that the interaction of ubiquitin with an unknown factor prevents sequestration of ubiquitylated cytosolic soluble proteins into autophagosomes for degradation. This is the first report indicating that ubiquitylation of cargo proteins hinder their autophagic degradation.

# Introduction

Protein turnover plays a critical role in cell survival. In eukaryotic cells, proteins are either synthesized on ribosomes soluble in cytoplasm or attached to the endoplasmic reticulum(ER), where they are properly modified and fold into active conformation by ER-resident modifying enzymes and chaperones. Misfolded proteins are destined for degradation, which protects cells from cytotoxicity and releases free amino acids for further protein synthesis. Two major pathways were found in eukaryotes to breakdown intracellular proteins. First is the ubiquitin-proteasome system (UPS) pathway, in which proteins are typically modified with ubiquitins and degraded via the proteasome (Johnson *et al.*, 1992; Johnson *et al.*, 1995; Pickart, 2001). The other is the massive degradation through autophagy, in which a portion of cytoplasm was sequestered and degraded by the vacuole in yeast or lysosomes in mammalian cells (Tsukada and Ohsumi, 1993; Levine and Klionsky, 2004). Both pathways serve to protect cells from cytotoxocity of misfolded proteins.

## Overview of the UPS pathway

Ubiquitin is a highly conserved small protein that expresses ubiquitously and serves multiple functions in eukaryotes (Hershko *et al.*, 2000; Chen and Sun, 2009). It was first described to participate in proteolysis by labeling substrate proteins to facilitate

their recognition by and degradation through the 26S proteasome. This proteolysis pathway is termed the UPS system (Hough *et al.*, 1986; Johnson *et al.*, 1992; Johnson *et al.*, 1995; Pickart, 2001). Ubiquitin was later shown to play multiple roles in regulation of multiple cellular processes, including endocytosis, signal transduction in immune system, and DNA repair (Chen and Sun, 2009). Conjugation of ubiquitin to target proteins is a complex process. Ubiquitin is first synthesized as an inactive precursor and subsequently processed by deubiquitinatin enzymes (DUBs) into the mature form that exposes its $76^{th}$ glycine residue at the C-terminus as the active site for the conjugation reaction. Next, for conjugation of ubiquitin to substrate proteins, ubiquitin must be sequentially processed by E1, E2 and E3 enzymes. First, ubiquitin is adenylated, which is catalyzed by the E1 enzymes using ATP as a substrate. The newly formed high energy bond between ubiquitin and AMP is soon attacked by a cystein residue at the active site of the E1 enzymes, resulting in the formation of a thioester bond between ubiquitin and the E1 enzymes. Next, Ubiquitin is passed to a cystein residue at the active site of the E2 enzymes, then finally to substrate proteins forming a isopeptide stable linkage bwtween the $76^{th}$ glycine of ubiquitin molecule and the ε-amino group of a lysine residue on target proteins. This latter reaction is catalyzed by the E3 enzymes (Kerscher *et al.*, 2006). Based on different structures discovered in the catalytic domains of E3 enzymes, the E3 enzymes could be further

divided into the HECT class and the RING class (Kerscher *et al.*, 2006). The HECT E3 enzymes, harboring an active cystein residue on the catalytic domain, form a thioester bond with ubiquitin molecule passing from E2 enzymes before conjugation of ubiquitin to target proteins. On the other hand, the RING E3 enzymes, which have no cystein group on the catalytic domain, harbor a RING motif that coordinates a pair of zinc ions. The RING E3 enzymes serve, at least in part, as scaffolds that bind thioester bond-linked ubiquitin E2 and target proteins, facilitating the direct transfer of ubiquitin from the E2 enzymes to target proteins. It was also shown that the RING E3 enzymes trigger subtle conformational changes in the bound E2 enzymes, stimulating ubiquitin release from the E2 enzymes and transfer to substrate proteins.

Due to the presence of seven lysine residues, ubiquitin could itself serves as a substrate for ubiquitylation, leading to the formation of polyubiquitin chian-modification on substrate proteins. The polyubiquitin chains, which are recognized by ubiquitin-interacting proteins for downstream signaling, serve multiple functions (Kerscher *et al.*, 2006; Ikeda and Dikic, 2008). Multiple topologies of polyubiquitin chains with different lysine linkage have been found (Ikeda and Dikic, 2008). It is known that the Lys48-linked polyubiquitin chains target substrates to the proteasomal degradation, while the Lys63-linked polyubiquitin chains label membrane proteins for endocytosis (Ikeda and Dikic, 2008). Although quite few,

some studies have also reported that cystein residues or the N-terminus of substrate proteins are also possible sites for ubiquitylation (Ciechanover and Ben-Saadon, 2004; Cadwell and Coscoy, 2005).

The 26S proteasome is a complex of ATP-dependent proteases that is capable of recognition and degradation of ubiquitylated proteins in eukaryotes (Coux *et al.*, 1996; Baumeister *et al.*, 1998). The 26S proteasome consists of two parts, the regulatory 19S particle and the hydrolytic 20S proteasome. The 20S proteasome is formed with two inner β-rings assembled as a proteolytic chamber and one outer α-ring on each side, which serves as the gate for the entry of substrates into the proteolytic chamber. The 19S regulatory particle could also be divided into two subcomplexes, the base and the lid. While the base subcomplex harbors the ATPase activity and the ubiquitin-binding domain, which is response for the denaturing of substrates as well as the opening of α-rings, function of the lid subcomplex is not fully known yet, which is only shows to have de-ubiquitylation activity so far (Murata *et al.*, 2009).

As earlier mentioned, proteins that are modified with Lys48 polyubiquitin chains will be degraded by proteasomes. The Lys48 polyubiquitin chains are first recognized by the 19S regulatory particle, which also denatures substrates and opens the α-rings of the 20S proteasome by the activity of the ATPase. After removing the poly-ubiquitin chains from substrates by the lid, substrates are sent into the proteolytic

chamber of the 20S proteasome for degradation and releasing free amino acids for reuse (Murata *et al.*, 2009).

**Overview of autophagy**

Autophagy, a conserved process for the degradation of long-lived proteins and organelles in eukaryotes, is a major pathway for protein turnover in response to environmental stresses (Tsukada and Ohsumi, 1993; Levine and Klionsky, 2004). The operation of autophagy could be dissected to several stages. First is the induction of autophagy, which is triggered by the deprivation of nitrogen source in yeast(Levine and Klionsky, 2004); under nutritional condition, Atg1 and Atg13 are hyper-phosphorylated through the action of the mTOR complex and dissociated from each other, leading to the conduction of a pathway, called the cytoplasm-to-vacuole targeting (Cvt) pathway, which is similar to autophagy but with a much lower transport capacity (Jung *et al.*, 2010; Kamada *et al.*, 2010). The Cvt pathway, which is now regarded as a specific type of selective autopahgy (Klionsky *et al.*, 1992; Scott *et al.*, 1997; Lynch-Day and Klionsky, 2010), is activated constitutively under nutritional condition for delivery of some vacuolar enzymes. Precursors of the vacuolar aminopeptidase Ⅰ (prApe1) are synthesized in cytosol and self-oligomerize into an electron dense structure, the Cvt complex. In addition to prApe1, the Cvt complex at

least contains another vacuolar enzymes, α-mannosidase. Atg19, the specific receptor for prApe1 and α-mannosidase transport, mediates the interaction between the Cvt complex and Atg11, which triggers the formation of Cvt vesicles leading to the transport of prApe1 and α-mannosidase to the vacuole. While as under nutrition limitation conditions, the activity of mTOR is inhibited, leading to the partial de-phosphorylation and subsequent association of Atg1, Atg13, and their associated proteins to form multi-protein complex, which stimulates the progression of autophagy (Jung *et al.*, 2010; Kamada *et al.*, 2010).

Once autophagy is activated, a double membrane sac structure termed isolation membrane starts to assemble, elongate, and expand from a specific cytosolic location, the pre-autophagosomal structure or the phagophore assembly site (PAS). The edge of the membrane structure eventually fuses to form a double membrane vesicle, called autophagosome, which sequesters a portion of cytoplasm including cytosolic proteins and organelles. The elongation of the isolation membrane into autophagosomes depends on two conjugation systems made of two ubiquitin-like proteins, Atg8 and Atg12 (Ohsumi, 2001). Atg12 is activated by the E1 like enzymes Atg7, subsequently transferred to the E2 like enzyme Atg10, and finally irreversibly conjugated to Atg5. Atg12-Atg5 conjugate binds with Atg16 to form a ~350 kD multimeric protein complex. Atg8, which is synthesized as a precursor form, is processed by Atg4

protease, sequentially interacted with Atg7 andATg13, and eventually conjugated to phosphatidylethanolamine (PE). It was proposed that the Atg12-Atg5-Atg16 complex acts as the E3 enzyme for the Atg8 conjugation system (Hanada *et al.*, 2007). The role of the Atg8-PE conjugate in autophagy is not clear yet, but it was proposed to be involved in the regulation of membrane tethering and hemifusion (Nakatogawa *et al.*, 2007). Other studies also show that Atg8 controls the expansion of isolation membrane and determines the size of autophagosomes (Xie *et al.*, 2008).

The membrane source of autophagosomes is an issue still under debate. PAS, the structure regarded as the site of vesicle formation during autophagy (Suzuki *et al.*, 2001; Kim *et al.*, 2002), has high concentration of phosphatidylinositol 3-phosphate (PI3P) required for autophagy progress that is synthesized in site by the phosphatidylinositol (PtdIns) 3-kinase complex I, which includes the PtdIns-3-kinase Vps34 and its regulatory components such as Vps15, Vps30/Atg6, and Atg14 (Kihara *et al.*, 2001). The transmembrane protein Atg9, which cycles between the PAS and peripheral structures, appears to regulate vesicle formation likely by, providing lipid components for the expansion of isolation membrane. Recently, some Rab GTPase and their guanine-nucleotide-exchange factors (GEFs) involved in the functions of the Golgi complex or traffic from ER to the Golgi complex are shown to contribute to the vesicle formation step during autophagy (Reggiori *et al.*, 2004; Geng *et al.*, 2010;

Lynch-Day *et al.*, 2010; van der Vaart *et al.*, 2010; Yen *et al.*, 2010). Moreover, mitochondria were shown to be one membrane source during autopahgy in mammalian cells (Hailey *et al.*, 2010). However, it is worth to mention that whether defect in autophagy is due to the block in specific transport steps that directly contributes to autophagosome formation or the impairment of membrane flow in the early secretory pathway that indirectly affects autophagy is not always easy to distinguish..

Autophagosomes sequester cytosolic proteins or organelles and transport cargoes from cytosol to vacuoles for degradation (Levine and Klionsky, 2004; Suzuki and Ohsumi, 2007; Xie *et al.*, 2008). In mammalian cells, autophagosomes were shown to move in a microtubule-dependent way (Monastyrska *et al.*, 2009). One of the homologues of Atg8 in mammalian cells termed microtubule-associated protein 1 light chain 3, MAP1-LC3 or simply LC3 in short, anchors autophagosomes to dynein, which carries autophagosomes along the microtubule from plus end to minus end. In addition, LC3 could associate with microtubule directly or indirectly via interaction with MAP1A and MAP1B, increasing the affinity between microtubule and autophagosomes that facilitates autophagosomes trafficking. Interestingly, although Atg8 is homologous to LC3 (28% identity to rat MAP1-LC3) (Lang *et al.*, 1998; Reggiori and Klionsky, 2002), microtubules are not required for bulk autophagy in

yeast. Instead, actin filaments were shown to be required for the Atg11-dependent selective types of autopahgy, but not nonspecific autophagy in yeast (Reggiori *et al.*, 2005).

Autophagosomes in the end fuse with the vacuole for degradation of cargoes. To target autophagosomes properly to the vacuole in yeast, the required molecular machinery includes the SNARE proteins Vam3, Vam7, Vti1, and Ykt6, the NSF, SNAP, and GDI homologs Sec17, Sec18, and Sec19, the Rab protein Ypt7, and members of the class C Vps/HOPS complex (Wang and Klionsky, 2003). After vacuole docking, the outer membrane of autophagosomes fuses with the vacuolar membrane, releasing inner vesicles, termed autophagic bodies, into the vacuolar lumen. The limlting membrane of autophagic bodies would be lysed by the vacuolar hydrolase Pep4, a process depends on the acidic environment of vacuolar lumen, releasing the transported cytoplasmic components for degradation (Kim *et al.*, 2001). Finally, the efflux of amino acids resulting from autophagic degradation to the cytosol is mediated by Atg22, Avt3, and Avt4, which are partially redundant vacuolar effluxers to recycle amino acids. The recycled amino acids help maintain energy homeostasis and protein synthesis under starvation (Yang *et al.*, 2006).

**The selectivity of autophagy**

Starvation-induced autophagy was first regarded as a non-selective process. However, increasing evidences have shown that cargo-recognition exists in the autophagic sequestration stages, in which some proteins and orga12nelles are shown to be degraded by autophagic process in a selective way (Kraft *et al.*, 2009b). For example, the Cvt pathway that is activated constitutively under nutrient rich condition is a selective pathway in which Atg19 not only recognizes the self-oligomerized prApe1 as cargo but also interacts with Atg11 to stimulate the sequestration of prApe1 into autophagosomes for delivery to the vacuole (Chang and Huang, 2007). Ribophagy, the selective degradation of ribosomes under starvation, is a process unique in the dependence of the ubiquitin protease Ubp3/Bre5 (Kraft *et al.*, 2009a). Mitophagy, the process autophagy that cells degrade excessive mitochondria under starvation, are shown to be a selective autophagy that depends on the interaction between Atg11 and a mitochondrial outer-membrane protein, Atg32 (Kanki and Klionsky, 2008; Kanki *et al.*, 2009; Okamoto *et al.*, 2009). Pexophagy, the degradation pathway for the turnover of excessive peroxisomes selectively under starvation, was shown to be an Atg11-dependent pathway, too (Bellu and Kiel, 2003; Sakai *et al.*, 2006). Cytosolic proteins are also shown to be degraded selectively by autophagy in mammalian cells, a process which is known to be mediated by ubiquitin-modification (Kirkin *et al.*, 2009b). Protein misfolding, which accompanies normal protein synthesis, could be

exacerbated by environmental stresses, such as oxidative stresses, starvation, and heat shock (Goldberg, 2003). Misfolded proteins are recognized by chaperones, promoting protein refolding or stimulating conjugation by ubiquitin for proteasomal degradation (Imai *et al.*, 2002; Kirkin *et al.*, 2009b). These proteins, if left unresolved by chaperone-dependent refolding or proteasomal degradation, will hamper cellular metabolism, leading to death of the cell (Kirkin *et al.*, 2009b). In response to the toxicity of misfolded proteins, it is reported that misfolded proteins from aggregates in a SQSTM1/p62 and NBR1 dependent process (Komatsu *et al.*, 2007; Seibenhener *et al.*, 2007; Kim *et al.*, 2008; Kirkin *et al.*, 2009a). During the process, misfolded proteins first are ubiquitylated, then the whole conjugate interacts with the UBA domain of SQSTM1/p62 and NBR1. The PB1 domain of SQSTM1/p62 and NBR1 mediates self-oligomerization, which facilitates the formation of aggregates of ubiquitylated misfolded proteins in the cytoplasm (Komatsu *et al.*, 2007; Seibenhener *et al.*, 2007; Kim *et al.*, 2008; Kirkin *et al.*, 2009a; Kirkin *et al.*, 2009b). While they are inaccessible to be cleaned by the 26S proteasomes, protein aggregates are selective substrates for autophagic degradation in mammalian cells (Kirkin *et al.*, 2009b). It is reported that the LIR motif of the p62/SQSTM1 and NBR1, which interacts with LC3, mediates protein aggregates to be recognized by the autophagy machinery, leading to the sequestration of protein aggregates into autophagosomes

and their transportation into lysosomes for degradation (Pankiv *et al.*, 2007; Seibenhener *et al.*, 2007).

As a consequence, ubiquitylation not only labels proteins for the UPS degradation but also marks cargo to be cleaned by autophagy in mammalian cells. Considering the evolution-conserved roles of ubiquitins and autophagy, we tried to verify the function of ubiquitin in autophagic degradation of cytosolic protein in yeast. To our surprise, we discovered that ubiquitylation hinder, rather than promote the autophagic degradation of cytosolic protein in yeast, which is opposite to that in mammalian cells. Moreover, we identified an ubiquitin mutant that not only prevents the degradation of ubiquitylated proteins by the 26S proteasomes but also eliminates the inhibitory effect of ubiquitylation on autophagic degradation. We proposed that ubiquitylated proteins might interact with an unknown ubiquitin-interacting protein in the cytosol that hinders the sequestration of cytosolic proteins into autophagosomes for transportation into vacuole and degradation. Our data clearly showed that ubiquitylation plays different roles in autophagic degradation of cytosolic proteins in yeast versus mammalian cells.

# Materials and Methods

## Strains and Media

The yeast *Saccharomyces cerevisiae* starins used in this study are liested in Table 1. Media used for growing yeast were listed below. SMD, 0.67% yeast nitrogen base without amino acids, 2% glucose, 0.5% casamino acid, supplemented with appropriate amino acids and vitamins; SD-N, 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose, supplemented with appropriate vitamins. Synthetic medium for yeast to grow overnight for proteasome inhibition were modified from a published report (Liu *et al.*, 2007), which consists of 0.17% yeast nitrogenous base without amino acids and ammonium sulfate, 0.1% proline, 2% glucose, supplemented with appropriate amino acids and vitamins.

## Plasmid construction

Plasmids used in this study are listed in Table 2.

To express free EGFP in yeast, the pRS414 (or pRS416)-$P_{Cu}$-EGFP-$T_{CYC1}$ plasmid with the *CUP1* gene promoter was constructed. Sequence encoding EGFP ORF was amplified by PCR. Two restriction enzymes sites, HindⅢ and ClaⅠ, were introduced to the ends of the PCR products. The resulting EGFP coding sequence was cloned into the same sites of the pRS414-$P_{Cu}$-$T_{CYC1}$ vector.

To express $Ub^{G76V}$-EGFPF in yeast, the pRS414 (or pRS416)-$P_{Cu}$-$Ub^{G76V}$-EGFP-$T_{CYC1}$ plasmid with the *CUP1* gene promoter was constructed. Sequence encoding ubiquitin ORF with the $76^{th}$ glycine residue replaced with valine was amplified by PCR. Two restriction enzyme sites, Spe Ⅰ and Xma Ⅰ, were introduced to the ends of the PCR products. The resulting ubiquitin coding sequence was cloned into the same sites of the pRS416-c-EGFP-$T_{CYC1}$ vector (previously constructed in our lab). Subsequently, the coding sequence of $Ub^{G76V}$-EGFP was amplified by PCR with the introduction of a Spe Ⅰ and a Cla Ⅰ restriction sites to the ends of the products. The PCR products were then subcloned into the same restriction sites of the pRS414 (or pRS416)-$P_{Cu}$-$T_{CYC1}$ vector.

To express $Ub^{I44A,G76V}$-EGFPF in yeast, the pRS414 (or pRS416) -$P_{Cu}$-$Ub^{I44A,G76V}$-EGFP-$T_{CYC1}$ plasmid with the *CUP1* gene promoter was constructed. Sequence encoding ubiquitin ORF with the $44^{th}$ isoleucine and the $76^{th}$ glycine residues replaced with alanine and valine, respectively, was amplified by PCR. Two restriction enzyme sites, Spe Ⅰ and Xma Ⅰ, were introduced to the ends of the PCR products. The resulting ubiquitin coding sequence was cloned into the same sites of the pRS416-c-EGFP-$T_{CYC1}$ vector (previously constructed in our lab). Subsequently, the coding sequence of $Ub^{I44A,G76V}$-EGFP was amplified by PCR with the introduction of a Spe Ⅰ and a Cla Ⅰ restriction sites to the ends of the sequence. The PCR products

were then subcloned into the same restriction sites of the pRS414 (or pRS416)-$P_{Cu}$-$T_{CYC1}$ vector.

To express Pgk1-EGFP in yeast, the pRS416-$P_{Cu}$Pgk1-EGFP-$T_{CYC1}$ plasmid with the *CUP1* gene promoter was constructed. Sequence encoding *PGK1* ORF was amplified by PCR. Two restriction sites, BamH Ⅰ and HindⅢ, were introduced to the ends of the PCR products. The resulting *PGK1* coding sequence was cloned into the same sites of the pRS416-$P_{Cu}$-EGFP-$T_{CYC1}$ plasmid.

**Western blot analysis**

Culture aliquots harvested for western blot analysis were first precipitated by 10% Trichloroacetic acid (TCA) for at least one hour and then washed with acetone three times. After discard of the acetone, air-dried pellets were lysed in sample buffer (1.25% SDS, 125mM Tris-Cl pH7.5, 12.5% glycerol, 0.0625% bromophenol blue and 1% β-mercaptoethanol) by vortex with silica beads (BioSpec Products, Inc., Bartlesville, OK) for 5 minutes and then boiled at 95℃ for another 5 minutes. Centrifugation was performed to spin down cell debris. Samples were subjected to SDS-PAGE, and then transferred to PVDF (polyvinylidene fluoride) membrane for antiserum analysis. Bands of interested proteins were quantified and further analyzed with Student's T-test.

**Fluorescent microscopy analysis**

For the analysis of cells under nutrient rich condition, culture grown to mid-log phase was harvested. Cells were washed by $ddH_2O$ once and then resuspended in $ddH_2O$ to be smeared on slides for fluorescent microscopy analysis. For the analysis of cells under starvation condition, cells of mid-log phase were transferred to SD-N for 4hr cultivation. Cells were then harvested and treated as samples of nutrient rich condition for fluorescent microscopy analysis.

**Inhibition of proteasome activity with the treatment of MG132**

Protocol for proteasome inhibition in yeast was modified from a published report (Liu *et al.*, 2007). Cells for proteasome inhibition were grown in synthetic medium overnight, and then diluted into fresh synthetic medium to 0.1 $OD_{600}$ for 10hr of cultivation till concentration at $OD_{600}$ = 0.6-0.8. 13.5 $OD_{600}$ of cells were then harvested and reintroduced into fresh synthetic medium containing 0.003% SDS for 3.5hr of cultivation. Next, 15 $OD_{600}$ of cells were harvested and shifted into SD-N containing 0.003% SDS and 75μM MG132 (Sigma-Aldrich, Inc.) for starvation.

For inhibition of protein synthesis and proteasome activity under starvation, cells were first treated as previous described and then shifted to SD-N containing 0.003% SDS, 75μM MG132, and 10μμg/ml Cycloheximide (Sigma-Aldrich, Inc.).

# Results

## Ubiquitylation triggers no protein aggregation in yeast

Ubiquitiylated red fluorescent protein (RFP) was used as a model to test the degradation of ubiquitylated protein by autophagy in mammalian cell (Kim *et al.*, 2008). As the N-terminus of RFP is modified with a single ubiquitin molecule in which the $Gly^{76}$ was changed into Val (G76V) to prevent the cleavage of the ubiquitin molecule from the ubiquitylated RFP by deubiquitinating enzymes (DUBs), it is shown that the pattern of the red fluorescence signal turned from the diffusive distribution to protein aggregates in cytosol by a p62 dependent pathway. Moreover, protein aggregates showing signal of red fluorescence were highly-colocalized with lysosomes, indicating that these protein aggregates were sequestered into autophagosomes and subsequently degraded in lysosomes.

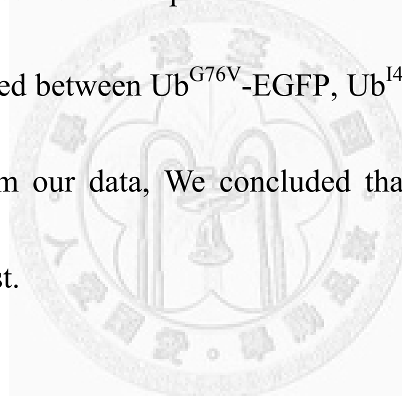
In order to clarify the role of ubiquitylation in the degradation of cytosolic protein via autophagy in budding yeast *Saccharomyces cerevisiae*, we expressed ubiquitin fusion degradation (UFD)-targeted EGFP, which was first reported as a powerful tool for elucidation of the UPS system(Dantuma *et al.*, 2000), for the analysis of protein aggregation and their degradation via autophagy (Figure 1A). Two N-terminus ubiquitylated EGFP were expressed. $Ub^{G76V}$-EGFP is a fusion protein that the EGFP is modified with an ubiquitin moiety habors a G76V mutation to prevent the cleavage

of ubiquitin from the fusion protein. $Ub^{I44A,G76V}$-EGFP is another fusion protein, in which the EGFP is modified with an ubiquitin moiety that the $Ile^{44}$ was mutated to Ala (I44A) in addition to the G76V mutation. From previous review, we know that $Ile^{44}$ is the key epitope in most interactions between ubiquitin-binding proteins and ubiquitin (Chen and Sun, 2009), for example, p62, which promotes autophagic degradation of ubiquitylated proteins in mammalin cells, and Rad23 and Dsk2, which transport ubiquitylated proteins to proteasomal degradation. We replaced $Ile^{44}$ with Ala to prevent the interaction between ubiquitin and ubiquitin-binding proteins. Our data showed that the I44A mutation blocked the proteasomal degradation prominently (Figure 3), suggesting that the I44A mutation did destroy the interaction between ubiquitin and ubiquitin-binding proteins effectively (see later paragraph for more description).

We decided to analyze the distribution of fluorescent signal in cells by fluorescent microscopy. We found that the distribution of free EGFP in WT cells under nutrient rich condition dispersed in cytosol (Figure 1B), which was similar to that of the free RFP in mammalian cells (Kim *et al.*, 2008). The vacuole showed up clearly with the absent of EGFP signal, showing that free EGFP was accumulated in cytosol instead of transported to the vacuole, where the massive degradation of proteins and organelles occurred.

To our surprise, the distribution of $Ub^{G76V}$-EGFP showed complete different phenotype to that of the ubiquitylated RFP in mammalian cells. $Ub^{G76V}$-EGFP dispersed throughout cytosol instead of forming protein aggregates (Figure 1B). The vacuole was also absent of fluorescent signal, showing that the $Ub^{G76V}$-EGFP was not the target of autophagy under nutrient rich condition. This result is different from that of the ubiquitylated RFP in mammalian cells, which forms protein aggregates in cytosol that is highly co-localized with lysosomes. The $Ub^{I44A,G76V}$-EGFP also distributed diffusively throughout cytosol with the lack of fluorescent signal in the vacuole (Figure 1B). $Ub^{G76V}$-EGFP, $Ub^{I44A,G76V}$-EGFP, and free EGFP were also expressed in *atg1*Δ and *pep4*Δ cells as autophagy-defective strains. Atg1 is a serine/threonine kinase required in the induction of autophagy and the formation of autophagosomes as previously described (Jung *et al.*, 2010; Kamada *et al.*, 2010). Pep4 is a vacuolar hydrolase charging for the breakdown of autophagic bodies releasing into the lumen of the vacuole (Levine and Klionsky, 2004). Autophagy is completely blocked in *atg1*Δ cells but remains its autophagosomes-transportation activity in *pep4*Δ strain. The distribution of $Ub^{G76V}$-EGFP, $Ub^{I44A,G76V}$-EGFP, and free EGFP expressed in *atg1*Δ and *pep4*Δ were similar to that of WT cells, indicating that ubiquitylation triggers no protein aggregation and its autophagic degradation in yeast under nutrient rich condition. However, it is possible that the strong signal of

diffusive fluorescent signal obscured the presence of protein aggregates in cytosol. We decided to treat cells with 0.1% Triton X-100, leading to the leakage of soluble cytosolic proteins from cytosol to outer environment. As a result, the structure of protein aggregates, if any, could be seen clearly. Mid-log phase WT cells were first treated with PBS solution containing 0.1% Triton X-100 for 30 minutes, then cells were transfer into PBS solution containing 4% paraformaldehyde for 40 minutes for fixation. Surprisingly, both three groups displayed similar fluorescent puncta in cytosol. Although how these florescent puncta were formed in cytosol is not clear, no difference could be observed between $Ub^{G76V}$-EGFP, $Ub^{I44A,G76V}$-EGFP, or free EGFP expressing WT cells. From our data, We concluded that ubiquitylation triggers no protein aggregation in yeast.



**Ubiquitylated EGFP is a substrate for autophagic degradation**

Non-selective autophagy is activated under starvation, in which cytosolic proteins are sequestered into autophagosomes, and then delivered to the vacuole for degradation (Levine and Klionsky, 2004). We decided to study the localization of ubiquitylated proteins in cells under starvation by fluorescent microscopy analysis. Cells grown to the mid-log phase were transferred to the nitrogen-starvation medium (SD-N) for 4hr of starvation before observation. Our data showed that $Ub^{G76V}$-EGFP and

Ub$^{I44A,G76V}$-EGFP, were the target of starvation-induced autophagy in WT cells, which were similar to that of free EGFP (Figure 2). Starvation stimulated the translocation of Ub$^{G76V}$-EGFP and Ub$^{I44A,G76V}$-EGFP from cytosol to the vacuole in WT cells, increasing the fluorescent signal in the vacuole. In *atg1*Δ cells which are lack of autophagy activity, starvation stimulated no transportation of Ub$^{G76V}$-EGFP, Ub$^{I44A,G76V}$-EGFP, and EGFP from cytosol to the vacuole (Figure 2). In *pep4*Δ cells expressing Ub$^{G76V}$-EGFP, Ub$^{I44A,G76V}$-EGFP or free EGFP, fluoresecent signal represented crowded autophagic bodies were detected clearly in the vacuole of *pep4*Δ cells after 4hr of starvation (Figure 2). This is the direct evidence showing that Ub$^{G76V}$-EGFP and Ub$^{I44A,G76V}$-EGFP were sequestered into autophagosomes as cargoes to delivered to the vacuole under starvation.

**Proteasomes, but not autophagy, degrade most ubiqutylated EGFP under starvation**

Our images indicated that Ub$^{G76V}$-EGFP and Ub$^{I44A,G76V}$-EGFP were transported to the vacuole under starvation by an autophagy-depedent pathway, we decided to analyze the different efficiency in their autophagic degradation by western blot analysis. Since EGFP has a long half-life in the vacuole, ubiquitylated EGFP transported to the vacuole was cleaved by hydrolases, releasing free EGFP in the

vacuolar lumen and can be detected with western blot by using anti-GFP antibody easily. In comparison, ubiquitylated EGFP targeted to proteasomal degradation would be digested into pieces, remaining no free EGFP. WT, *atg1*Δ and *pep4*Δ strains expressing $Ub^{G76V}$-EGFP or $Ub^{I44A,G76V}$-EGFP were grown in SMD medium till the mid-log phase, and then transferred into SD-N medium for starvation treatment. Culture aliquots were collected at 0hr, 1hr, 2hr, 4hr and 6hr during a period of 6hr starvation and precipitated with 10%TCA before western blot analysis. Using Pgk1 as the loading control, the decrease of fusion proteins and the augmentation of free EGFP allow us to monitor the difference between the degradation of $Ub^{G76V}$-EGFP and $Ub^{I44A,G76V}$-EGFP under starvation.

$Ub^{G76V}$-EGFP decreased rapidly in WT strain as cells were shifted to the SD-N medium (Figure 3A). At the 2hr of starvation, a band corresponding to the 25kDa of free EGFP was observed and maintained in similar levels at the following time points (Figure 3A). Interestingly, the degradation of $Ub^{I44A,G76V}$-EGFP in WT cells under starvation showed different pattern to that of $Ub^{G76V}$-EGFP. $Ub^{I44A,G76V}$-EGFP was maintained in similar levels under starvation. Free EGFP which could be detected even at the very beginning of starvation was accumulated prominently (Figure 3A), indicating that $Ub^{I44A,G76V}$-EGFP, which was blocked in proteasomal degradation, became a good substrate for autophagic degradation. Surprisingly, the degradation of

$Ub^{G76V}$-EGFP in *atg1*Δ cells was even faster than that in WT cells with no generation of free EGFP (Figure 3B), indicating that $Ub^{G76V}$-EGFP was degraded by proteasomes effectively in *atg1*Δ cells. However, $Ub^{I44A,G76V}$-EGFP expressing in *atg1*Δ cells was also maintained in similar levels under starvation accompanied no generation of free EGFP. On the other hand, $Ub^{G76V}$-EGFP expressing in *pep4*Δ cells was degraded in a much lower rate compared to that in WT and *atg1*Δ cells (Figure 3C), indicating that $Ub^{G76V}$-EGFP which was sequestered into autophagic bodies were accumulated in the vacuolar lumen of cells, keeping $Ub^{G76V}$-EGFP from the digestion of proteasomes. In addition, $Ub^{I44A,G76V}$-EGFP was maintained in relatively high levels throughout starvation (Figure 3C), demonstrating that $Ub^{I44A,G76V}$-EGFP, which was blocked in proteasomal degradation, was accumulated in autophagic bodies.

Two conclusions could be drawn from our data. First, the activity of proteasomes hinders the autophagic degradation of$Ub^{G76V}$-EGFP. Second, I44A is the exact mutant that destroys the interaction between ubiquitin and ubiquitin-interacting proteins in proteasomal degradation.

**Autophagic degradation of $Ub^{G76V}$-EGFP was slower than that of $Ub^{I44A,G76V}$-EGFP**

We decided to inhibit the activity of proteasomal degradation by treating cells with

MG132, short peptide aldehydes that block active sites of the 26S proteasome (Lee and Goldberg, 1996). Unfortunately, due to the multiple drug-resistance of yeast, the impermeability of the plasma membrane hampered the inhibition of MG132 treatment in WT cells. Increased drug permeability, like mutant strain (*erg6*Δ, *pdr5*Δ), is necessary for the treatment of MG132 (Liu *et al.*, 2007). Fortunately, several methods have been developed to inhibit proteasome activity in WT cells (Pannunzio *et al.*, 2004; Liu *et al.*, 2007). To inhibit proteasome activity, cells were grown in specific synthetic medium with proline as the nitrogen source for 10hr till the mid-log phase and then shifted to fresh medium containing 0.003% SDS for additional 3.5hr cultivation. For starvation, cells were transferred into SD-N medium containing 0.003% SDS and 75μM MG132 for 6hr of cultivation. Culture aliquots were collected and treated as previous described for western blot analysis.

The level of the $Ub^{G76V}$-EGFP was accumulated dramatically in WT cells under starvation, while the level of the $Ub^{I44A,G76V}$-EGFP was remained constantly in WT cells under starvation (Figure 4A). Quantification of these bands showed that the level of the $Ub^{G76V}$-EGFP was accumulated remarkably compared to that of $Ub^{I44A,G76V}$-EGFP, which was significantly higher after 2hr of starvation (Figure 4A). On the other hand, while the level of free EGFP from the cleavage of $Ub^{G76V}$-EGFP, which could be detected after 2hr of starvation, was increased gradually over times,

free EGFP from the cleavage of $Ub^{I44A,G76V}$-EGFP was detected after 1hr of starvation with a rapid accumulation rate (Figure 4A). This result suggested that ubiquitylated EGFP was synthesized and degraded in WT cells at the same time under starvation, thus higher level of $Ub^{G76V}$-EGFP indicated that $Ub^{G76V}$-EGFP was degraded in a much slower rate than that of $Ub^{I44A,G76V}$-EGFP by autophagic degradation. Moreover, the I44A mutation destroyed the delay of ubiquitylated EGFP in autophagic degradation, suggesting the participation of an unknown ubiquitin-interacting factor in this process.

In autophagy-defective strains, we found no difference between $Ub^{G76V}$-EGFP and $Ub^{I44A,G76V}$-EGFP in autophagic degradation. In *atg1*Δ cells, the level of fusion proteins remained constant under starvation with no difference between $Ub^{G76V}$-EGFP and $Ub^{I44A,G76V}$-EGFP (Figure 4B). Moreover, no free form EGFP was detected, showing that the transport of both $Ub^{G76V}$-EGFP and $Ub^{I44A,G76V}$-EGFP to the vacuole was blocked in *atg1*Δ cells. *pep4*Δ cells showed similar result to that of *atg1*Δ cells (Figure 4C), in which the levels of fusion proteins decreased slightly that nearly remained constant under starvation with no prominent difference between $Ub^{G76V}$-EGFP and $Ub^{I44A,G76V}$-EGFP. No free EGFP was detected either, indicating that no ubiquitylated EGFP was digested by vacuolar hydrolases in *pep4*Δ cells. These results suggested that the synthesis of new ubiquitylated EGFP was blocked in *atg1*Δ

and *pep4*Δ cells, probably by the shortage of the nutrient-supply in autophagy-defective cells. Ubiquitylated EGFP remains stable under starvation, suggesting that levels of ubiquitylated EGFP in WT cells were primarily due to the equilibrium between autophagic degradation and protein synthesis.

**Low level of ubiquitylated EGFP is not a substrate for autophagic degradation**

To eliminate the effect of new-synthesized protein in our analysis of ubiquitylated EGFP degradation under starvation, we decided to treat cells with Cycloheximide (CHX), an inhibitor of protein synthesis in eukaryotes produced by the bacterium *Streptomyces griseus*. Cells were treated and collected as previous described except the treatment of starvation, in which 10 μg/ml of CHX was added into the SD-N medium in addition to 0.003% SDS and 75μM MG132 for the inhibition of protein synthesis and proteasome activity in cells under starvation. Astonishingly, quantification from the western blot analysis showed that the level of $Ub^{G76V}$-EGFP processed a gradual decrease over starvation in WT cells with no appearance of free EGFP. In comparison, $Ub^{I44A,G76V}$-EGFP was maintained in similar levels over starvation with the gradual increase of free EGFP, indicating that $Ub^{I44A,G76V}$-EGFP was processed by autophagic degradation normally (Figure 5A). In *atg1*Δ cells (Figure 5B), $Ub^{G76V}$-EGFP was decreased in a much higher rate, in which the level of

fusion protein remained less than half of the initial level at the end of the 6hr starvation, than that of WT cells. No free EGFP was detected from the degradation of $Ub^{G76V}$-EGFP in *atg1*Δ cells. The level of $Ub^{I44A,G76V}$-EGFP also decreased under starvation, however, in a much lower rate than that of $Ub^{G76V}$-EGFP with no free EGFP detected as well.

From these data, we proposed that the decrease of $Ub^{G76V}$-EGFP in WT cells under the treatment of MG132 and Cycloheximide under starvation was due to proteasome activity that was not completely blocked by treatment of MG132, while the remaining $Ub^{G76V}$-EGFP was escaped from autophagic degradation by an unknown mechanism which was destroyed by the I44A mutation. Moreover, our data suggested that 26S proteasome might maintain a higher activity in *atg1*Δ cells than WT cells, which degraded ubiquitylated EGFP in a higher rate in autophagic-defective cells than WT cell.

**Delay of ubiquitylated EGFP in autophagic degradation is not unique to specific genetic background**

To test whether the delay of ubiquitylated EGFP in autophagic degradation is a strain specific pathway, we expressed $Ub^{G76V}$-EGFP and $Ub^{I44A,G76V}$-EGFP in BY4742 strain. Cells expressing $Ub^{G76V}$-EGFP or $Ub^{I44A,G76V}$-EGFP are grown to mid-log

phase and treated as previous described for the inhibition of proteasome activity. Data collected from BY4742 strain showed similar result to that of the SEY6210 (WT) strain (Figure 6), in which $Ub^{G76V}$-EGFP was accumulated in a higher rate than that of $Ub^{I44A,G76V}$-EGFP under starvation. A delay in the detection of free EGFP from the cleavage of $Ub^{G76V}$-EGFP compared to that of $Ub^{I44A,G76V}$-EGFP was similar to data from SEY6210 strain. These data showed that the delay of ubiquitylated EGFP in autophagic degradation is not a strain-specific situation.

**Autophagic degradation of ubiquitylated EGFP is slower that of other cytosolic proteins**

Our data showed that autophagic degradation of $Ub^{G76V}$-EGFP was hampered compared to that of $Ub^{I44A,G76V}$-EGFP. However, we could not rule out a possibility that I44A mutation created a super substrate facilitating autophagic degradation of ubiquitylated EGFP. To verify that I44A mutant is actually the mutation which destroyed the delay of ubiquitylated EGFP in autophagic degradation, we analyzed the difference between ubiquitiylated EGFP and other cytosolic proteins in autophagic degradation under starvation..

We expressed a fusion protein Pgk1-EGFP in WT cells as an indicator for non-selective autophagic degradation, in which Pgk1 is C terminally modified with a

EGFP that would be released after the cleavage of fusion protein by vacuolar hydrolases in the vacuole. Pgk1 is a 3-phosphoglycerate kinase that catalyzes transfer of high-energy phosphoryl groups from 1,3-bisphosphoglycerate to ADP to produce ATP, which mainly located in cytoplasm of cells and was used as the internal control in western blot analysis. Pgk1-EGFP expressed in yeast as a fusion protein was delivered to the vacuole under starvation for hydrolases digestion into fragments, releasing free EGFP accumulated in the vacuole. Thus the level of endogenous Pgk1 may not be affected by the cleavage of Pgk1-EGFP.

We first analyzed the distribution of Pgk1-EGFP in cells by fluorescent microscopy. WT, *atg1*Δ, and *pep4*Δ cells expressing Pgk1-EGFP were grown in SMD till mid-log phase as the sample of nutrient rich condition for fluorescent microscopy analysis. Images of WT, *atg1*Δ and *pep4*Δ cells under nutrient rich condition showed similar phenotype, in which Pgk1-EGFP dispersed throughout cytosol under nutrient rich condition with the vacuole absent of fluorescent signal (Figure 7A). Next we transferred mid-log phase cells into SD-N for 4hr of starvation and then analyzed the distribution of Pgk1-EGFP in cells under starvation by fluorescent microscopy (Figure 7A). Images of WT cells showed that the signal of Pgk1-EGFP was detected in the vacuole, indicating that starvation triggered the transport of Pgk1-EGFP from cytosol to the vacuole. Images from *atg1*Δ cells showed that starvation triggers no

transport of Pgk1-EGFP from cytosol to the vacuole, indicating that autophagy played critical role in the transport of Pgk1-EGFP from cytosol to the vacuole in WT cell. Moreover, accumulated autophagic bodies labeled with fluorescent signal were detected in the vacuole of *pep4*Δ cells, confirming that Pgk1-EGFP was sequestered into autophagosomes for autophagic degradation.

The level of Pgk1-EGFP degradation was further determined by western blot analysis. Cells expressing Pgk1-EGFP were treated as previous described, in which cells were cultivated in SD-N medium containing 75μM MG132 for a period of 6hr starvation. The level of Pgk1-EGFP in WT cells remained constant over starvation followed with an increase of free EGFP which was first detected after 1hr of starvation (Figure 7B). In *atg1*Δ and *pep4*Δ cells, PgK1-EGFP was kept in a constant level with no free EGFP was generated (Figure 7B). Quantification of these bands showed that Pgk1-EGFP expressing in WT cells was maintained in similar level with $Ub^{I44A,G76V}$-EGFP expressing in WT cells (Figure 7C). The accumulation of free EGFP from the cleavage of Pgk1-EGFP also displayed a similar pattern to that of $Ub^{I44A,G76V}$-EGFP in WT cells (Figure 7C). However, WT cells significantly accumulated more $Ub^{G76V}$-EGFP than Pgk1-EGFP under starvation, showing that Pgk1-EGFP was more readily to be degraded by autophagy in WT cells (Figure 7C). In *atg1*Δ cells and *pep4*Δ cells, Pgk1-EGFP, $Ub^{G76V}$-EGFP, and $Ub^{I44A,G76V}$-EGFP

were maintained in similar levels under starvation (Figure 7D, 7E).

Based on these data, we clearly demonstrated that compared to other cytosolic proteins, $Ub^{G76V}$-EGFP was delayed in autophagic degradation.

# Discussion

## Ubiquitylation triggers no protein aggregation in yeast.

Our data suggested that ubiquitylation stimulates no protein aggregation in budding yeast. It is known that SQSTM1/p62 and NBR1 trigger ubiquitylated protein aggregation in mammalian cells, which are further selectively degraded by autophagy (Kim *et al.*, 2008; Kirkin *et al.*, 2009a). However, here we showed that signal of $Ub^{G76V}$-EGFP and $Ub^{I44A,G76V}$-EGFP dispersed throughout cytosol in yeast, similar to that of free EGFP (Figure 1). This suggested that ubiquitylation plays no role in protein aggregation in yeast. Although some fluorescent punta which might represent protein aggregates were observed after treating cells with 0.1% triton X-100 and 4% paraformaldehyde, no difference could be detected between cells expressing $Ub^{G76V}$-EGFP, $Ub^{I44A,G76V}$-EGFP, or free EGFP. This indicated that the formation of these fluorescent puncta is not an ubiquitin-dependent pathway. From these data, we hypothesize that no SQSTM1/p62 or NBR1 analogs present in yeast.

## Delay, rather than acceleration, of $Ub^{G76V}$-EGFP in autophagic degradation

Ubiquitylation triggers not only proteasomal degradation but also lysosomal degradation of proteins in mammalian cells. This has been regarded as a protective pathway for cell survival under environmental stresses. However, this mechanism

seems not to exist in yeast. Moreover, we showed that ubiquitylation partially hinders EGFP from autophagic degradation (Figure 4). This result supports our hypothesis that no SQSTM1/p62 or NBR1 analogs are present in yeast. First, from images of fluorescent microscopy (Figure 2), we showed that $Ub^{G76V}$-EGFP, $Ub^{I44A,G76V}$-EGFP, and free EGFP would be translocated from cytosol to the vacuole in response to starvation in an autophagy-dependent pathway, which is also supported by the emergence of free EGFP in WT cells expressing $Ub^{G76V}$-EGFP or $Ub^{I44A,G76V}$-EGFP in western blot analysis (Figure 3). However, after75μM MG132 was used to block proteasome activity under starvation, $Ub^{G76V}$-EGFP started to accumulate in WT cells, which was significantly higher than the level of $Ub^{I44A,G76V}$-EGFP after 1hr of starvation. Moreover, free EGFP cleaved from $Ub^{I44A,G76V}$-EGFP in WT cells was accumulated in a higher rate than that from $Ub^{G76V}$-EGFP (Figure 4A). Compared to the degradation rate of Pgk1-EGFP, we demonstrated that $Ub^{G76V}$-EGFP, but not $Ub^{I44A,G76V}$-EGFP, is delayed in autophagic degradation (Figure 7). This is the direct evidence showing that ubiquityltion, which targets protein to proteasomal degradation, delays autophagic degradation of EGFP, indicating that ubiquitylated proteins could somehow escape from the non-selective sequestration of cytoplasm into autophagosomes for vacuolar degradation by an unknown pathway.

**Low level of ubiquitylated EGFP is not a substrate for autophagy**

To eliminate the effect of new-synthesized protein in our analysis of ubiquitylated EGFP degradation under starvation, we decided to treat cells with CHX and MG132 to inhibit protein synthesis and proteasome activity under starvation. Surprsingly, $Ub^{G76V}$-EGFP was degraded gradually in WT cells under starvation accompanied no free EGFP (Figure 6A). However, free EGFP was still detected in $Ub^{I44A,G76V}$-EGFP expressing cells (Figure 6A), indicating that low level of $Ub^{G76V}$-EGFP was targeted to proteasomal degradation rather than autophagy. Combined with previous results, we concluded that low amount of $Ub^{G76V}$-EGFP would be destined to proteasomal degradation instead of delivering to vacuole under starvation, whereas high amount of $Ub^{G76V}$-EGFP that exceeds the threshold would be sequestered into autophagosomes for autophagic degradation.

**I44A mutation destroys the delay of ubiquitylated EGFP in autophagic degradation**

We hypothesized that an unknown ubiquitin-interacting factor which binds to $Ub^{G76V}$-EGFP prevents its autophagic degradation. This hypothesis is supported by our data that an additional I44A mutation on ubiquitin moiety, which was shown to block proteasomal degradation of $Ub^{I44A,G76V}$-EGFP effectively (Figure 3A), also

destroyed the delay of ubiquityled EGFP in autophagic degradation. This indicated that the release of $Ub^{I44A,G76V}$-EGFP from the unknown ubiquitin-interacting factor allows the sequestration of the fusion protein into autophagosomes for degradation. Moreover, limited amount of the ubiquitin-interacting factor determines the threshold that low amount of $Ub^{G76V}$-EGFP would be recognized and kept away from isolation membrane whereas exceeded $Ub^{G76V}$-EGFP would be sequestered into autophagosomes and delivered to the vacuole under starvation. On the one hand, this unknown ubiquitin-interacting factor probably mediates proteasomal degradation of $Ub^{G76V}$-EGFP. Dual roles might be played by this unknown ubiquitin-interacting factor in degradation of ubiquitylated proteins.

**The competition between proteasome and autophagy for $Ub^{G76V}$-EGFP as a substrate**

Our data demonstrated that $Ub^{G76V}$-EGFP was a substrate preferred to be degraded by proteasomes rather than autophagy (Figure 3, 5). Under nutritional stage, new synthesized $Ub^{G76V}$-EGFP is soon degraded by proteasomes, maintaining this protein in a low level with no free EGFP generated. After cells are shifted to SD-N medium, although $Ub^{G76V}$-EGFP is still targeted to proteasomes, the activation of autophagy triggers the sequestration of cytoplasm into autophagosomes for vacuolar degradation,

leading to some degradation of Ub$^{G76V}$-EGFP via autophagy, releasing free EGFP (Figure 3A). This is supported from images of fluorescent microscopy that showed the translocation of fluorescent signal from cytosol to the vacuole in WT cells under starvation but not in *atg1*Δ cells (Figure 2). Paradoxically, our data also showed that the decrease of Ub$^{G76V}$-EGFP was similar in WT and *atg1*Δ cells with proteasome activity (Figure 3A, B), suggesting that first, autophagy may not contribute much to the degradation of Ub$^{G76V}$-EGFP under starvation, and second, proteasome activity might be up-regulated in *atg1*Δ cells.

It is interesting to point out that fusion proteins, especially Ub$^{G76V}$-EGFP, were degraded in a higher rate in *atg1*Δ than WT cells when CHX and MG132 were used to treat cells under starvation (Figure 5A, B). This supported our hypothesisi that proteasome activity is up-regulated in autophagy-defective cells. Moreover, no free EGFP was detected in WT cells (Figure 5A), suggesting that low level of Ub$^{G76V}$-EGFP would be targeted to proteasomal degradation rather than autophagy. This could be explained by our model that an unknown ubiquitin-interacting factor mediates proteasomal degradation of ubiquitylated proteins and delays their elimination by autophagy under starvation. It is now clear that multiple steps are required in protein degradation through the UPS system (Funakoshi *et al.*, 2002; Richly *et al.*, 2005; Ye, 2006; Dantuma *et al.*, 2009). The substrate protein, by the

activity of E1, E2 and E3 enzymes, is first modified by one or two ubiquitin moieties. This oligoubiquitylated substrate is then interacted with the $Cdc48^{Ufd1/Npl4}$ complex which further recruits Ufd2 as an “E4” enzyme extending the oligoubiquitin chain by a few extra ubiquitin moieties. Subsequently, Rad23 (or Ddi1, Dsk2) is recruited to the complex by the activity of Ufd2, which binds to the ubiquitylated substrate and delivers it to the Rpn10, one component of the 19S proteasomal cap, for further degradation. We propose that the unknown ubiquitin-interacting factor is one of these proteins mediating the recognition of ubiquitylated protein by proteasomes. Cdc28, Rad23 and Ddi1 were shown to interact with one or two ubiquitin-modified substrate, while Dsk2 was shown to bind to polyubiquitin chain, preferentially to Lys28 linked chain, via their UBA domain (Bertolaet *et al.*, 2001; Funakoshi *et al.*, 2002; Richly *et al.*, 2005; Dantuma *et al.*, 2009). In addition, $Ub^{I44A,G76V}$-EGFP, which was poorly interacted with the UPS machinery, was shown to be freely involved into autophagic degradation. We believe that the interaction of ubiquitin moiety with these ubiquitin binding factors led $Ub^{G76V}$-EGFP to proteasomal degradation and delayed their sequestration into autophagosomes, probably by a resisting signal harboring in the UPS system responding for the “cargo-recognition” process in starvation-induced autophagy, which was originally thought to be a non-selective process.

It is interesting to note that Korolchuk et al. (2009) have reported that autophagy

inhibition compromises degradation of ubiquitin-proteasome pathway substrates in mammalian cells, in which they showed that autophagy inhibition increases levels of proteasome substrates. By the interaction of poly-ubiquitin chain with excessive p62, ubiquitylated substrates which destined for proteasomal degradation originally become protein aggregates accumulated in cytosol. (Korolchuk *et al.*, 2009). This is far different from our result, in which we proposed that proteasome activity is up-regulated in autophagy-deficient cells, and ubiquitylated proteins targeted to proteasomes are resistant to autophagic degradation. Combined with our fluorescent images (Figure 1), we suggested that this is the another evidence showing that mammalian p62 and NBR1 analogs are absent in budding yeast, leading to the fact that proteasome machinery, but not autophagy, is the pathway that eliminates ubiquitylated proteins in yeast. Autophagy are regarded as homologous pathways among eukaryotes that share similar machinery, while our study suggested that the role of ubiquitylation in autophagic degradation of soluble proteins is quite different from mammalian cell to budding yeast.

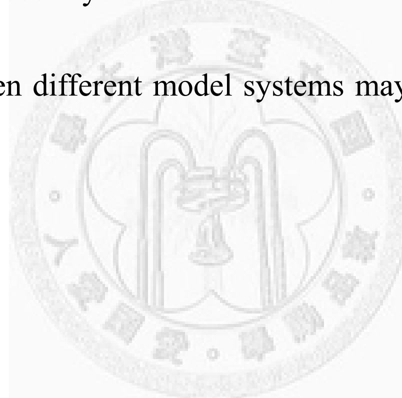
**The first report that ubiquitylation hinders autophagic degradation of cytosolic soluble proteins**

UPS system and autophagy are two parallel routes for protein degradation in

mammalian cells, in which autopahgy was regarded as a protective pathway cleaning up misfolded proteins that are failed to be eliminated by proteasomes. It is known that the impairment of constitutive autophagy causes cytoplasmic accumulation of ubiquitylated inclusion bodies accompanied with severe liver injuries and neurodegeneration.in mammals (Komatsu *et al.*, 2007). However, here we showed that ubiquitylated proteins are mainly degraded by proteaomes and was hindered from autophagic degradation in budding yeast. A great puzzle was raised, how can yeast deal with the accumulation of misfolded proteins under stresses without massive degradation of ubiquitylation-dependent autophagy? We have shown that no mammalian p62 or NBR1 analogs are presented in yeast to compete ubiquitylated proteins with proteasomal machinery. However, it is not clear whether p62 and NBR1 are evolved independently in mammals or lost in budding yeast. Moreover, we could not rule out a possibility that there exist a complementary pathway which is independent of ubiquitylation in regulating level of misfolded proteins by autopahgy in yeast. Thus, roles of autophagy in the clearance of misfolded proteins under stresses must be studied. Budding yeast has been used to model neurodegeneration elucidating mechanism underlying these complex diseases and developing novel therapeutics (Khurana and Lindquist, 2010). However, our study has suggested different roles of ubiquitylation in autophagic degradation of soluble protein between

yeast and mammalian cells, which should now be taken into consideration in working with yeast as a model of neurodegenerative diseases. Nevertheless, some proteins, for example, $PrP^{SC}$ in Prion diseases, are self-aggregation with no ubiquitylation required. Further analysis of how protein aggregates are degraded by autophagy in yeast would help us understanding cell toxicity in humans.

Finally, this is the first report that ubiquitylation does not promote autophagic degradation of cytosolic protein. The different scenario in autophagic degradation of ubiquitylated protein between yeast and mammalian cells reminds us that the conserved pathway between different model systems may display great difference in detail.



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## Tables

Table 1. Yeast strains used in this study

| Strain | Description | Genotype | Reference |
|---|---|---|---|
| SEY6210 | WT | *MATα his3-Δ200 leu2-3, 112 lys2-801 trp1-Δ901 ura3-52 suc2-9ΔGAL* | (Robinson *et al.*, 1988) |
| WHY1 | *atg1Δ* | SEY6210 *atg1Δ::HIS5 S.p.* | (Shintani *et al.*, 2002) |
| TVY1 | *pep4Δ* | SEY6210 *pep4Δ::LEU2* | (Kim *et al.*, 2001) |
| BY4742 | | *MATα his3Δ leu2Δ lys2Δ ura3Δ* | ResGen/Invitrogen |

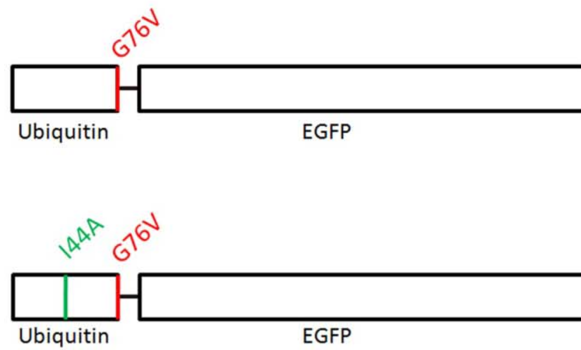
Table 2. Plasmids used in this study

| Plasmid | Description | Reference |
|---|---|---|
| pRS414-$P_{Cu}$-EGFP-$T_{CYC1}$ | Expression of free EGFP, *CUP1* promoter | This study |
| pRS414-$P_{Cu}$-$Ub^{G76V}$-EGFP-$T_{CYC1}$ | Expression of $Ub^{G76V}$-EGFP, *CUP1* promoter | This study |
| pRS414-$P_{Cu}$-$Ub^{I44A,G76V}$-EGFP-$T_{CYC1}$ | Expression of Ub $^{I44A,G76V}$-EGFP, *CUP1* promoter | This study |
| pRS416-$P_{Cu}$-$Ub^{G76V}$-EGFP-$T_{CYC1}$ | Expression of $Ub^{G76V}$-EGFP, *CUP1* promoter | This study |
| pRS416-$P_{Cu}$-$Ub^{I44A,G76V}$-EGFP-$T_{CYC1}$ | Expression of Ub $^{I44A,G76V}$-EGFP, *CUP1* promoter | This study |
| pRS416-$P_{Cu}$-Pgk1-EGFP-$T_{CYC1}$ | Expression of Pgk1-EGFP, *CUP1* promoter | This study |

# Figures

## Figure 1.

**A**




**B**

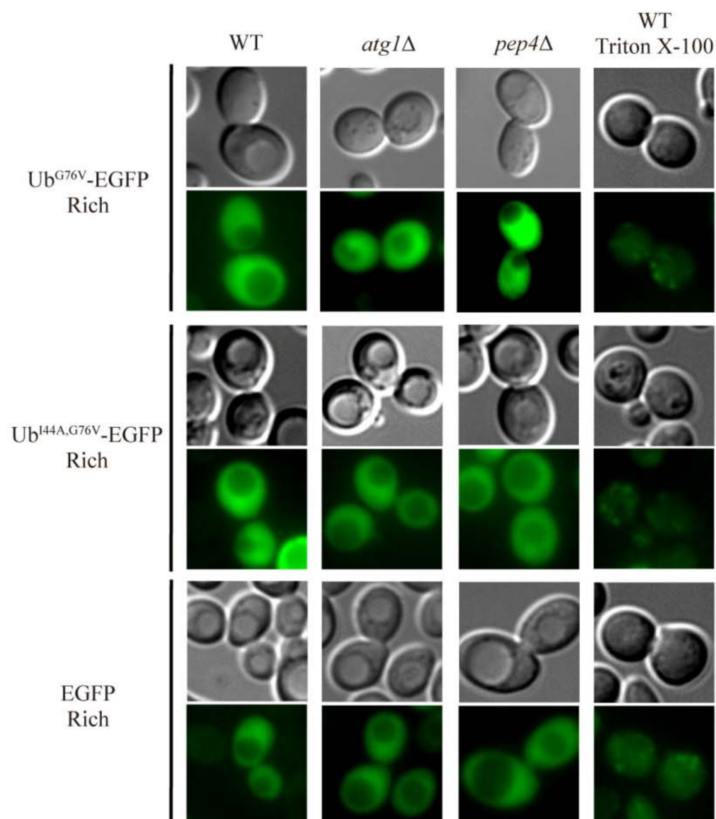


**Figure 1. Ubiquitylation triggers no protein aggregation in yeast.** (A) Two ubiquitylated EGFP, Ub$^{G76V}$-EGFP and Ub$^{I44A,G76V}$-EGFP, were constructed to express in yeast. (B) Ub$^{G76V}$-EGFP and Ub$^{I44A,G76V}$-EGFP distributed diffusively in WT, *atg1*Δ, and *pep4*Δ cells under nutrient rich condition, which were similar to that of the EGFP. Cells grown to mid-log phase were harvested for fluorescent microscopy analysis. WT cells of the right most panel were treated with 0.1% TritonX-100 for 30min following by 4% Paraformaldehyde for 20min before fluorescent microscopy analysis, which showed the fluorescent puncta in Ub$^{G76V}$-EGFP, Ub$^{I44A,G76V}$-EGFP, and EGFP expressing cells.

**Figure 2.**

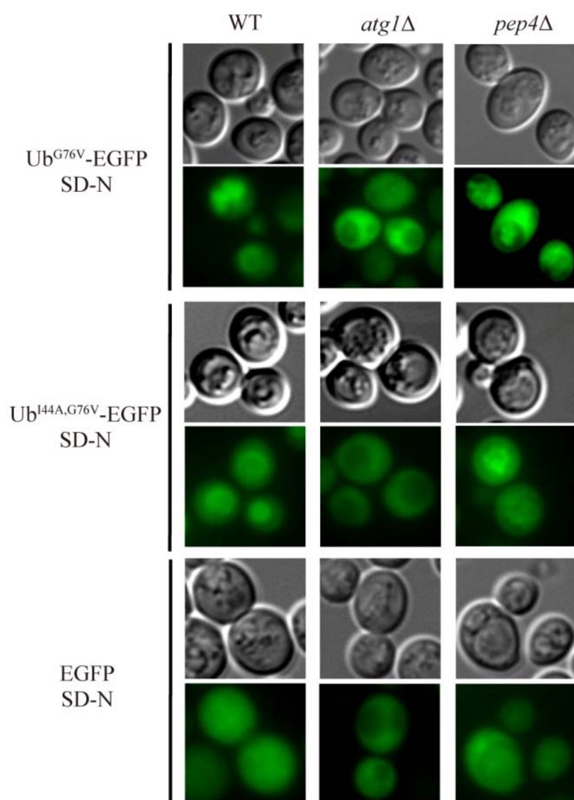



**Figure 2. Ubiquitylated EGFP is a substrate for autophagic degradation.** Ub$^{G76V}$-EGFP, Ub$^{I44A,G76V}$-EGFP, and free EGFP were found to be transported to the vacuole of WT cells under starvation, which was blocked in *atg1*Δ cells. Moreover, autophagic bodies with fluorescent signal were found to be accumulated in the vacuole of *pep4*Δ cells, indicating that this starvation-induced transportation is an autophagy-dependent pathway. Cells expressing Ub$^{G76V}$-EGFP, Ub$^{I44A,G76V}$-EGFP, or free EGFP were grown to mid-log phase and transferred to the SD-N medium. After 4hr of starvation, cells were harvested for fluorescent microscopy analysis.

**Figure 3**

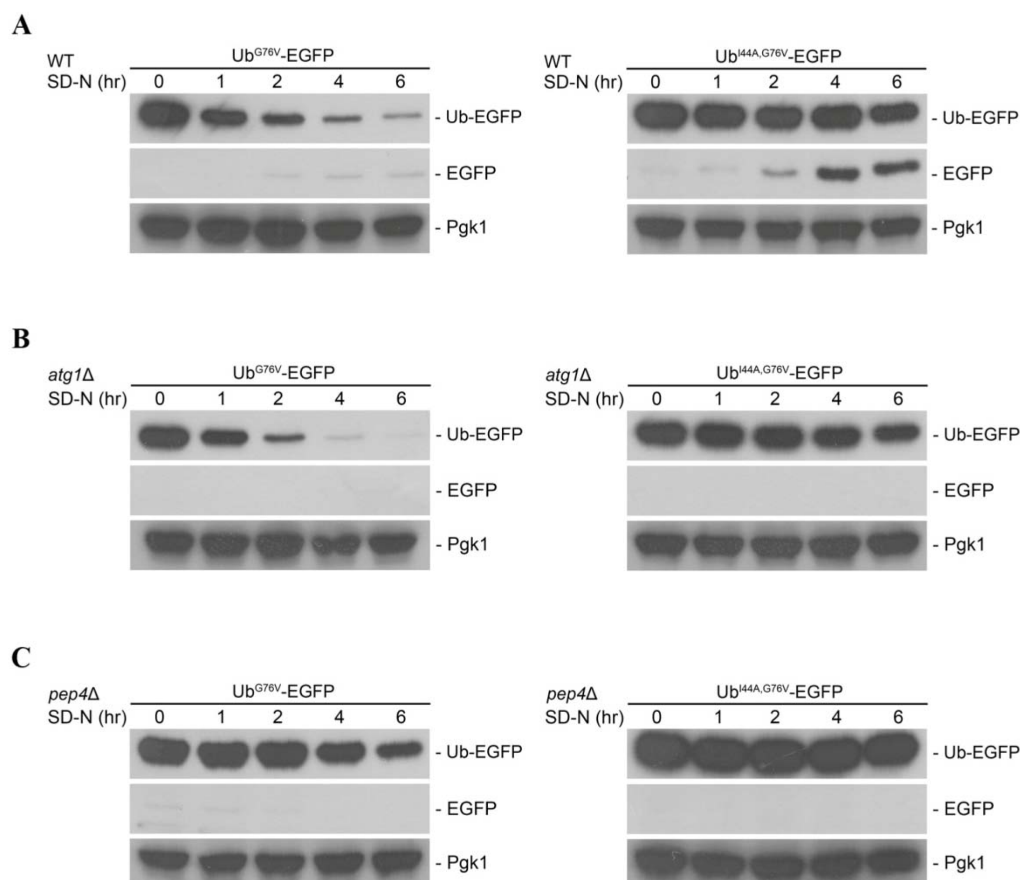
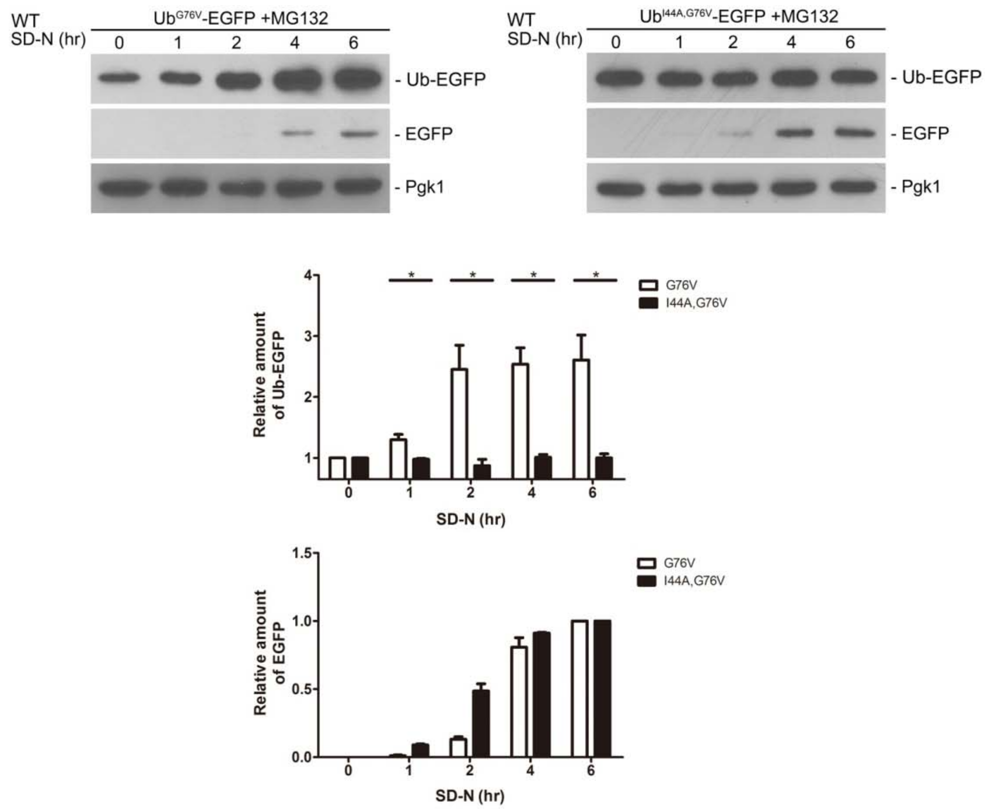



**Figure 3. Proteasomes, but not autophagy, degrade most ubiqutylated EGFP under starvation.** (A) In WT cells, Ub$^{G76V}$-EGFP was shown to be degraded rapidly under starvation accompanying with small amount of free EGFP accumulation, while Ub$^{I44A,G76V}$-EGFP was maintained in a constant level accompanying with rapid accumulation of free EGFP. Cells for western blot analysis were grown in SMD to mid-log phase, and then transferred to SD-N medium for starvation. Culture aliquots were collected at 0hr, 1hr, 2hr, 4 hr and 6hr during a period of 6hr starvation, following by 10%TCA treatment for protein precipitation. Samples were subjected to

western blot analysis, in which anti-GFP antibody (α-GFP) were used to detect Ub-EGFP and free EGFP. Levels of Pgk1, which were detected by anti-Pgk1 antibody (α-Pgk1), were used as the internal control. (B) In *atg1*Δ cells, $Ub^{G76V}$-EGFP was degraded in a higher rate than that of the WT cell under starvation, while $Ub^{I44A,G76V}$-EGFP was remained in a constant level. However, no free EGFP was detected from the cleavage of both fusion proteins in *atg1*Δ cells. *atg1*Δ cells expressing $Ub^{G76V}$-EGFP or $Ub^{I44A,G76V}$-EGFP were treated and analyzed as WT cells. (C) In *pep4*Δ cells, $Ub^{G76V}$-EGFP was degraded in a much slower rate than that of the *atg1*Δ cells, while $Ub^{I44A,G76V}$-EGFP was maintained in a similar level under starvation. No free EGFP was detected from the degradation of both fusion proteins. *pep4*Δ cells expressing $Ub^{G76V}$-EGFP or $Ub^{I44A,G76V}$-EGFP were treated and analyzed as WT cells.

**Figure 4**

**A**

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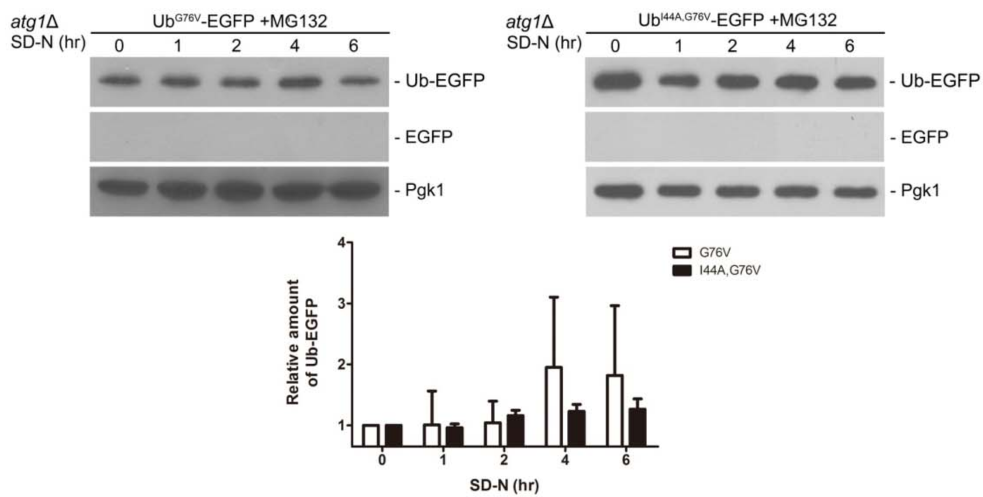



**C**

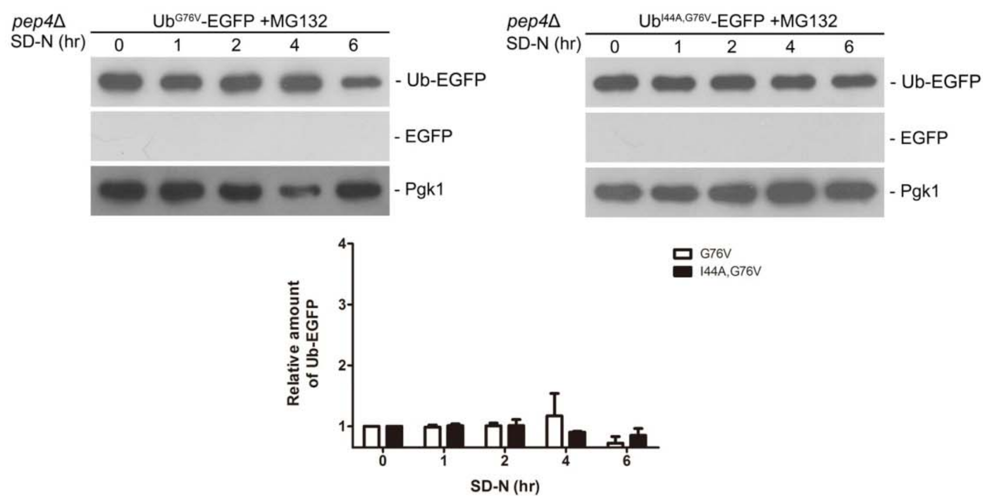
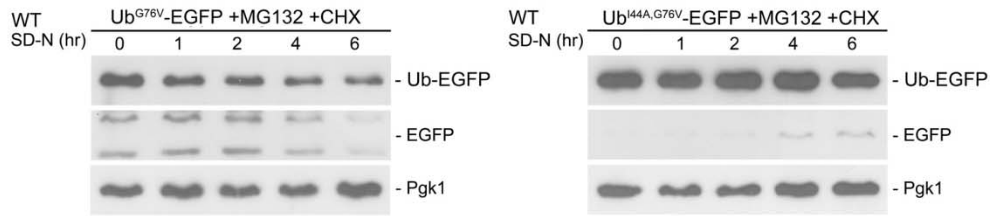
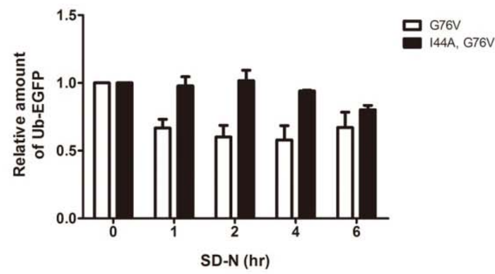


**Figure 4. Autophagic degradation of Ub$^{G76V}$-EGFP was slower than that of Ub$^{I44A,G76V}$-EGFP.** (A) Ub$^{G76V}$-EGFP was degraded in a slower rate than that of Ub$^{I44A,G76V}$-EGFP in WT cells treated with MG132 under starvation. Ub$^{G76V}$-EGFP was accumulated in WT cells under starvation with the generation of free EGFP after 4hr of starvation. In comparison, Ub$^{I44A,G76V}$-EGFP was maintained in a steady level, which was lower than that of Ub$^{G76V}$-EGFP obviously after 1hr of starvation. Moreover, free EGFP from the cleavage of Ub$^{I44A,G76V}$-EGFP was accumulated more rapidly than Ub$^{G76V}$-EGFP expressing cells. Cells expressing Ub$^{G76V}$-EGFP or Ub$^{I44A,G76V}$-EGFP for MG132 treatment were treated as described in Material and Methods. Experiments were repeated three times, in which relative levels of bands representing Ub-EGFP or free EGFP were quantified and analyzed by Student's T-test. (B) In *atg1*Δ cells treated with MG132, Ub$^{G76V}$-EGFP and Ub$^{I44A,G76V}$-EGFP were maintained in similar levels accompanied no generation of free EGFP under starvation. (C) Ub$^{G76V}$-EGFP and Ub$^{I44A,G76V}$-EGFP were maintained in similar levels accompanied no free EGFP was generated in *pep4*Δ cells treated with MG132 under starvation, which is similar to that of *atg1*Δ cells.

**Figure 5.**

**A**

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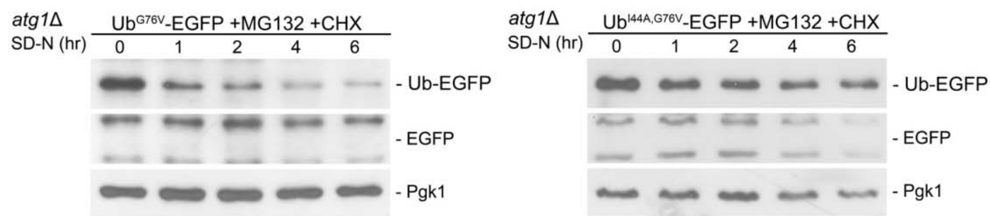


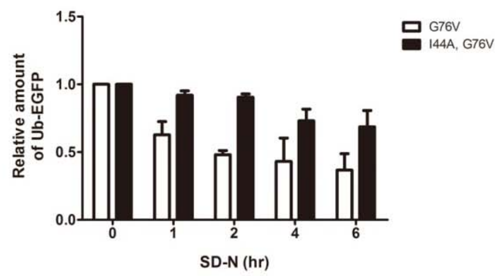



**Figure 5. Low level of ubiquitylated EGFP is not a substrate for autophagic degradation.** (A) WT cells treated with 75μM MG132 and 10 μμg/ml Cycloheximide (CHX) showed that Ub$^{G76V}$-EGFP was degraded gradually with no generation of free EGFP, while free EGFP was accumulated in Ub$^{I44A,G76V}$-EGFP expressing WT cells under starvation. Cells expressing Ub$^{G76V}$-EGFP or Ub$^{I44A,G76V}$-EGFP for CHX and MG132 treatment were treated and analyzed as Materials and Methods described. (B) No free EGFP was detected in *atg1*Δ cells treated with 75μM MG132 and 10 μg/ml Cycloheximide. However, Ub$^{G76V}$-EGFP was degraded quickly from relative level 1 to about 0.4. In addition, *atg1*Δ cells also showed higher degradation rate of Ub$^{I44A,G76V}$-EGFP than that of the WT cells. *atg1*Δ cells expressing Ub$^{G76V}$-EGFP or Ub$^{I44A,G76V}$-EGFP were treated and analyzed as previously described.

**Figure 6.**

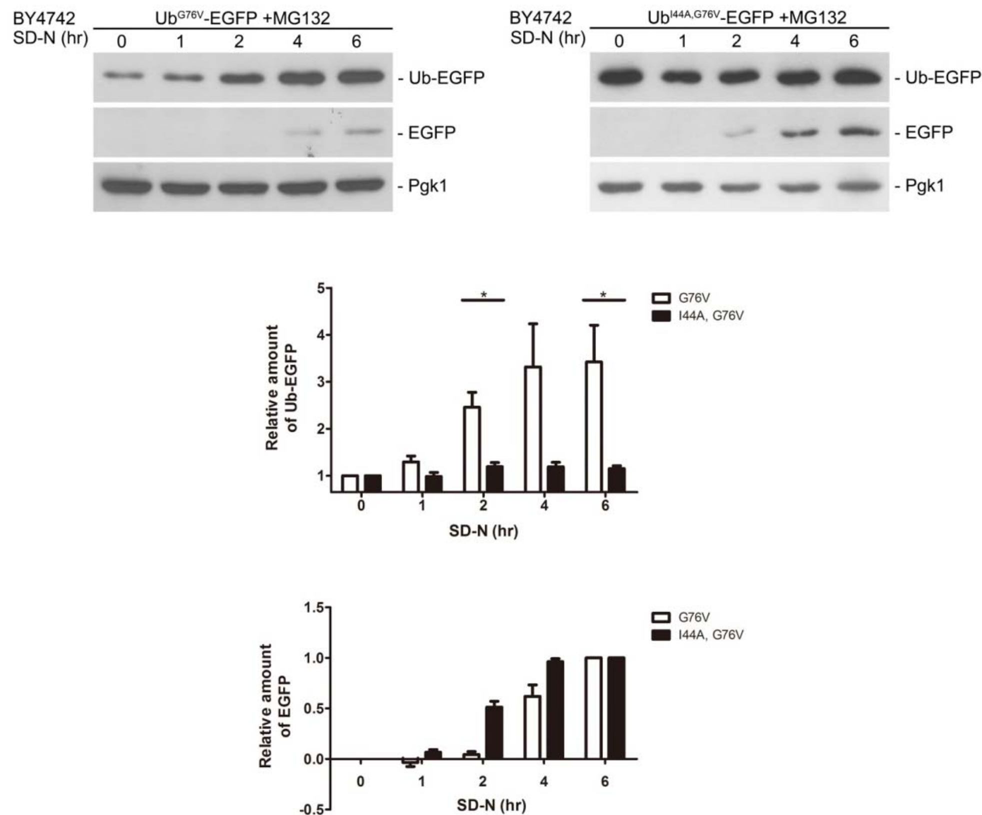
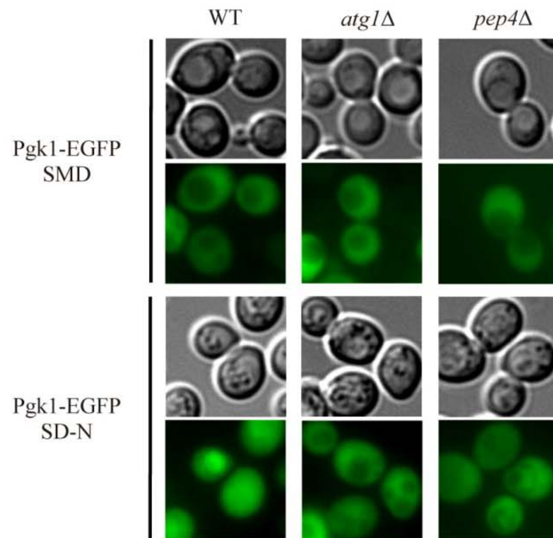



**Figure 6. Delay in autophagic degradation of ubiquitylated EGFP is not unique to specific genetic background.** Under the treatment of MG132, Ub$^{G76V}$-EGFP and Ub$^{I44A,G76V}$-EGFP expressing in BY4742 were degraded in similar phenotypes to that of WT(SEY6210) cells under starvation. Cells were treated and analyzed as previously described.

**Figure 7.**

**A**

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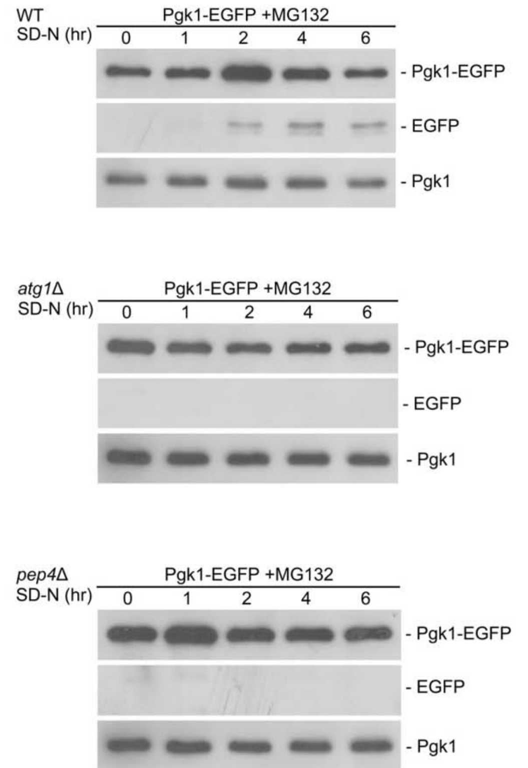
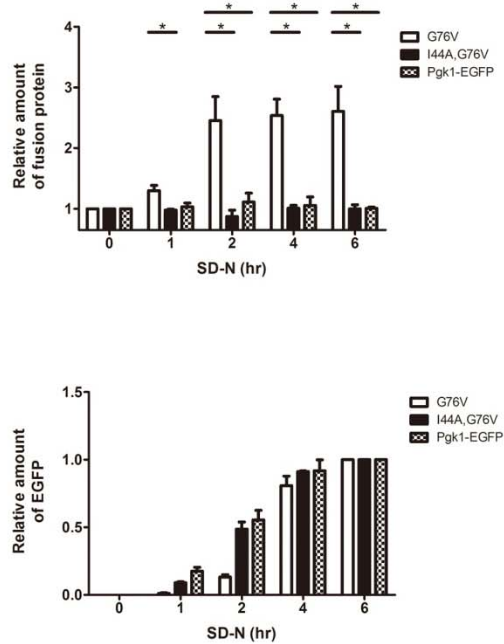
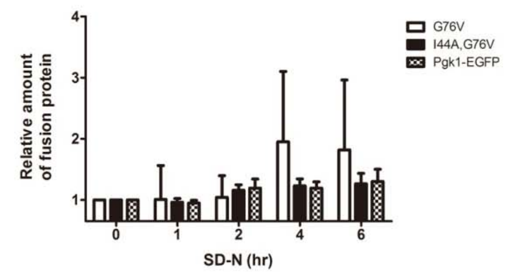



**C**




**D**




**E**

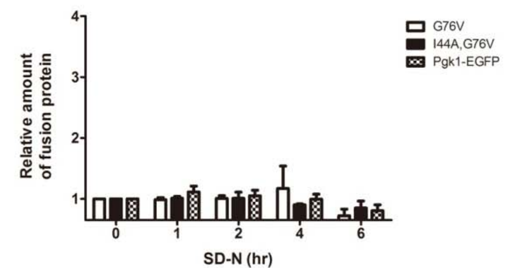


**Figure 7. Autophagic degradation of ubiquitylated EGFP is slower that of other cytosolic proteins.** (A) Under nutrient rich condition, Pgk1-EGFP expressed in WT, *atg1*Δ, and *pep4*Δ cells showed dispersed distribution in cytosol. Under starvation treatment, Pgk1-EGFP was translocated from cytosol to the vacuole in WT but not *atg1*Δ cell. Moreover, autophagic bodies with fluorescent signal were accumulated in the vacuole of *pep4*Δ cells, indicating that the transportation of Pgk1-EGFP from cytosol to the vacuole was an autophagic-dependent pathway. Cells for fluorescent microscopy analysis were treated as Materials and Methods described. (B) Pgk1-EGFP expressed in WT cells was maintained in similar levels under starvation which accompanied the accumulation of free EGFP after 2hr of starvation. However, Pgk1-EGFP was maintained in similar levels in *atg1*Δ and *pep4*Δ cells under starvation with no generation of free EGFP. (C) Data from the quantification of western blot showed that the degradation rate of Pgk1-EGFP was similar to that of $Ub^{I44A,G76V}$-EGFP rather than $Ub^{G76V}$-EGFP in WT cells. (D) In *atg1*Δ cells, Pgk1-EGFP was maintained in similar levels under starvation, which is similar to that of $Ub^{G76V}$-EGFP and $Ub^{I44A,G76V}$-EGFP. (E) In *pep4*Δ cells, Pgk1-EGFP was maintained in similar levels under starvation, which is similar to that of $Ub^{G76V}$-EGFP and $Ub^{I44A,G76V}$-EGFP. Cells for western blot analysis were treated as Materials and Methods described. The degradation of Pgk1-EGFP and generation free EGFP were

quantified from the western blot analysis and plotted with the data of $Ub^{G76V}$-EGFP and $Ub^{I44A,G76V}$-EGFP from Figure 4 for comparison.