博士論文番号：1081203

Isolation and functional analysis of yeast ubiquitin ligase Rsp5 variants that alleviate the toxicity of human α-synuclein

（ヒト α-シヌクレインの毒性を緩和する
酵母ユビキチンリガーゼ Rsp5 変異体の単離と機能解析）

Indah Wijayanti

Laboratory of Applied Stress Microbiology
Graduate School of Biological Sciences
Nara Institute of Science and Technology

Prof. Hiroshi Takagi

Submitted on 2014/11/14

TABLE OF CONTENTS

ABSTRACT

Stress induces denaturation, generates abnormal proteins, and leads to growth inhibition or cell death. Such abnormal proteins are mainly degraded via autophagy or the ubiquitin-proteasome system. In the budding yeast *Saccharomyces cerevisiae*, Rsp5 is the only essential HECT-type ubiquitin ligase belonging to the highly conserved Nedd4 family. Our laboratory previously revealed that Rsp5 is a key enzyme involved in the degradation of stress-induced abnormal proteins for yeast cell growth under various stress conditions. Overexpression of human α-synuclein, a small lipid binding protein implicated in several neurodegenerative diseases (e.g. Parkinson's disease), in *S. cerevisiae* leads to cell death due to various intracellular defects, including accumulation of reactive oxygen species (ROS). In this study, to understand the molecular mechanism of Rsp5-mediated detoxification of α-synuclein, I isolated and analyzed new Rsp5 variants, which protect yeast cells from α-synuclein toxicity as a model stress, using error-prone PCR-based random mutagenesis.

First, I confirmed that α-synuclein toxicity was enhanced due to the accumulation of α-synuclein in the Δ*vps1* and Δ*end3* mutants, but not in the autophagy-deficient Δ*atg5* mutant, demonstrating that α-synuclein undergoes degradation mediated specifically by the endocytotic pathway. Hence it is intriguing to see whether Rsp5 regulates the degradation of α-synuclein. Previously, our laboratory showed that two mutations in the WW domains of Rsp5, each of which contains two tryptophan residues that directly bind to the proline-rich motifs in substrate proteins, conferred altered tolerance to toxic proline analogues on yeast cells. While *rsp5*^{A401E} cells are sensitive compared to wild-type cells, *RSP5*^{T357A} cells are more tolerant than wild-type cells. This phenotype correlates with the fact that the ubiquitination of the general amino acid permease Gap1 by Rsp5 is impaired in *rsp5*^{A401E} and enhanced in *RSP5*^{T357A}. Here, I found that α-synuclein toxicity in *rsp5*^{A401E} cells was stronger than in wild-type cells, although there was no significant improvement of α-synuclein tolerance by *RSP5*^{T357A} cells. In addition, α-synuclein was accumulated in *rsp5*^{A401E} cells faster and higher than in wild-type cells. These results suggest that Rsp5 recognizes α-synuclein in a different way from Gap1. Thus, I constructed a plasmid library by introducing random mutagenesis into the DNA region encoding the WW domains of Rsp5 to isolate novel Rsp5 variants with higher tolerance to α-synuclein.

In error-prone PCR, manganese chloride and isopropanol were used to reduce the fidelity of DNA polymerase with lower base-pairing specificity. As a result, mutation was introduced into 1.7 bases per 1 kb on the average. The PCR products of

the WW domains were cloned into the centromere-based low-copy number plasmid pRS415, and approximately 30,000 independent colonies of *Escherichia coli* transformants were collected as the random-mutagenized plasmid library of *RSP5*. After this library was introduced into *rsp5*^{A401E} cells overexpressing human α-synuclein-encoding cDNA, several transformants could grow faster than *rsp5*^{A401E} cells carrying the empty vector only. Among them, I successfully isolated four novel Rsp5 variants, Thr255Ala in the WW1 domain, Asp295Gly in between the WW1 and WW2 domains, Pro343Ser in the WW2 domain, and Asn427Asp in the vicinity of the WW3 domain, which showed enhanced tolerance to α-synuclein. These mutated *RSP5* genes also conferred tolerance to α-synuclein when integrated into the *RSP5* locus on the chromosome.

To further characterize these Rsp5 variants, I first examined the growth phenotypes under various conditions. All four variants did not significantly confer tolerance to toxic amino acid analogues, as did *RSP5*^{T357A}. This result supports our hypothesis on the substrate-specific recognition mechanism requiring Rsp5. Intriguingly, I also found that only *RSP5*^{T255A} cells showed sensitivity to high temperature (39°C), but increased tolerance to acetic acid compared with that of wild-type cells, suggesting that *RSP5*^{T255A} and the other three mutants are phenotypically differently categorized. Next, I tested whether the mutations actually alleviate the toxicity caused by α-synuclein. First, the intracellular ROS level elevated during the overexpression of α-synuclein was significantly decreased in the *RSP5*^{D295G}, *RSP5*^{P343S}, and *RSP5*^{N427D} mutants, but not by the *RSP5*^{T255A} mutant. These three mutations (*RSP5*^{D295G}, *RSP5*^{P343S}, and *RSP5*^{N427D}) accelerated the degradation of α-synuclein after shutting off its transcription. Co-immunoprecipitation analysis revealed that only the P343S substitution strongly enhanced the physical interaction between Rsp5 and α-synuclein. Furthermore, this mutation also raised the level of ubiquitinated α-synuclein. These results demonstrate that α-synuclein is indeed detoxified through the enhanced substrate recognition of the P343S variant Rsp5 toward α-synuclein. In contrast, the *RSP5*^{T255A} mutation was suggested to alleviate α-synuclein toxicity and confer tolerance to acetic acid via unknown mechanisms.

In conclusion, I identified novel amino acid changes in the WW domains or their linker regions of Rsp5. Among them, *RSP5*^{P343S} specifically enhanced the recognition, ubiquitination, and degradation of α-synuclein. Considering that the *RSP5*^{T357A} mutation increases the ubiquitination activity selectively toward Gap1, all of these results support the hypothesis that the WW domains of Rsp5 play a pivotal role in recognition of the substrate proteins. This study will contribute to understanding the molecular basis of how disused proteins are specifically recognized and effectively removed, which is a key regulatory mechanism of stress response and adaptation in eukaryotic cells.

GENERAL INTRODUCTION

➢ Yeast stress responses

The budding yeast *Saccharomyces cerevisiae* has been widely used in the fermentation industries to produce various useful metabolites, representatively ethanol and carbon dioxide. Under fermentation processes or industrial yeast production, yeast cells are exposed to various environmental stresses, such as high concentrations of ethanol, freezing, desiccation, and high osmotic pressure. These stress conditions induce several dysfunctions of proteins, including denaturation and misfolding (Gaser et al., 2008), generate reactive oxygen species (ROS) (Davidson et al., 1996; Moraitis & Curran, 2004), and lead to growth inhibition or cell death. To avoid these difficulties, transcription of the genes encoding protein chaperones and antioxidant enzymes is quickly induced in response to stresses (Morano et al., 2012). Furthermore, as denatured proteins that cannot be properly refolded are targeted for degradation, the proteolytic mechanisms are reinforced upon stresses. Therefore, improvement of yeast stress tolerance through enhancing those cellular functions would greatly contribute to the fermentation industries.

➢ Ubiquitin system

Aberrant proteins associated with denaturation, misfolding, or aggregation in living organisms lead to growth inhibitition or cell death. Therefore, regulated proteolysis has an essential role in all organisms (Sullivan et al., 2003). The ubiquitin system is responsible for much of the regulated proteolysis in the cell (Hershko and Ciechanover, 1998; Campello et al., 2013). This is a multicomponent complex system that identifies and specifically degrades unnecessary proteins of all eukaryotic cells, and is involved in cell growth and differentiation (Moon et al., 2004), DNA replication and repair (Erdenize and Rothstein, 2000; Lipp et al., 2009), glioma cell cycle control (Vlachostergios et al., 2012), angiogenesis (Rahimi, 2012), morphogenesis (Hamilton and Zito, 2013), environmental stress responses (Hiraishi et al., 2009), and immune response (Kloetzel, 2004).

The degradation of a protein through ubiquitin system involves three types of different enzymes; (1) E1 enzymes known as ubiquitin-activating enzymes that modify ubiquitin into a reactive state, (2) E2 enzymes known as ubiquitin-conjugating enzymes that carry ubiquitin to E3 and the substrate proteins and (3) E3 enzymes as ubiquitin ligases that play an important role in recognizing the substrate proteins (Fig. 1) (Hershko and Ciechanover, 1998). First, an ubiquitin molecule is activated by E1 in an ATP-dependent fashion. This activation followed by the formation of a thioester bond between a cysteine residue in the active center of E1 and the C-terminus of ubiquitin. Secondly, the activated ubiquitin is then transferred to a cysteine residue located in the active center of E2. Finally, E2 binds to E3, which recognizes the substrate protein and conjugate ubiquitin to it. As for cytosolic proteins, the ubiquitinated unnecessary proteins are shuttled to the proteasome complex to undergo degradation. In contrast, the ubiquitinated plasma membrane proteins are endocytosed and trafficked into the lysosomes/vacuoles, where they undergo complete proteolysis (Rotin and Kumar, 2009; Lauwers et al. 2010).

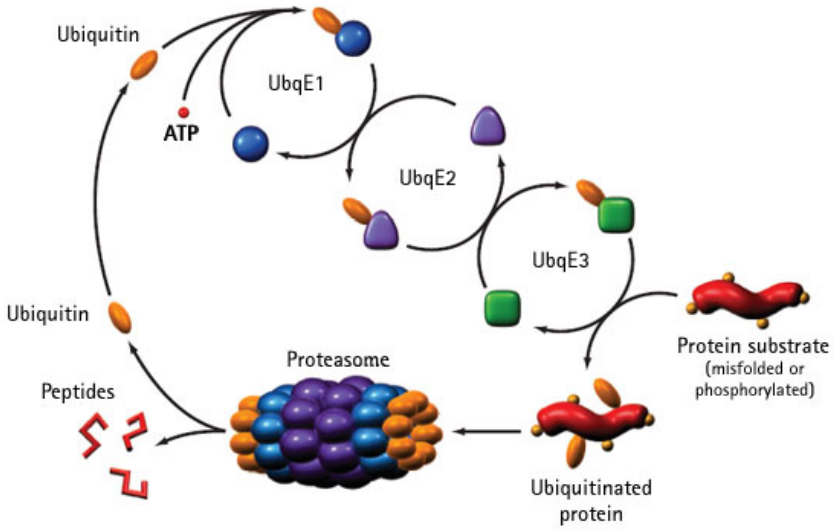


Figure 1. Ubiquitin–proteasome system (UPS). Intracellular misfolded, damaged, and obsolete proteins are degraded by the UPS in a process in which an E1 ubiquitin-activating enzyme first binds to ubiquitin (Ub) with ATP hydrolysis. The so-activated ubiquitin is then transferred to an E2 ubiquitin-conjugating enzyme, which carries the E2- bound ubiquitin to a substrate protein that is specifically bound to an E3 ubiquitin protein ligase. After several rounds of ubiquitin molecule addition, degradation of the polyubiquitinated substrate occurs inside the proteasome, releasing as products free peptides and reusable ubiquitin moieties. Ubiquitin recycling is carried out with deubiquitinating enzymes (DUBs). The two α- and β-rings of the proteasome 20S core particle are composed of seven subunits, which are labeled in purple and cyan, respectively (Campello et al, 2013).

E3 plays a key role in this ubiquitin-mediated proteolytic cascade by acting as one of the specific recognition factors of the system (Campello et al., 2013). There are >1,000 E3s in cells that link ubiquitin to proteins in a highly regulated manner (Lecker et al., 2006). E3s generally belong to one of two families. The really interesting new gene (RING)-type E3s are thought to act primarily as molecular scaffolds to bring together E2 charged with ubiquitin and a substrate targeted for ubiquitination (Rotin and Kumar, 2009). In contrast, the homologous to the E6-AP carboxyl terminus (HECT)-type E3s form a covalent thioester intermediate with ubiquitin before transferring ubiquitin to the substrate (Hershko and Ciechanover, 1998; Pickart, 2001). A conserved cysteine residue located within the active site of the HECT catalytic domain forms a thioester bond with ubiquitin upon its transfer from E2 enzymes (Huibregtse et al., 1995). Different types of ubiquitin modifications adopt distinct structural conformations, and these structural differences are functionally important because they target proteins for different fates in the cell.

- **Ubiquitin ligase Rsp5**

Among E3 enzymes, Rsp5 is the only yeast member of the highly conserved mammalian Nedd4 family of HECT-type E3 ligases (Tardiff et al., 2013). Numerous studies demonstrated that Rsp5 is an essential ubiquitin ligase in *S. cerevisiae* involved in various cellular functions, including endocytosis, multivesicular body sorting, RNA export, transcription, lipid biosynthesis, mitochondrial inheritance, and protein catabolism (Belgareh-Touze et al., 2008; Shearwin-Whyatt et al., 2006). The ability of Rsp5 to act as a multifunctional E3 in yeast is, at least in part, due to its capacity to modify different substrates with distinct mono- and poly-ubiquitin signals. Several proteins that function in endocytosis, such as Rvs167, are monoubiquitinated in an Rsp5-dependent manner (Rotin et al., 2000; Stamenova et al., 2004). In contrast, Rsp5 targets a number of cellular proteins for polyubiquitination, including the large subunit of RNA polymerase II, the vacuolar membrane protein Sna3, and the mRNA nuclear export factor Hpr1 (Chang et al., 2000; MacDonald et al., 2012; Gwizdek, et al., 2005). Rsp5 catalyzes Lys63 linkages of ubiquitin to diverse membrane proteins and thereby regulate endosomal trafficking, not proteasomal degradation (Rotin and Kumar, 2009; Lauwers et al., 2010).

How can Rsp5 recognize those various substrate proteins? A series of

evidence indicates that the WW protein-interaction domains play a pivotal role in substrate recognition. All Nedd4 family members possess an N-terminal Ca^{2+}-dependent lipid/protein binding (C2) domain, two to four WW domains, and a C-terminal catalytic HECT domain (Rotin et al., 2000). In Rsp5, a C2 domain, three WW domains, and a HECT domain are identified (Huibregtse et al., 1997; Ciechanover, 1998; Lauwers et al., 2010). The WW domain is a small (40 amino acids) protein-interacting module named after the presence of two conserved tryptophan residues (W), which are spaced 20-22 amino acids apart within the sequence. Its structure consists of 3 β strands (antiparallel β sheet), with a hydrophobic binding surface (Sudol and Hunter, 2000). WW domains are divided into four groups according to their binding specificities (Bedford et al., 2000); Group I binds the PXY, LPXY, and PPXY motifs, Group II binds to the PPLP motif, Group III recognizes the PPR motif, and Group IV binds to short sequences containing phosphoserine or phosphothreonine followed by proline (Lu et al., 1999; Shcherbik et al., 2002). However, since all three WW domains in Rsp5 are categorized in Group I, the mechanism responsible for the substrate specificity of Rsp5 has been obscure.

- **Human α-synuclein toxicity**

Parkinson's disease (PD) is the most common and fatal neurodegenerative movement disorder that affects individual elderly people. Almost 10 million people in the world are affected with PD (Dorsey et al., 2007), and more than 4% of the population is affected by the age of 85 years (Franssens, et al., 2010). This disease is characterized by resting tremor, muscular rigidity, bradykinesia, and postural instability (Polymeropoulos et al., 1997).

Numerous studies demonstrated that PD is related to the aggregation and misfolding of a small lipid-binding protein α-synuclein (α-syn) in human brain (Taylor et al., 2002, Takalo et al., 2013). Human α-syn is a naturally unfolded protein containing 140 amino acids (~14 kDa) (Jakes et al., 1994), which is thought to regulate cell differentiation, synaptic plasticity, and dopaminergic neurotransmission (Liang et al., 2008). It has been known that the toxicity of human α-syn is due to its aggregation and misfolding that lead to the defects in endoplasmatic reticulum (ER)-to-Golgi trafficking, mitochondrial functions, stress responses, and lipid/sterol biosynthesis (Auluck et al., 2010). Feng et al., (2010) reported that α-syn induces

formation of SDS-stable oligomers, generation of intracellular aggregates, alterations in membrane conductance reminiscent of leak channels, and subsequent cytotoxicity in a dopaminergic-like cell line. The α-syn toxicity is also intimately connected to mitochondrial dysfunction (Butler et al., 2012), ER stress (Smith et al., 2005), and Golgi fragmentation (Gosavi et al., 2002). The general structure of synucleins involves a series of imperfectly repeated KTKEGV repeats at the N-terminal and an acidic C-terminal tail (Fig. 2) (Cookson, 2005). The N-terminal repeats have been described to share similarities to those found in apolipoproteins and this domain is believed to form similar amphipathic helices in lipid membranes (Clayton and George, 1998). The capacity of α-syn to bind freely to such membranes has already been shown (Conway et al., 2000).

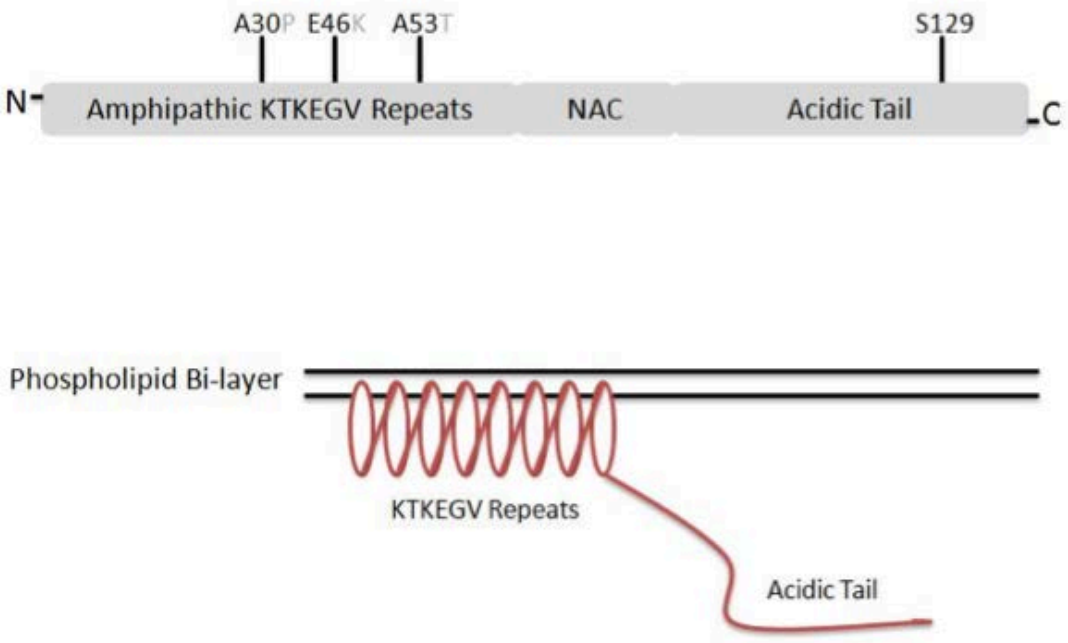


Figure 2. Primary structure of α-synuclein with KTKEGV repeats. The structure itself can be divided into three unequal regions: the imperfect KTKEGV repeats believed to form a helical structure on phospholipid membranes, the non-Aβ component (NAC) seen in Alzheimer disease-associated plaques, and a non-membrane-associated acidic c- terminal tail. Upon binding to membranes, α-synuclein assumes a helical conformation due to the amphipathic nature of the KTKEGV repeats. All three amino acid substitution sites associated with familial forms of PD (A30P, E46K, and A53T) can be found in the amphipathic repeat region (Clayton and George, 1998; Cookson, 2005).

The clearance of aggregated or misfolded α-syn plays an important role in order to maintain cellular integrity. However, the exact clearance mechanisms are still controversial; several studies reported that α-syn is seemingly degraded through the ubiquitin-proteasomal system (Webb et al., 2003; Yamada et al., 2006), the authophagy-lysosomal pathway (Cuervo et al., 2004; Crews et al., 2010), macroauthophagy (Spencer et al., 2009; Yu et al., 2009), and chaperon-mediated autophagy (Martinez-Vizente et al., 2008; Vogiatzi et al., 2008).

To gain insights related to the α-syn toxicity, *S. cerevisiae* has been used widely as a model organism (Cebollero and Reggiori, 2009; Dixon et al., 2005; Soper et al., 2008; Zabrocki et al., 2008; Vamvaca et al., 2009; Outerio and Lindquist, 2003; Sharma et al., 2006). In yeast cells, ectopic expression of human α-syn affects various cellular phenomena, such as stress responses, including ROS generation (Flower et al., 2005), cell death (Buttner et al., 2008), membrane cell interaction (Flower et al., 2007), signal transduction (Zabrocki et al., 2008), vesicular trafficking (Vamvaca et al., 2009), protein quality-control system (Liang et al., 2008), protein synthesis (Gitler et al., 2009), and lipid metabolism (Rappley et al., 2009).

More recently, Tofaris et al. (2011) reported that the Nedd4 ubiquitin ligase promotes degradation of α-syn via the endosomal-lysosomal pathway. They also demonstrated that purified yeast Rsp5, as well as Nedd4, ubiquitinates α-syn *in vitro*, and the ligase activity of Rsp5 is required for degradation of α-syn and protects against inclusion formation in *S. cerevisiae* cells. These data identified α-syn as a novel substrate protein of Nedd4/Rsp5. Therefore, the α-syn toxicity might be utilized as a model stress caused by accumulated unnecessary or disused proteins to be specifically recognized and effectively removed through the functions of Rsp5. The study of α-syn degradation mechanism might aid not only in elucidating how Nedd4-family ubiquitin ligases recognize different substrate proteins, but also in establishing a novel gene-therapy strategy for neurodegenerative diseases including PD.

CHAPTER I

Isolation of novel Rsp5 variants with higher tolerance to human α-synuclein

I.1. Introduction

Rsp5, which is the only yeast member of the highly conserved mammalian Nedd4 family of HECT-type ubiquitin ligases, participates in many biological events through ubiquitination of various target proteins. Among them, the proteolytic control of general amino acid permease Gap1 is well understood. Rsp5 is required for endocytosis of the general amino acid permease Gap1 when yeast cells have a good nitrogen source, such as ammonium (De Craene et al., 2001). Our laboratory previously isolated an *S. cerevisiae* mutant, which carries a single amino acid change, Ala401Glu, in the WW3 domain of Rsp5. In *rsp5*^{A401E} cells, Gap1, which primarily transports the toxic proline analogue L-azetidine-2-carboxylic acid (AZC) into yeast cells (Springael & André, 1998), remained stable and active on the plasma membrane, without undergoing ubiquitination, leading to hypersensitivity to AZC (Hoshikawa et al., 2003). Additionally, *rsp5*^{A401E} cells showed severer sensitivity than wild-type cells to the stresses that induce protein denaturation, such as high-growth temperature, ethanol, and heat-shock treatment (Hoshikawa et al., 2003). This suggests that Rsp5 is a key enzyme involved in degradation and/or repair of stress-induced abnormal proteins under stress conditions (Haitani and Takagi, 2008), and also that the WW domains play an essential role in interaction with those substrate proteins.

While *rsp5*^{A401E} cells are more sensitive to AZC than wild-type cells, the *RSP5*^{T357A} mutation, which has the Thr357Ala substitution in the WW2 domain, enhances AZC tolerance. These phenotypes correlate with the fact that the ubiquitination of Gap1 by Rsp5 is impaired and enhanced in *rsp5*^{A401E} and *RSP5*^{T357A} cells, respectively (Hoshikawa et al., 2003; Sasaki and Takagi, 2013). It is unclear, however, whether *RSP5*^{T357A} cells show enhanced tolerance to stresses caused by accumulation of aberrant proteins, oppositely to *rsp5*^{A401E} cells. Given the WW domains as the origin of substrate specificity, the T357A variant might act as a hyperactive form of Rsp5 selectively toward Gap1. If a specific hyperactive form for each substrate exists, it might be proven that substrate-specific conformational

changes of the WW domain might account for the molecular recognition mechanism of Rsp5.

The main purpose of this chapter is isolation of novel hyperactive forms of Rsp5. To achieve this, I constructed a random-mutagenized DNA library of the WW domains of Rsp5 by error-prone PCR. As for a model substrate of Rsp5, human α-synuclein (α-syn), a small lipid-binding protein linked genetically and neuropathologically to Parkinson's disease (PD), was adopted. Rsp5 and its mammalian ortholog Nedd4 were recently reported to directly ubiquitinate α-syn (Tofaris et al., 2011). Thus, isolation of the hyperactive Rsp5 variants toward α-syn might provide the basis for future therapeutics in most forms of PD, and potentially, other neurodegenerative diseases.

I.2. Materials and methods

I.2.1. Strains and plasmids

The *S. cerevisiae* strains, plasmids, and oligonucleotide primers used in this study are shown in Tables 1, 2, and 3, respectively. BY4741 wild-type and Δ*atg5* disruptant strains were purchased from EUROSCARF. The *RSP5*^{T357A} and *rsp5*^{A401E} mutants, and the other single gene disruptants (Δ*end3*, Δ*vps1*, and Δ*pep4*) were constructed previously (Sasaki and Takagi, 2013).

The other point base mutations A763G, A884G, C1028T, and A1285G, corresponding to Thr255Ala, Asp295Gly, Pro343Ser, and Asn427Asp, respectively, were introduced using QuikChange II XL site-directed mutagenesis kit (Stratagene) with pRS415-*RSP5* and oligonucleotide primers Rsp5-T255A-F and Rsp5-T255A-R, Rsp5-D295G-F and Rsp5-D295G-R, Rsp5-P343S-F and Rsp5-P343S-R, and Rsp5-N427D-F and Rsp5-N427D-R, respectively. The 3.4-kb PCR fragments of *RSP5* open reading frames (ORFs) with 0.5-kb upstream and downstream sequences and *Hin*dIII and *Eco*RI-recognition sites were subcloned into the *Hin*dIII/*Eco*RI-digested pRS415 or pRS406. The two-step method was used for replacements of the chromosomal *RSP5* gene in BY4741 with *RSP5*^{T255A}, *RSP5*^{D295G}, *RSP5*^{P343S}, or *RSP5*^{N427D} mutant allele on pRS406 to generate strains YI001, YI002, YI003, or YI004, respectively. The pRS415-*CgHIS3-MET15* plasmid (constructed by Dr. Morigasaki, unpublished)

was introduced into these strains to complement the auxotrophic genes to generate YI005, YI006, YI007, and YI008, respectively.

For construction of α-syn overexpression plasmid, a galactose-inducible multicopy plasmid pYES2 (Invitrogen) was used. α-Syn cDNA was amplified by PCR using pcDNA-α-syn (provided by Prof. Mochizuki) as a template and oligonucleotide primers A-syn-F and A-syn-R, and cloned into the *Kpn*I/*Xba*I site of pYES2 to construct pYES2-α-syn-HA. To fuse a GFP tag in the C-terminus of α-syn, two-step fusion PCR amplification was employed. First α-syn was amplified by A-syn-F and A-syn-R primer set, then the gene fragment encoding GFP was amplified from plasmid pFA6a-GFP-loxP_kan_loxP (constructed by Dr. Sasaki, unpublished) using primers GFP-F and GFP-R. Two fragments of α-syn and GFP ORFs were then combined by PCR amplification using primers A-syn-F and GFP-R. The unique 1.3-kbp fragment of α-syn-GFP was cloned between the *Kpn*I and *Xba*I sites of pYES2 to construct pYES2-α-syn-GFP.

I.2.2. Culture media

The culture media used for growth of *S. cerevisiae* were a nutrient rich (YPD) medium (1% yeast extract, 2% peptone, and 2% glucose), a synthetic complete (SC) medium containing 1.7 g/L yeast nitrogen base without amino acids and ammonium sulfate, 5 g/L ammonium sulfate, 20 g/L glucose, and 2 g/L drop-out mix amino acid powder lacking uracil (for SC-Ura) or lacking L-leucine and uracil (SC-Leu-Ura), and a minimal medium synthetic dextrose (SD). Yeast strains were also cultured on SD+Am agar plates containing 1 mM L-azetidine-2-carboxylic acid (L-proline analogue), 0.5 μg/mL L-canavanine (L-arginine analogue), 2 mg/mL DL-norleucine (L-methionine analogue), and 5 μg/mL fluoro-DL-phenylalanine (L-phenylalanine analogue). All of the amino acid analogues were obtained from Sigma-Aldrich. The *E. coli* recombinant strains were grown in Luria–Bertani (LB) medium (Sambrook & Russell, 2001) containing ampicillin (50 μg/mL). If necessary, 2% agar was added to solidify the medium. To examine yeast response under various stresses, Rsp5 variants also cultured on YPD medium containing LiCl, acetic acid, NaCl, or furfural at 25^{o}C or 39^{o}C (high temperature). SC medium containing galactose (SC-Gal) was used to induce overexpression of α-syn.

Table 1. Yeast strains used in this study.

Strains	Genotype	Source or reference
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
TSY235	BY4741 *RSP5*^{T357A}	Sasaki and Takagi, 2013
TSY259	BY4741 *rsp5*^{A401E}	Sasaki and Takagi, 2013
Δ*atg5*	BY4741 Δ*atg5::kanMX*	EUROSCARF
TSY260	BY4741 Δ*end3::natNT2*	Sasaki and Takagi, 2013
TSY261	BY4741 Δ*vps1::hphNT1*	Sasaki and Takagi, 2013
TSY265	BY4741 Δ*pep4::hphNT1*	Sasaki and Takagi, 2013
YI001	BY4741 *RSP5*^{T255A}	This study
YI002	BY4741 *RSP5*^{P343S}	This study
YI003	BY4741 *RSP5*^{D295G}	This study
YI004	BY4741 *RSP5*^{N427D}	This study
YI005	BY4741 *RSP5*^{T255A} [pRS415-*cgHIS3-MET15*]	This study
YI006	BY4741 *RSP5*^{D295G} [pRS415-*cgHIS3-MET15*]	This study
YI007	BY4741 *RSP5*^{P343S} [pRS415-*cgHIS3-MET15*]	This study
YI008	BY4741 *RSP5*^{N427D} [pRS415-*cgHIS3-MET15*]	This study
FW1808	BY4741 *RSP5*^{L733S}	Haitani and Takagi, 2008

Table 2. Plasmids used in this study.

Plasmids	Description	Source
pRS415	*CEN*, *LEU2*	Stratagene
pRS415-*RSP5*	Wild-type *RSP5* in pRS415	This study
pRS415-*RSP5*^{T255A}	*RSP5*^{T255A} in pRS415	This study
pRS415-*RSP5*^{D295G}	*RSP5*^{D295G} in pRS415	This study
pRS415-*RSP5*^{P343S}	*RSP5*^{P343S} in pRS415	This study
pRS415-*RSP5*^{N427D}	*RSP5*^{N427D} in pRS415	This study
pRS415-*CgHIS3-MET15*	*CgHIS3* and *MET15* in pRS415	Unpublished
pRS406	*URA3*	Stratagene
pRS406-*RSP5*^{T255A}	*RSP5*^{T255A} in pRS406	This study
pRS406-*RSP5*^{D295G}	*RSP5*^{D295G} in pRS406	This study
pRS406-*RSP5*^{P343S}	*RSP5*^{P343S} in pRS406	This study
pRS406-*RSP5*^{N427D}	*RSP5*^{N427D} in pRS406	This study
pcDNA-α-syn	Human α-syn cDNA in pcDNA3.1(+)	Provided by Prof. Mochizuki
pYES2	2μ, *URA3*, *GAL1*-promoter	Invitrogen
pYES2-α-syn-HA	α-Syn tagged with HA in pYES2	This study
pYES2-α-syn-GFP	α-Syn tagged with GFP in pYES2	This study
pFA6a-GFP-loxP_kan_loxP	GFP-loxP_kan_loxP in pFA6a	Unpublished

Table 3. Oligonucleotide primers used in this study.

Oligonucleotides	Sequence
Rsp5WW(AatII)-F	5’-ACTACCTTCGTCAAGTCCGC-3’
Rsp5WW(BglII)-R	5’-CTGGAAACACCACCGTAATC-3’
A-syn-F	5’-GGGGGTACCATGGATGTATTCATGAAAG-3’ (*Kpn*I restriction site is underlined)
A-syn-R	5’-CCCTCTAGAATTA*agcgtaatctggaacatcgtatgggta*GGCTTCAG GTTCGTAGTC-3’ (*Xba*I restriction site is underlined; HA-tag sequences are shown in italic font)
GFP-F	5’-TACGAACCTGAAGCCTCTAGAATGAGTAAAGGAGAA GAA-3’ (*Xba*I restriction site is underlined)
GFP-R	5’-ATGCGGCCCTCTAGATTATTTGTATAGTTCATCCAT-3’ (*Xba*I restriction site is underlined)
Rsp5-T255A-F	5’-AACACAAGGACTGCCACTTGGAAAC-3’
Rsp5-T255A-R	5’-GTTTCCAAGTGGCAGTCCTTGTGTTATG-3’
Rsp5-D295G-F	5’-GTTTCCAAGTGGCAGTCCTTGTGTTATG-3’
Rsp5-D295G-R	5’-TGTTACAGAGGAATTACCTGATGATCCACCAGG-3’
Rsp5-P343S-F	5’-GAGCAGCGATTTACTTCAGAAGGAAGAGCTT-3’
Rsp5-P343S-R	5’-AAGCTCTTCCTTCTGAAGTAAATCGCTGCTCC-3’
Rsp5-N427D-F	5’-TCATCGCTAGACCAAGATGTTCCACAATACAAG-3’
Rsp5-N427D-R	5’-TGTATTGTGGAACATCTTGGTCTAGCGATG-3’
RSP5-RT-F	5’-GGGTTTCTTGGCGTGGTTAAC-3’
RSP5-RT-R	5’-TTCATCCAAATGGCCCAAAA-3’
A-syn-RT-F	5’-GGAGCAGGGAGCATTGCA-3’
A-syn-RT-R	5’-GCCCAACTGGTCCTTTTTGA-3’
ACT1-RT-F	5’-TCTACTACCGCCGAACGTGAA-3’
ACT1-RT-R	5’-CAAAGCGACGTAGCAAAGTTTCT-3’

I.2.3. PCR-based random mutagenesis of the WW domains

DNA fragments corresponding to the WW domains in the *RSP5* gene were amplified from plasmid pRS415-*RSP5* by error-prone PCR with oligonucleotide primers Rsp5WW(AatII)-F and Rsp5WW(BglII)-R. Twenty microliters of T8 mutation cocktail (consisted of 0.5 μL of propanol, 0.625 μL of 20 mM $MnCl_2$, and distilled water) were mixed with 20 ng of DNA template (pRS415-*RSP5*), 10 μM of each primer (Rsp5WW(AatII)-F and Rsp5WW(BglII)-R), 10 mM dNTPs, 5 U/μL Ex-

Taq DNA polymerase, 5 µL of 10x buffer, and distilled water to bring the total volume to 50 µL. PCR program was 1 cycle of 94°C, 2 min, and 25 cycles of 94°C, 30 sec; 60°C, 30 sec; and 68°C, 1 min. The unique 1 kb amplified band was digested with *Aat*II and *Bgl*II and ligated to the *Aat*II/*Bgl*II site in pRS415-*RSP5*. The ligated DNA was used to transform *Escherichia coli* DH5α on LB solid medium containing ampicillin, and plasmid DNA prepared from approximately 30,000 ampicillin-resistant colonies was used as the mutagenized plasmid library. The construction of the library is summarized in Fig. 3.

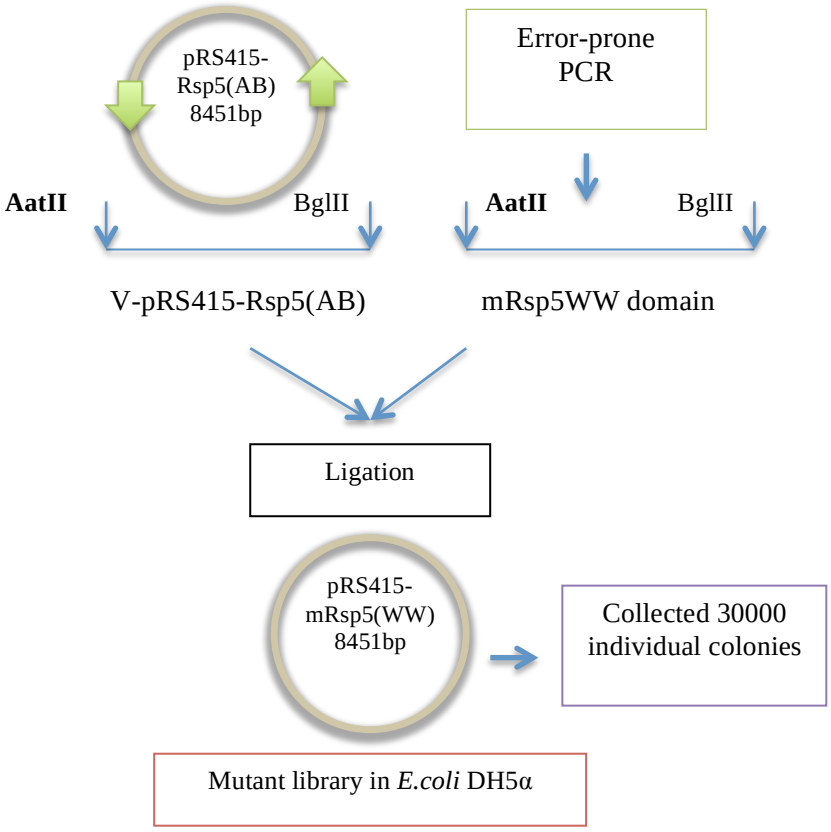


Figure 3. Construction of the WW domain-mutagenized plasmid library of Rsp5.

I.2.4. Spotted test for growth analysis

To evaluate toxicity of α-syn, yeast cells were precultured in SC-Ura for overnight and inoculated into the same medium. To assay stress tolerance of the *RSP5* mutants, YPD medium was used instead of SC medium. In both cases, initial OD_{600} was 0.25. About 4 h later, when the OD_{600} value reached 1.0, approximately 10^6 cells

were harvested. A serial dilution from 10^{-1} to 10^{-4} was spotted to the indicated media plates and incubated at 25^{o}C unless otherwise indicated.

I.2.5. Growth analysis in liquid medium

To evaluate growth inhibition of the novel Rsp5 variants when α-syn was overexpressed, I examined the growth curve of each variant expressing from the plasmid or integrated in the genome as well. Yeast cells harboring α-syn tagged with HA were cultivated in SC-Ura for overnight and inoculated into the galactose-containing medium to induce α-syn overexpression. Initial OD_{600} was adjusted at 0.15 for each strain and samples were taken every 4 h to measure OD_{600}, until the end of logarithmic phase (28 h).

I.2.6. Fluorescence microscopy

Yeast cells harboring pYES2-α-syn were cultivated to the mid-log growth phase in SC-Ura liquid medium, and then overexpression of α-syn was induced by transferring cells into the galactose-containing medium. Upon observation, cells were harvested by centrifugation and concentrated 10 times. Cells were observed immediately without fixation under a fluorescence microscope (Axiovert 200M, Carl Zeiss), and images were captured with an HBO 100 Microscope Illuminating System (Carl Zeiss) and processed using Adobe Photoshop Elements 2.0 (Adobe Systems).

I.2.7. Western blot analysis

Yeast cells were cultured to the mid-log growth phase in SC-Ura liquid medium. To induce the overexpression of α-syn, cells were transferred into the galactose-containing medium. Approximately 10^{7} cells were harvested by centrifugation. Pelleted cells were disrupted with glass beads in 10% trichloroacetic acid. Supernatants obtained after centrifugation for 5 min at 3000 × *g* were boiled in two-fold concentration of sample buffer (50mM Tris-HCl [pH 8.0], 2% SDS, 0.0125% BPB, and 2.25% glycerol) for 3 min at 95^{o}C. One microgram of protein was then loaded onto a 15% SDS-polyacrylamide gel. The proteins were transferred to PVDF membranes and then blocked with 5% skim milk (wt/vol) in 1 × TBST buffer

(20 mM Tris-base, 150 mM NaCl, hydrochloric acid to neutralize the solution to pH 7.6 and 0.1% Tween-20). Proteins were reacted with an anti-GFP mouse antibody (Roche) at 1:2,000 dilutions or an anti-α-syn mouse monoclonal antibody (BD Transduction Laboratories) at 1:4,000 dilutions, and 3-phosphoglycerate kinase (Pgk1) was detected by an anti-Pgk1 mouse antibody (Invitrogen) at 1:10,000 dilutions as a primary antibody. An HRP-conjugated anti-mouse IgG was used as a secondary antibody at 1: 2,000 dilutions. The protein targets were detected by Pierce ECL Western Blotting Substrate (Thermo Scientific) and visualized using Fuji LAS4000 imager (GE Healthcare).

I.2.8. mRNA transcription level assay by RT-PCR

Yeast cells were cultivated to the exponential growth phase (OD_{600} of 1.0) in SC-Ura liquid medium at 25^{o}C. Overexpression of α-syn was then induced with transferring the cells into the galactose-containing medium. After induction for 3 h, the cells were harvested and washed, and the whole-cell extracts were prepared by vortexing the cells with glass beads. Total RNA from *S. cerevisiae* was isolated by the RNeasy Mini Kit (Qiagen) and incubated with the RNase-free DNase set (Qiagen). Two micrograms of total RNA was reverse-transcribed using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems) following the supplier's guidelines. cDNA were amplified with the gene-specific primers and analyzed by real-time quantitative PCR performed with a 7300 Real-Time PCR System (Applied Biosystems). The mRNA levels of the target genes were normalized to those of *ACT1*, which encodes β-actin. The cycle threshold (Ct) value for each reaction was determined using the 7300 Real-time PCR System software package (Applied Biosystems). The Ct value was used to calculate the mean fold change via the $2^{-\Delta\Delta Ct}$ methods for each sample in triplicate (Livak & Schmittgen, 2001). For each gene tested, the transcription level measured in non-stressed wild-type cells was used as a standard.

I.3. Results

I.3.1. Toxicity conferred to yeast cells by overexpression of human α-synuclein

S. cerevisiae is a simple and powerful tool to examine the cellular toxicity mechanisms of human α-syn *in vivo* (Cookson, 2009) and to find novel factors that influence its toxicity in mammalian cells (Outerio and Lindquist, 2003). In this study, first I tested whether overexpression of human α-syn caused toxicity in yeast cells (Fig. 4A). In wild-type strain BY4741, plasmid pYES2-α-syn-GFP did not significantly affect cell growth on SC-Ura agar medium. In contrast, on the galactose-containing medium that induces the expression of α-syn under the control of the *GAL1* promoter, pYES2-α-syn-GFP caused a severe growth defect.

Microscopic observation indicated that overexpression of α-syn-GFP was localized on the plasma membrane at first, and gradually formed intracellular inclusions (Fig. 4B). This process started with the appearance of small foci around the plasma membrane, which grew into larger cytoplasmic aggregates, similar to Lewy Body in the PD brain (Outeiro and Lindquist, 2003). Thus, these results suggest that overexpression of α-syn confers toxicity on yeast and mammalian cells through the similar mechanism.

Next, I examined the degradation system of α-syn in yeast. *S. cerevisiae* cells employ major two proteolytic pathways as do other eukaryotic cells; autophagy and the ubiquitin-mediated system. Therefore, I tested the growth of BY4741 wild-type strain overexpressing α-syn and its disruptants of an autophagy-related gene (*Δatg5*), vacuolar proteinase A (*Δpep4*), dynamin-like GTPase required for vacuolar sorting protein (*Δvps1*), and an endocytosis-related gene (*Δend3*). Although deletion of the *ATG5* or *PEP4* gene did not significantly change the tolerance to α-syn overexpression, the *VPS1* or *END3* gene-disrupted cells show severe growth inhibition during overexpression of α-syn (Fig. 5). Western blot analysis also revealed that the accumulation of α-syn was enhanced in yeast cells defective in vacuolar protein sorting pathway (Δ*vps1*) or endocytosis (Δ*end3*) (Fig. 6). Together, these results suggest that degradation of α-syn in yeast cells requires the endocytotic pathway and protein-sorting pathway from Golgi to vacuoles.

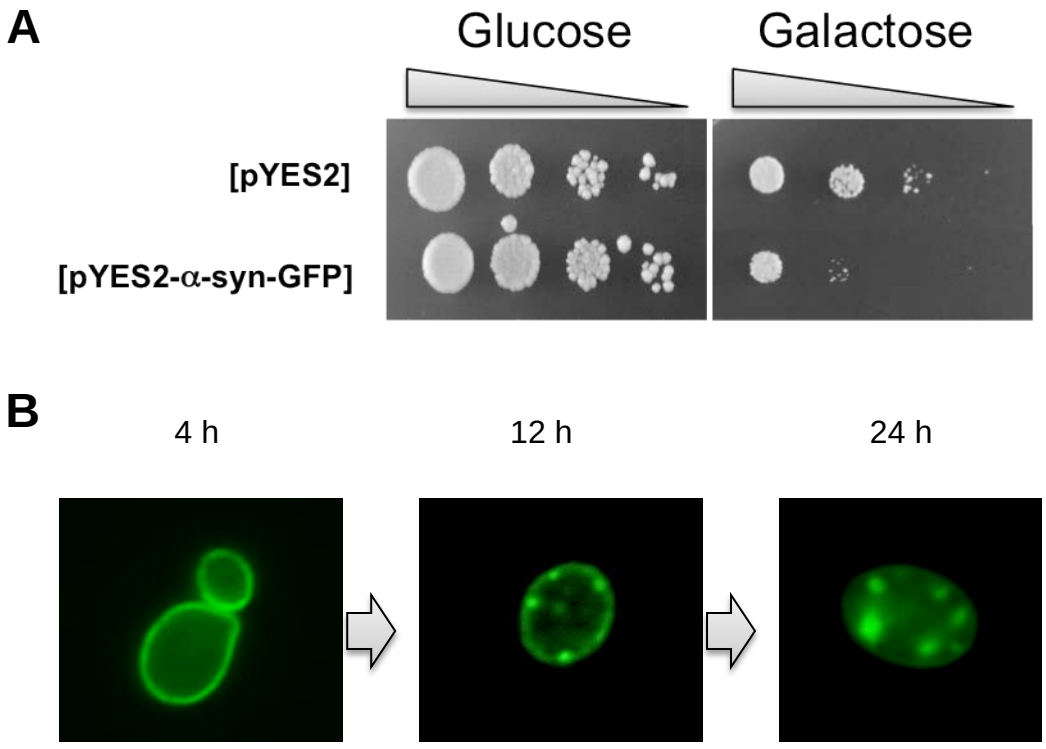


Figure 4. Overexpression of human α-synuclein. (A) Spotting growth analysis of yeast cells (BY4741). (B) Microscopic observation of α-synuclein overexpression. Shown is a time and expression-dependent redistribution of α-synuclein-GFP fusion proteins in BY4741 cells at 4, 12 and 24 h after induction of its expression. Initially, (~ 4h) α-synuclein is localized at the plasma membrane. On prolonged induction (~ 12h), α-synuclein starts to form nuclei and small membrane localized inclusions. These inclusion bodies often convert into larger cytoplasmic ones at later time points (~ 24 h).

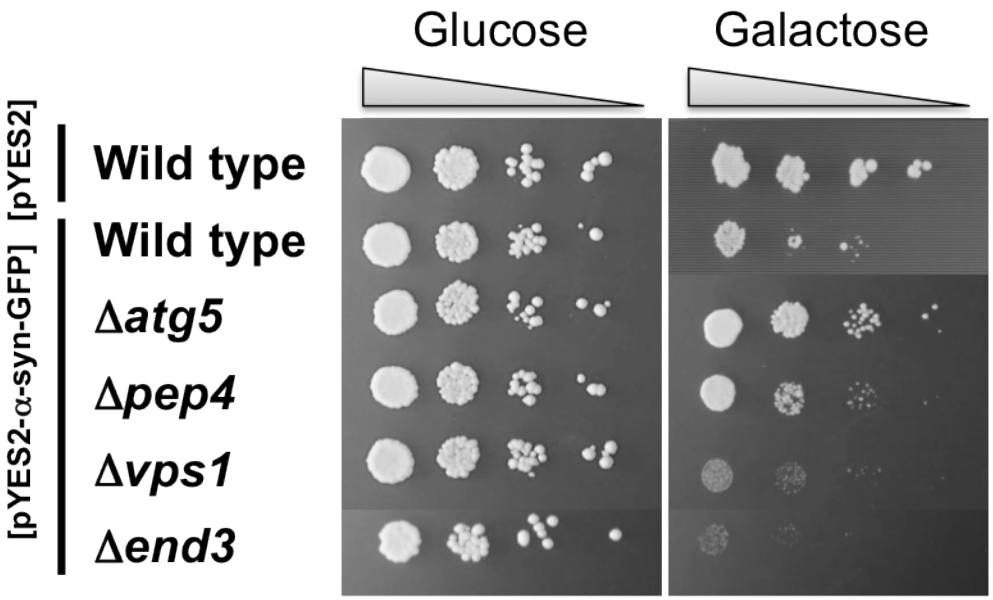


Figure 5. Spotting growth analysis of yeast cells with defect on certain degradation pathway. Four-fold serial dilution of BY4741 strain and BY4741 with defect on autophagy-related gene (*Δatg5*), vacuolar proteinase A (*Δpep4*), dynamin-like GTPase required for vacuolar sorting protein (*Δvps1*), and endocytosis (*Δend3*), harboring α-synuclein-GFP fusion spotted on the plate containing glucose (α-synuclein off) and galactose (α-synuclein on).

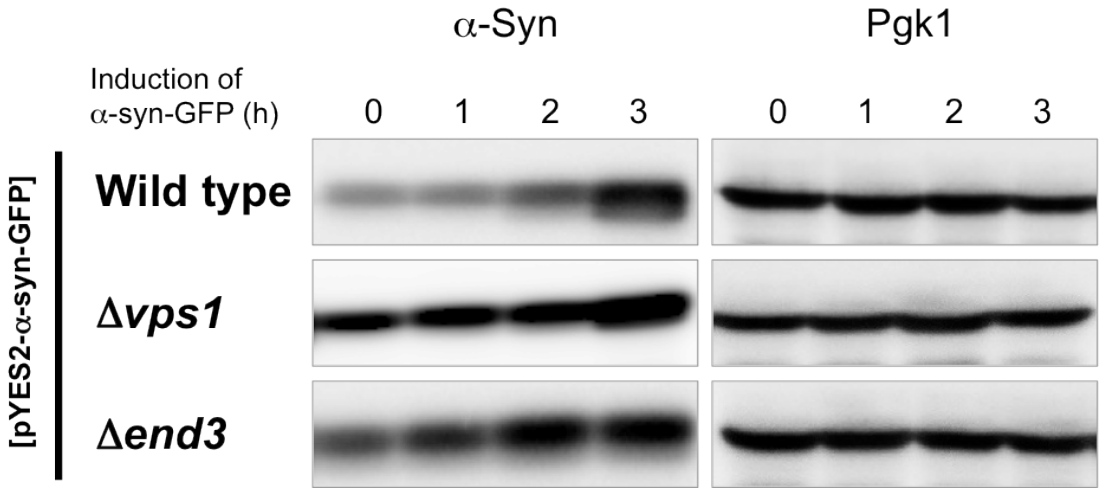


Figure 6. α-Synuclein accumulation in yeast cells with defect on the protein-sorting pathway from Golgi to vacuole (*Δvps1*) gene and endocytosis (*Δend3*). BY4741, *Δvps1*, and *Δend3* cells transformed with multicopy plasmid carrying α-synuclein-GFP were induced in the galactose-containing medium for 3 h. Accumulation of α-synuclein was observed at 0, 1, 2, and 3 h after induction. Pgk1 was used as a protein-loading control.

I.3.2. α-Syn toxicity in *rsp5*^{A401E} and *RSP5*^{T357A} mutant cells

Previously, our laboratory isolated two mutations in the WW domains of Rsp5, both of which influence endocytosis and degradation of Gap1. A loss-of-function *rsp5*^{A401E} mutant has a point mutation in the non-conserved alanine residue of the WW3 domain, and is hypersensitive to AZC, as well as to various stresses (Hoshikawa et al. 2003). On the other hand, *RSP5*^{T357A}, a point mutation in the highly conserved threonine residue of the WW2 domain, renders yeast cells less sensitive toward AZC. These phenotypes are associated with the fact that ubiquitination of Gap1 by Rsp5 is attenuated in *rsp5*^{A401E} (Hoshikawa et al., 2003) and enhanced in *RSP5*^{T357A} (Sasaki and Takagi, 2013). Hence, I first checked the growth phenotypes of these two mutants against overexpression of α-syn (Fig. 7). As a result, both mutants were unexpectedly more sensitive to overexpression of α-syn than wild-type cells. Accumulation of α-syn was higher and faster in *rsp5*^{A401E} cells than wild-type cells (Fig. 8). Taken together, these results suggest that the recognition mechanism of α-syn by Rsp5 is likely different from that of Gap1, although the function of the WW domain is required for downregulation of α-syn,

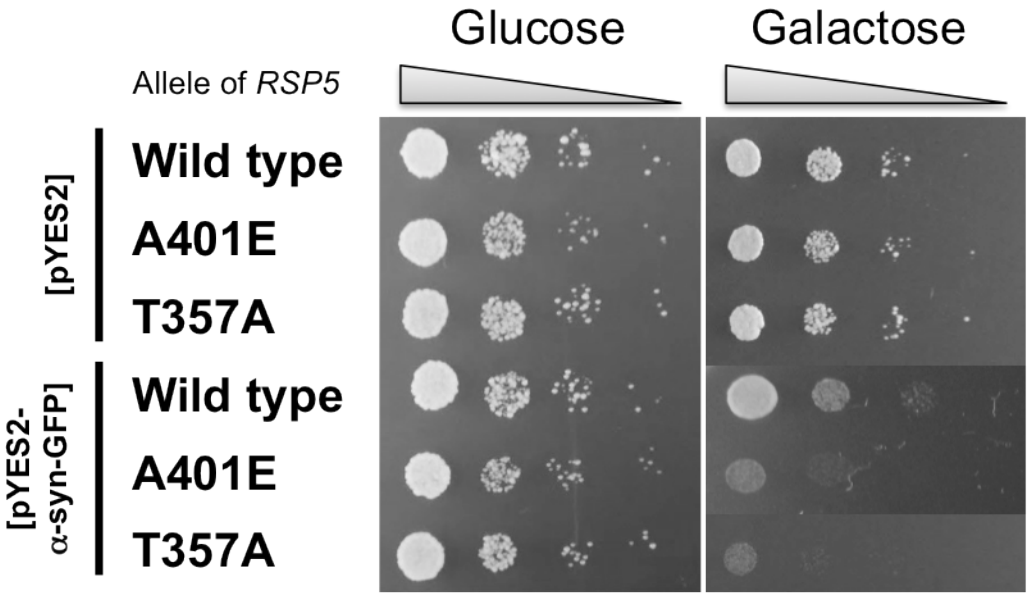


Figure 7. Toxicity of α-synuclein in yeast cells carrying *RSP5* (WT), mutated *rsp5*^{A401E} and *RSP5*^{T357A} integrated into the genomic *RSP5* locus. α-Synuclein was overexpressed with multicopy plasmid pYES2 under the control of the inducible *GAL1* promoter. Pictures are taken after 2 days incubation at 25^{o}C.

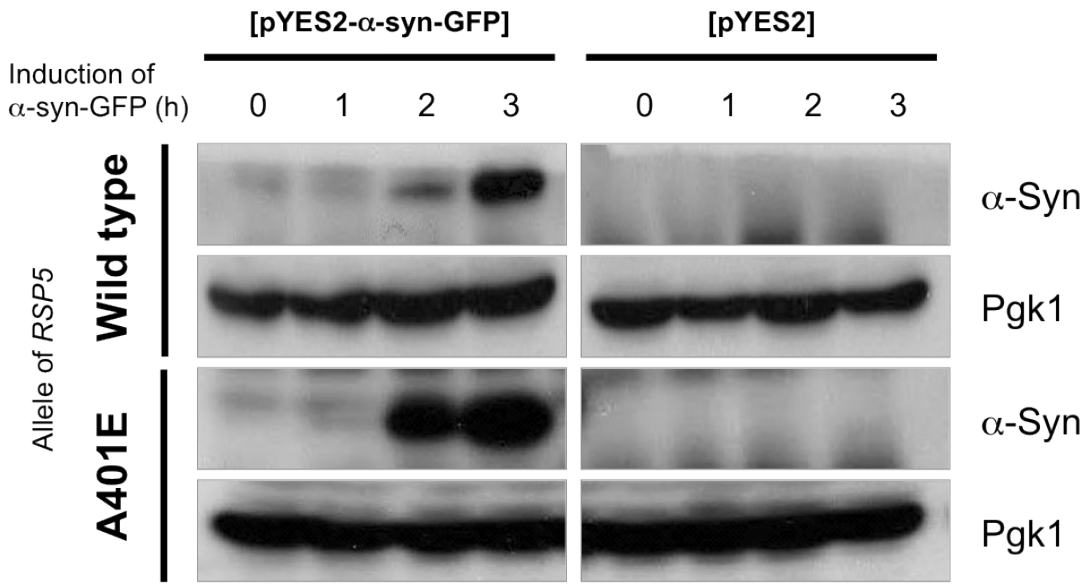


Figure 8. Expression level of α-synuclein in yeast cells harboring mutation on *rsp5*^{A401E} compared with the wild-type *RSP5* gene. Pgk1 was used as a protein-loading control.

I.3.3. Construction of the plasmid library of Rsp5 by random mutagenesis

The results described above indicate that overexpression of human α-syn confers toxicity on yeast cells, and the Rsp5-mediated endocytic pathway is involved in preventing α-syn accumulation. However, our previously isolated hyperactive *RSP5*^{T357A} mutation did not alleviate the α-syn toxicity (Fig. 7), raising the possibility that α-syn might be recognized by Rsp5 in a different manner from Gap1. I thus constructed a plasmid library in which the DNA regions encoding the WW domains of Rsp5 are randomly mutagenized in order to isolate novel Rsp5 variants that reduce the toxicity of α-syn. To generate diverse mutations in the WW domains, I performed error-prone PCR with T8 mutation cocktail, in which manganese chloride and isopropanol were used to reduce the fidelity of DNA polymerase with lower base-pairing specificity. Random mutations were introduced only into the regions containing three WW domains (amino acid residues 229-424), and as a result, 1.7 bases on the average were mutated per 1 kb. The PCR products of the WW domains were cloned into plasmid pRS415, and approximately 30,000 independent colonies of *E. coli* transformants were collected as the random-mutagenized plasmid library of *RSP5* (Fig. 9).

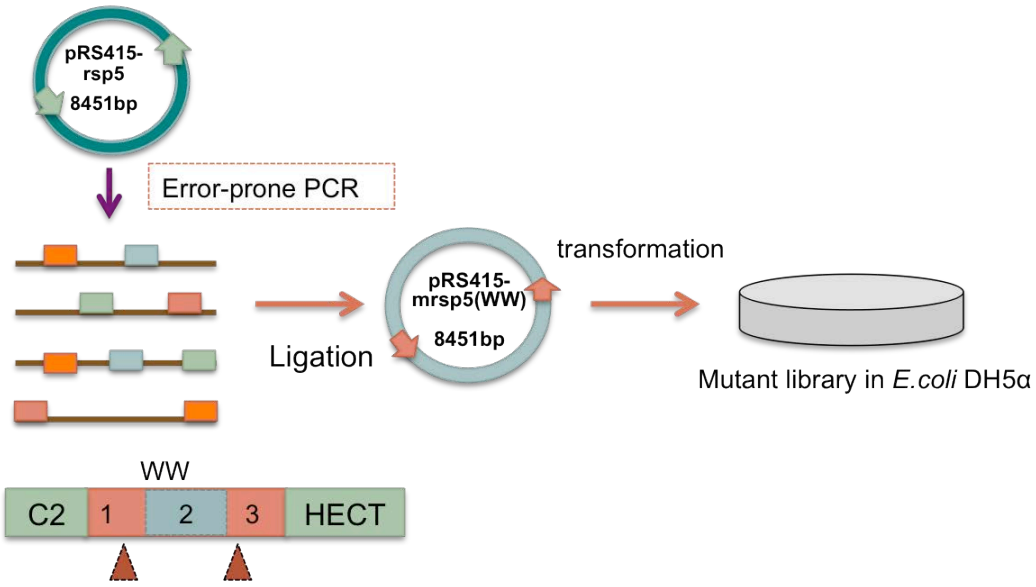


Figure 9. Schematic representation for construction of the mutagenized plasmid library. Error-prone PCR was carried out with mutation cocktail containing manganese and isopropanol to generate mutations in the WW domains of Rsp5. Plasmid pRS415-Rsp5 with *Aat*II and *Bgl*I restriction sites on its WW domain were used as a template for PCR.

I.3.4. Screening of novel Rsp5 variants with higher tolerance to α-synuclein

The plasmid library of Rsp5 WW domains was transformed into *S. cerevisiae* *rsp5*^{A401E} cells, and serial screenings were performed shown in the chart below (Fig. 10). I used this mutant as a host cell because the α-syn toxicity was amplified compared with wild-type cells. I expected that the colonies, which grew better than the cells expressing wild-type *RSP5* when α-syn was overexpressed, could contain the mutation(s) that reduce the α-syn toxicity.

The candidates obtained were then re-screened by patching each colony onto the galactose-containing agar plates, which was used for overexpression of α-syn. This patching step was repeated twice. The plasmids were extracted from the candidate colonies, shuttled to *E. coli* DH5α cells and re-introduced to *S. cerevisiae* *rsp5*^{A401E} cells to test the plasmid dependency. After the enhanced growth phenotype was confirmed, the plasmids were sequenced to identify the point-mutation sites in the DNA regions encoding the WW domains. The screening results are summarized in Table 4.

Consequently, four novel *RSP5* mutants, *RSP5*^{T255A}, *RSP5*^{D295G}, *RSP5*^{P343S}, and *RSP5*^{N427D}, were identified as candidates for the hyperactive *RSP5* mutants toward α-syn. The *rsp5*^{A401E} cells expressing these Rsp5 variants showed improved growth under conditions of α-syn overexpression compared with those expressing wild-type Rsp5 (Fig. 11). I also confirmed the proper expression levels of α-syn mRNA (derived from *SNCA/PARK1* cDNA) and the *RSP5* mRNA in these mutant cells in the galactose-containing medium (Fig. 12). Interestingly, the overexpression of α-syn doubled the *RSP5* mRNA levels in all strains, suggesting that an unknown mechanism is involved in the transcriptional control of the *RSP5* gene.

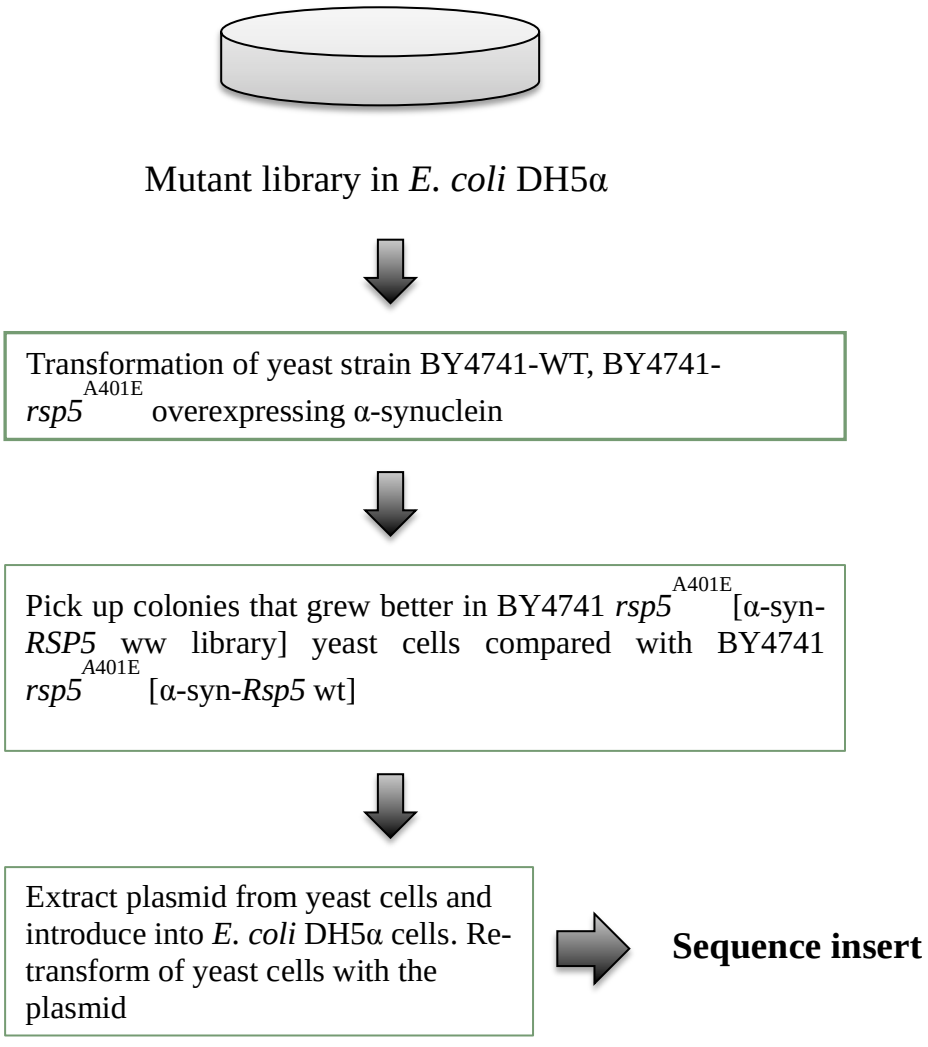


Figure 10. Flow chart of the screening method to isolate novel Rsp5 variants that improve its enzymatic activity using α-synuclein as a model stress. Three steps of screening process were done.

Table 4. Screening results to isolate novel Rsp5 variants.

First screening	2,800 colonies
Second screening	107 candidates
Third screening	22 candidates (18 candidates were wild-type), 4 mutants.
Mutants: **Thr255Ala**	1 candidate has a point mutation A → G, at the position of 255, Threonine (ACC) → Alanine (GCC), T255A in the 1st WW domain (a conserved residue in the WW domains)
Pro343Ser	1 candidate has a point mutation C → T, at the position of 343, Proline (CCA) → Serine (TCA), P343S in the 2nd WW domain (a non-conserved residue among the WW domains)
Asp295Gly	1 candidate has a point mutation A → G, at the position of 295, Aspartte (GAT) → Glycine (GGT), D295G in the non WW domain (between the 1st and 2nd WW domains)
Asn427Asp	1 candidate has a point mutation A → G, at the position of 427, Asparagine (AAT) → Aspartate (GAT), N427D in the non WW domain (between the 3rd WW domain and the HECT domain)

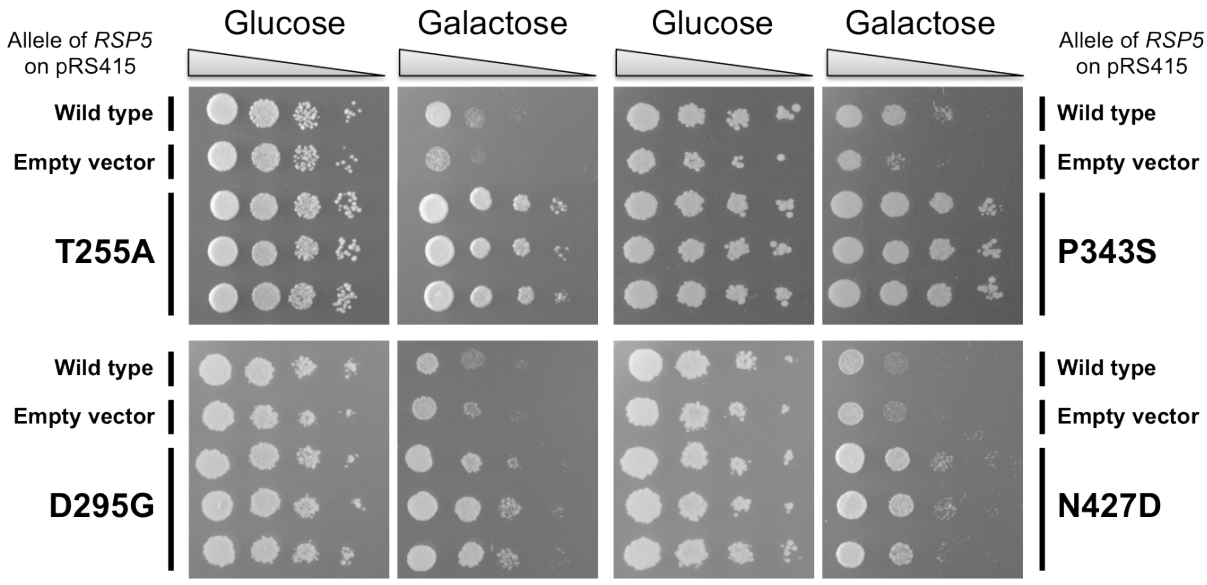


Figure 11. Novel Rsp5 variants obtained from colonies, which confer tolerance to overexpression of α-synuclein.

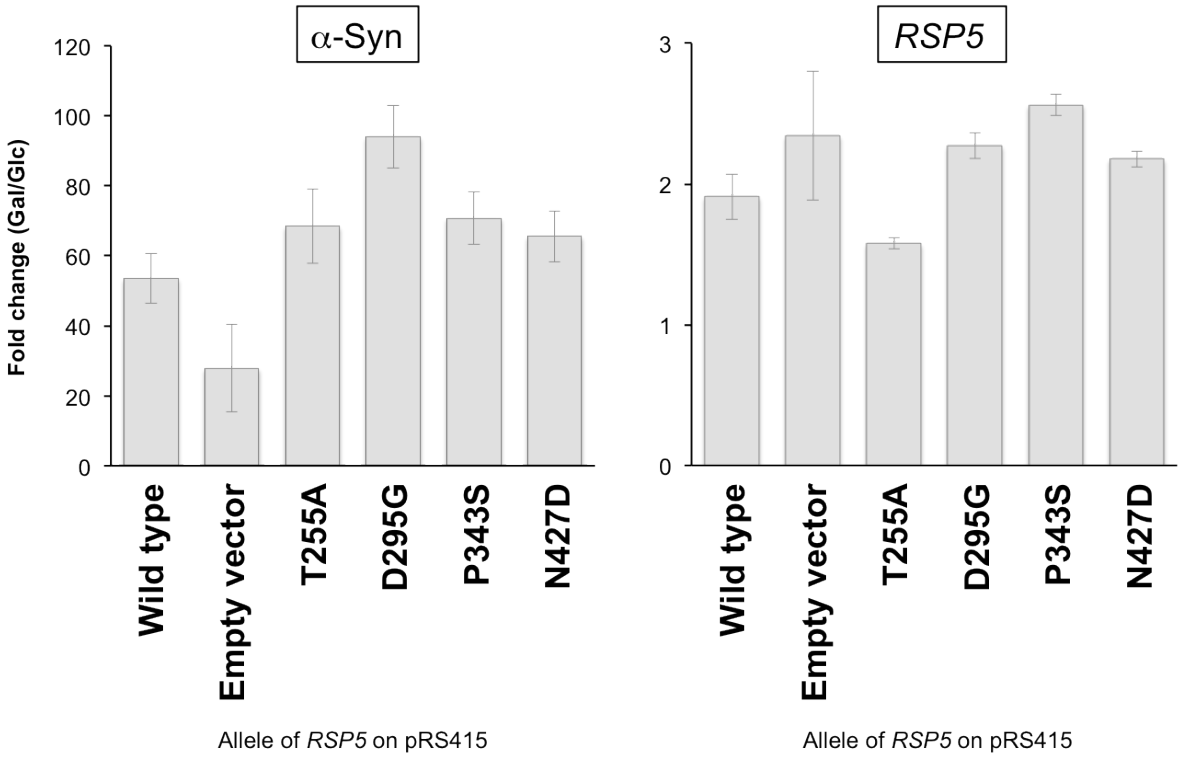


Figure 12. The mRNA transcription levels of the *SNCA/PARK1* gene encoding α-synuclein and the *RSP5* gene after induction of α-synuclein for 3 h.

Next, I measured the growth curves of *rsp5*^{A401E} cells expressing novel Rsp5 variants (Fig. 13) and of the genome-integrated *RSP5* mutants (Fig. 14) in the galactose-containing liquid medium to induce overexpression of α-syn. Both experiments confirmed that three mutants (*RSP5*^{D295G}, *RSP5*^{P343S}, and *RSP5*^{N427D}) remarkably enhanced the growth when α-syn was overexpressed. However, the *RSP5*^{T255A} mutation did not confer growth improvement in cells with the *rsp5*^{A401E} background (Fig. 13), although a significant enhancement in its growth was observed in wild-type cells (Fig. 14).

The mutated sites of *RSP5*^{T255A}, *RSP5*^{D295G}, *RSP5*^{P343S}, and *RSP5*^{N427D} were summarized in Fig. 15. These four sites have no apparent relationship; two sites (Thr255 and Pro343) are located within the WW domains, while the other two positions (Asp295 and Asn427) are located between or beside the WW domains; Thr255 is highly conserved among the other WW domains, but Pro343 is not conserved. None of these mutated sites is obviously associated with known mutation sites Thr357 and Ala401 (Hoshikawa et al., 2003; Sasaki and Takagi, 2013).

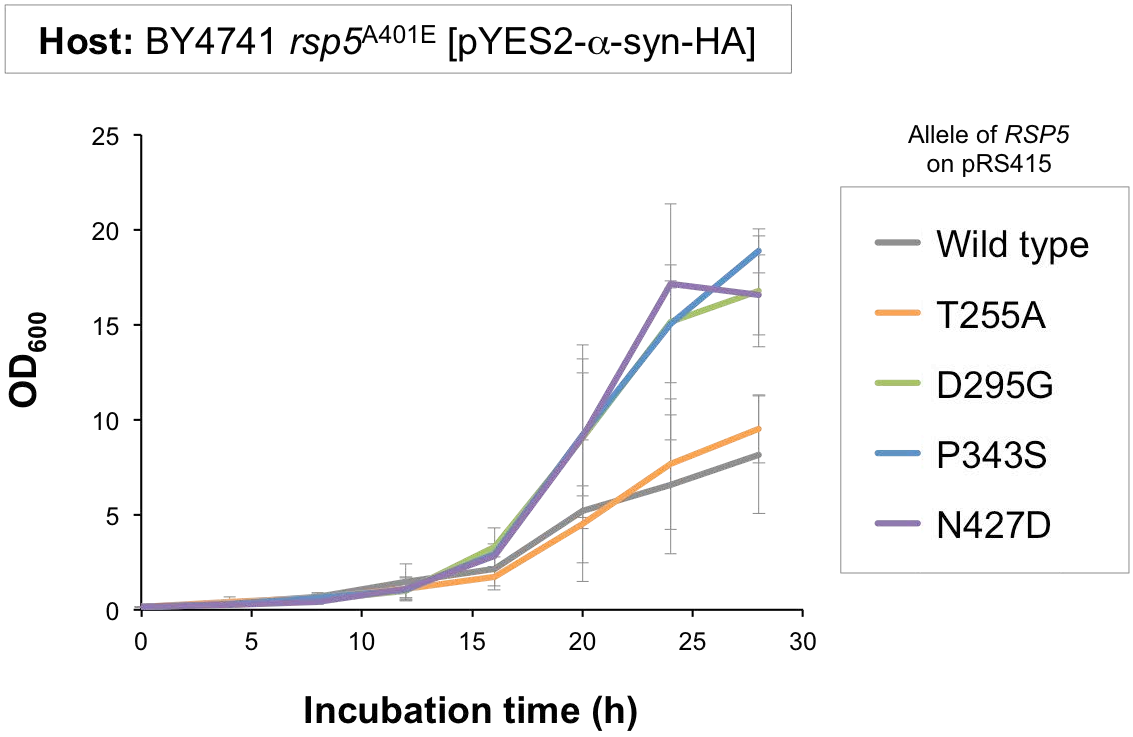


Figure 13. Growth curve of the Rsp5 variants with background of BY4741-*rsp5*^{A401E}. α-Synuclein was overexpressed in the galactose-containing medium under the control of the *GAL1* promoter. OD_{600} was measured by every 4 h.

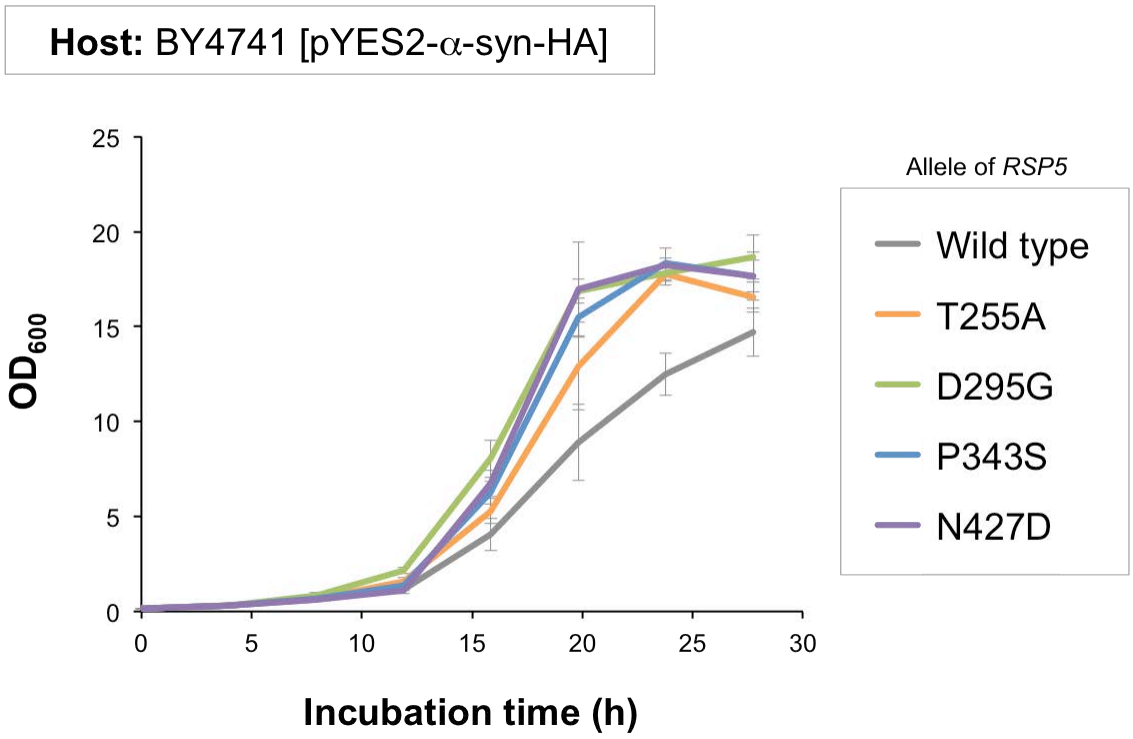
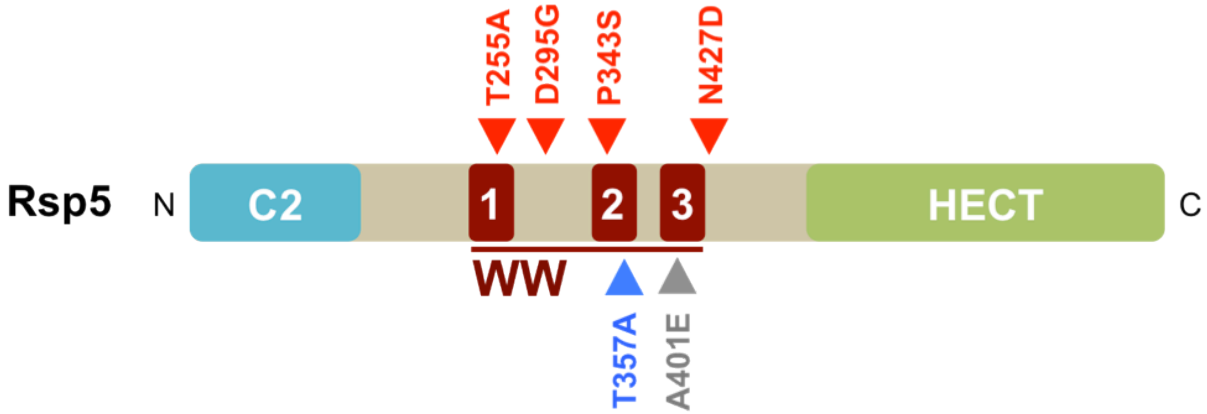


Figure 14. Growth curve of BY4741 (WT), Rsp5^{T255A}, Rsp5^{D295G}, Rsp5^{P343S}, and Rsp5^{N427D}. Yeast cells were grown overnight in SC-ura liquid medium. Initial OD_{600} of 0,15 was cultivated in the galactose-containing medium to overexpress α-synuclein. OD_{600} was measured by every 4 h.




```
  1  MPSSISVKLV AAESLYKRDV FRSPDPFAVL TIDGYQTKST SAAKKTLNPY
 51  WNETFKFDDI NENSILTIQV FDQKKFKKKD QGFLGVVNVR VGDVLGHLDE
101  DTATSSGRPR EETITRDLKK SNDGMAVSGR LIVVLSKLPS SSPHSQAPSG
151  HTASSSTNTS STTRTNGHST SSTRNHSTSH PSRGTAQAVE STLQSGTTAA
201  TNTATTSHRS TNSTSSATRQ YSSFEDQYGR LPPGWERRTD NFGRTYYVDH
251  NTRTTTWKRP TLDQTEAERG NQLNANTELE RRQHRGRTLP GGSSDNSSVT
301  VQVGGGSNIP PVNGAAAAAF AATGGTTSGL GELPSGWEQR FTPEGRAYFV
351  DHNTRTTTWV DPRRQQYIRT YGPTNTTIQQ QPVSQLGPLP SGWEMRLTNT
401  ARVYFVDHNT KTTTWDDPRL PSSLDQNVPQ YKRDFRRKVI YFRSQPALRI
451  LPGQCHIKVR RKNIFEDAYQ EIMRQTPEDL KKRLMIKFDG EEGLDYGGVS
501  REFFFLLSHE MFNPFYCLFE YSAYDNYTIQ INPNSGINPE HLNYFKFIGR
551  VVGLGVFHRR FLDAFFVGAL YKMMLRKKVV LQDMEGVDAE VYNSLNWMLE
601  NSIDGVLDLT FSADDERFGE VVTVDLKPDG RNIEVTDGNK KEYVELYTQW
651  RIVDRVQEQF KAFMDGFNEL IPEDLVTVFD ERELELLIGG IAEIDIEDWK
701  KHTDYRGYQE SDEVIQWFWK CVSEWDNEQR ARLLQFTTGT SRIPVNGFKD
751  LQGSDGPRRF TIEKAGEVQQ LPKSHTCFNR VDLPQYVDYD SMKQKLTLAV
801  EETIGFGQE*
```

Figure 15. The positions of amino acid substitutions and the sequence related to the domains in the Rsp5 molecule. Blue; the C2 domain, red; the WW domains, Green; the HECT domain.

Additionally, I checked the AZC tolerance of novel mutants (Fig. 16). Our previously found mutant *RSP5*^{T357A} clearly enhanced the AZC tolerance. On the other hand, *RSP5*^{T255A}, *RSP5*^{D295G}, *RSP5*^{P343S}, and *RSP5*^{N427D} mutant cells did not show any significant differences in growth compared with wild-type cells. These results suggest that the Rsp5-mediated mechanisms for tolerance toward AZC and toward the α-syn toxicity are genetically dissected. Thus, *RSP5*^{T255A}, *RSP5*^{D295G}, *RSP5*^{P343S}, and *RSP5*^{N427D} were identified as the novel mutants specifically alleviating the α-syn toxicity.

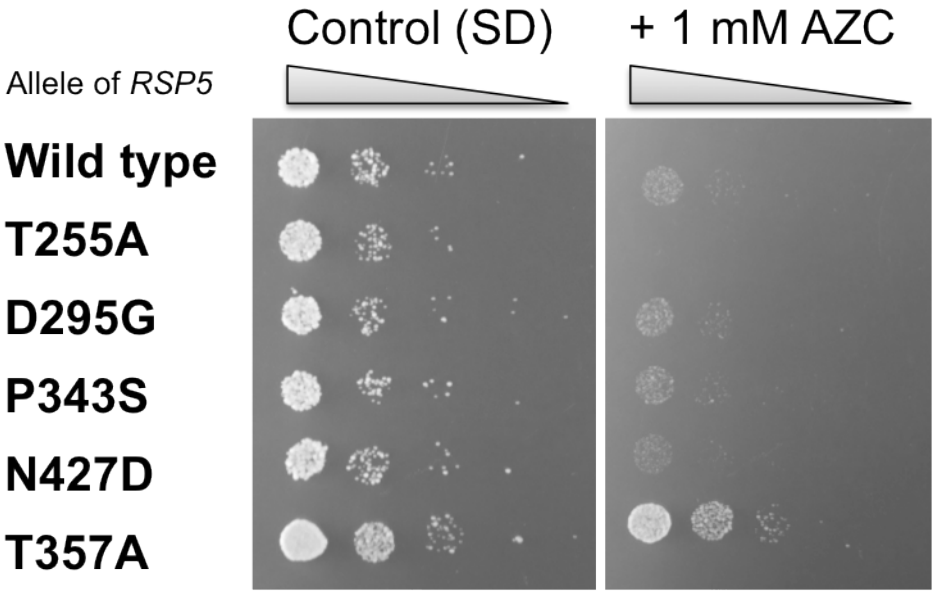


Figure 16. Growth phenotype of yeast cells on the AZC-containing agar medium. *S. cerevisiae* strain BY4741 (WT), *RSP5*^{T357A} (T357A), and novel *RSP5* mutants harboring high-copy number plasmid (2 μm) pYES2-α-syn-GFP and the complement auxotrophic plasmid pRS415-CgHIS3-MET15 were cultivated in SD liquid medium. Approximately 10^6 cells and serial dilution of 10^{-1} to 10^{-4} were spotted onto the SD agar plate in the absence or presence of 1 mM AZC. Plates were incubated at 25^{o}C for 2 days.

I.3.5. Growth phenotypes of novel Rsp5 variants under stress conditions

To understand the physiological importance of the novel mutations in the WW domains of Rsp5, I examined their effects on yeast growth phenotypes under various stress conditions (Fig. 17). Among the phenotypes examined, only the *RSP5*^{T255A} mutant cells were more tolerant to acetic acid and less tolerant to high-temperature stress (39^{o}C) than wild-type cells. There were no significant differences in the growth phenotypes between the other three mutants and wild-type cells. These results suggest that *RSP5*^{T255A} and the other three variants are phenotypically differently categorized.

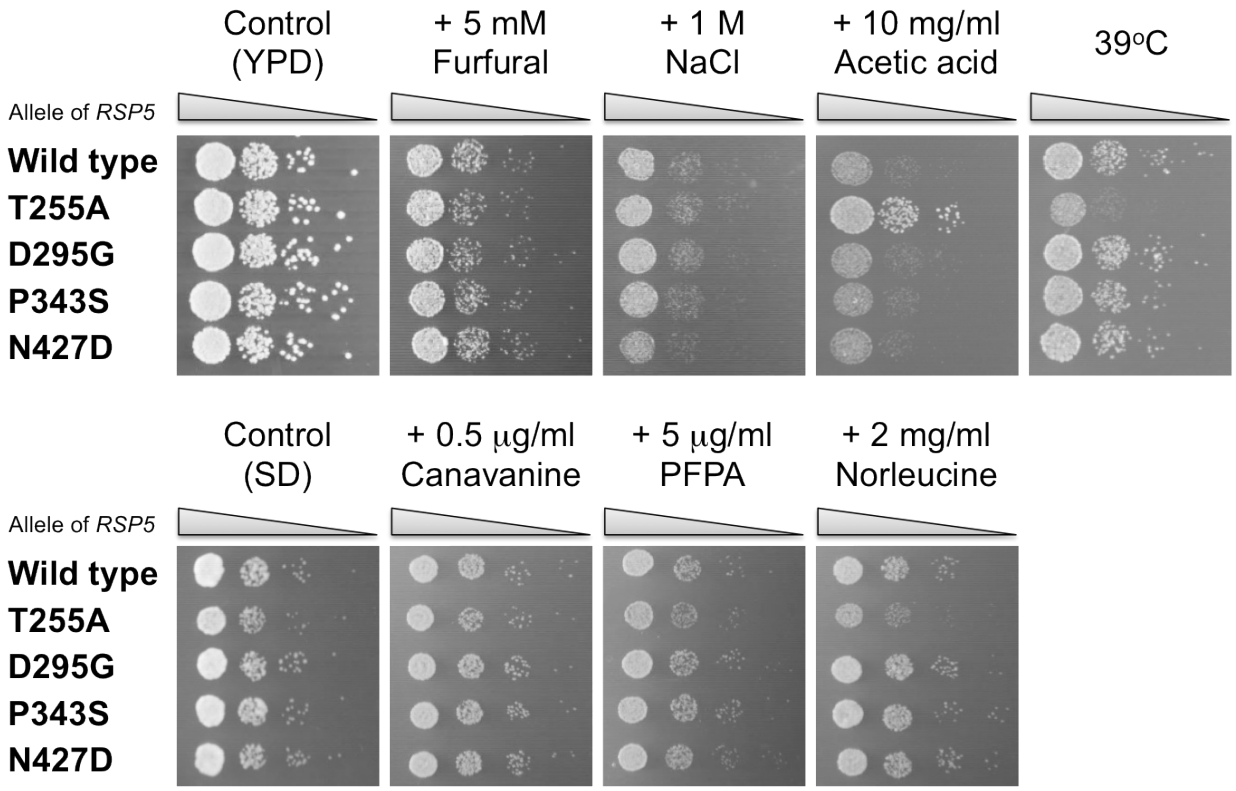


Figure 17. Growth phenotypes of Rsp5 variants under various stress conditions. The T255A mutant showed significance tolerance to acetic acid and high sensitivity against high temperature stress (39°C) compared to wild-type and other mutant cells. This point mutation might have different function in regulating the stress response requiring Rsp5.

I.4. Discussion

In this chapter, I isolated novel variants of Rsp5 that alleviated the α-syn toxicity from the random-mutagenized library of *RSP5* constructed by PCR-based mutageneis. Error-prone PCR to create the library of mutagenized genes is based on substantially increasing of the overall error frequency of Taq DNA polymerase (Labrou, 2010). This method is simple and powerful, and does not require information how the protein structure is related to its function in advance. Genetic selection or high-throughput screening subsequently identifies the mutants responsible for improved enzymatic properties (Stemmer, 1994; Kuchner and Arnold, 1997). Thus, random mutagenesis has been widely used in both basic and applied studies, such as cytosine deaminase from *Agrobacterium* (Kim et al., 2004), xylanase from

Streptomyces halstedii JM8 (Diaz et al., 2004). Previously, our laboratory isolated a hyperactive Rsp5 variant toward Gap1 (T357A), using the error-prone PCR method (Haitani et al., 2009; Sasaki and Takagi, 2013).

As a model stress, I used the toxicity of α-syn, a small lipid-binding protein. First, I verified the toxicity of α-syn in yeast cells by the spotted test and the microscopic observation (Fig. 4). In a haploid yeast strain BY4741, which I used for all the experiments in this study, obvious growth inhibition and inclusion body formation were clearly observed when α-syn was overexpressed. These results differ from Sharma et al. (2006), in which they reported no toxicity of α-syn in S. cerevisiae strains TSY623, BY4741, and BY4743. In my study, the different points with the work by Sharma et al. (2006) are (i) the tag they used to check the growth of BY4741 strain is GFP, instead of HA-tag in my experiment; (ii) in the spot test, they adjusted OD_{600} to 2.0 and spotted cell suspensions with five-fold serial dilution, whereas I adjusted OD_{600} to 1.0 and spotted with ten-fold serial dilution; (iii) induction time of α-syn was not shown in their microscopic analysis, therefore, if induction time was prolonged, the aggregation of α-syn might be also occurred in their experiments. In accordance with Volles and Lansbury (2007), the toxicity of α-syn was clearly demonstrated in another laboratory strains W303 and FY23 (Flower et al., 2005).

The mechanisms of α-syn degradation have been controversial. In mammalian cells, clearance of α-syn was reported to occur via both the ubiquitin-proteasomal system (Webb *et al*., 2003; Yamada *et al*., 2006) and the authophagy-lysosomal pathway (Cuervo *et al.*, 2004; Crews *et al.*, 2010). In yeast, it was also shown that α-syn was degraded via both proteasome and autophagy (Zabrocki et al., 2005). However Tofaris et al., (2011) reported that blockage of proteasomal function fail to prevent α-syn degradation by NEDD4 and recent study conducted by Petroi et al.,(2012) demonstrated that treatment with MG132, the proteasomal inhibitor could not prevent the clearance of α-syn in yeast. The finding of multiple routes for α-syn degradation in yeast, such as proteasome and autophagy, apparently associate to the fraction of α-syn, whether in form of cytosolic protein, fibril or aggregate related to its stage of pathobiology in cells (Ebrahimi-Fakhari et al., 2012).

In this study, however, I showed that yeast cells lacking the genes related to the vacuolar protein sorting (Δ*vps1*) or endocytosis (Δ*end3*) exhibit enhanced α-syn toxicity, but an autophagy mutant Δ*atg5* did not alleviate the toxicity (Figs. 5 and 6). Consistently, I proved that the function of the WW domains of Rsp5 is required for

effective degradation of α-syn, by using the *rsp5*^{A401E} mutant (Figs. 7 and 8). These results suggest that the degradation pathway of α-syn in yeast mainly requires the ubiquitination-mediated endocytosis-vacuolar pathway. Similar results were obtained from the previous works (Tofaris et al., 2011; Willingham et al., 2003).

Although the known hyperactive form of Rps5 (*RSP5*^{T357A}) was tolerant against AZC (Fig. 7), it did not exhibit tolerance toward α-syn overexpression (Fig. 8). And vice versa, the novel Rsp5 variants were tolerant to the α-syn toxicity, but they did not show enhanced tolerance against AZC. These results strongly suggest that the point mutations in the WW domains affect the substrate specificity of Rsp5. Based on this, I hypothesize that the WW domains play a pivotal role in the recognition of various substrate proteins. Furthermore, among the novel mutants that show enhanced tolerance to α-syn, only *RSP5*^{T255A} exhibited distinct phenotypes: sensitivity to high-temperature stress and resistance to acetic acid stress (Fig. 17). Tolerance to acetic acid found in *RSP5*^{T255A} cells is particularly intriguing, because this phenotype might be useful for improving the fermentation properties with acetic acid treatment in food and brewing industries or in bioethanol production. The mechanism underlying how Rsp5 regulates acetic acid tolerance is now under investigation in our laboratory. Such a study also might provide more information concerning the role of the WW domains in the substrate-specific recognition mechanisms by Rsp5.

CHAPTER II

Functional analysis of novel Rsp5 variants with higher tolerance to human α-synuclein

II.1. Introduction

The WW domains responsible for substrate specificity of Rsp5, mutation in the WW domain often causes temperature sensitivity (Dunn & Hicke, 2001). In addition to the importance of WW domain as substrate binding module, the WW domains, but not C2, are important for internalization of Fur4 and Ste2 and for fluid phase endocytosis (Gajewska et al., 2001; Dunn & Hicke, 2001). Mutation of either the Sna3 PPXY motif or the Rsp5 WW3 domain or reduction in the amount of Rsp5 results in mistargeting of Sna3 to multiple mobile vesicles and prevents its sorting to the endosomal pathway (Stawiecka-Mirota et al., 2007). In particular, the WW3 domain (amino acids 387-424) and the WW2 domain are required for the essential in vivo function of Rsp5 (Chang et al., 2000; Wang et al., 2001). The mutation at position 401 of Rsp5 WW3 domain render sensitivity to various stresses, including high temperature, LiCl, AZC, and many other amino acid analogue (Hoshikawa et al., 2003), while mutation in Rsp5 WW2 domain T357, affect the hyperactivity to down-regulate Gap1 to vacuole through ubiquitination (Sasaki and Takagi, 2013).

In Chapter I, I revealed that the detoxification of α-synuclein (α-syn) in *S. cerevisiae* requires the function of the WW domains of Rsp5 (Figs. 7 and 8), and the subsequent the endocytic and protein-sorting pathways from Golgi to vacuoles (Figs. 5 and 6). By a genetic screening established in this study, I isolated four novel Rsp5 variants, T255A in the WW1 domain, D295G in between the WW1 and WW2 domains, P343S in the WW2 domain, and N427D in the vicinity of the WW3 domain, which showed enhanced growth during the overexpression of α-syn (Figs. 11, 13, 14, and 15). Thus, the main purpose of this chapter is to examine whether these variants actually enhance the clearance of α-syn, such as interaction, ubiquitination, and degradation.

II.2. Materials and methods

II.2.1. Measurement of intracellular ROS level

Intracellular ROS levels were determined by flow cytometric analyses using a fluorescent probe dichlorofluorescin diacetate (DCFDA). Cells were grown to the mid-log phase in SC-Ura liquid medium for overnight. Approximately 1×10^7 cells were harvested by centrifugation at 3,000 × *g* for 5 min, suspended in 1 mL of 20 μM DCFDA in PBS buffer, and incubated at 30°C for 30 min in the dark place. Stained cells were then exposed to 0.5 mM H_2O_2 stress as a positive control or transferred to the galactose-containing medium to overexpress α-syn by making a 1:20 dilutions. Fluorescence of the oxidized fluorescent DCFDA probe was measured by the Accuri C6 flow cytometer (BD Biosciences) using the FL-1 filter (green fluorescence) at 0, 3, and 6 h after inoculation. A minimum of 20,000 events was collected for each experiment. Data analysis was performed using the CFlow software for Accuri Cytometer (BD Biosciences). Results were expressed as median fluorescence intensity (MFI) of the cells. The mean values of three independent experiments were statistically analyzed using Student's *t*-test.

II.2.2. α-Synuclein degradation assay

Degradation of α-syn after shutoff of its gene expression was analyzed by Western blot. Cells were cultivated in SC-Ura medium to the logarithmic growth phase, and then overexpression of α-syn was induced by the addition of galactose for 3 h. The shutoff of gene expression was done with changing the medium to SC-Ura. The levels of α-syn remaining in the cells were analyzed at 0, 3, and 6 h after shutoff. Approximately 1×10^8 cells were collected, suspended in 10% trichloroacetic acid (TCA), and disrupted with glass beads in a Multibead shocker (Yasui Kikai). Proteins in the whole cell extracts were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (15% (w/v) polyacrylamide), transferred to a 0.45-mm PVDF membrane, blocked with 5% (w/v) powdered milk at 4°C for overnight, and reacted with an anti-α-syn antibody (BD Transduction Laboratories) at 1:4,000 dilutions, an anti-Rsp5 rabbit polyclonal antibody (supplied by Dr. Huibregste) at 1:4,000 dilutions, an anti-Pgk1 mouse antibody (Invitrogen) at 1:10,000 dilutions, or an anti-

GAPDH rabbit antibody at 1: 10,000 dilutions, as a primary antibody for 30 min. After several washing steps, the membrane was incubated for 60 min with HRP-conjugated anti-mouse or anti-rabbit IgG as a secondary antibody at 1: 2,000 dilutions, and washed three times (30 min/wash). The target proteins were detected by Pierce ECL Western Blotting Substrate (Thermo Scientific) and visualized using Fuji LAS4000 imager (GE Healthcare). Quantification of the signals was performed using ImageJ software.

II.2.3. Co-immunoprecipitation analysis

Yeast cells harboring pYES2-α-syn-GFP were cultivated to the exponential growth phase in SC-Ura medium at 25°C. After 3 h induction of α-syn with galactose, cells were harvested, suspended in ice-cold lysis buffer B88 (20 mM HEPES, 150 mM potassium acetate, 5 mM magnesium acetate, 250 mM sorbitol, pH 6.8) containing protease inhibitors cocktail (Sigma) and 1% Triton X-100, and then disrupted by rigorously agitating with glass beads using a Multibead shocker (Yasui Kikai) at 4°C seven times for 30 sec with 30-sec intervals between each burst. Unbroken cells were removed by centrifugation at 3000 × *g* for 5 min. The cleared lysate was mixed with the anti-Rsp5 rabbit polyclonal antibody and incubated at 4 °C for 90 min with gentle rotation. Protein G-Sepharose beads (GE Healthcare) washed three times with ice-cold B88 buffer containing 1% Triton X-100 were added, and the incubation was continued at 4°C for another 1 h. The beads were washed five times with B88 containing 1% Triton X-100 and then heated in SDS sample buffer (Tris-HCl [pH 8.0], 2% SDS, 0.0125% BPB and 2.25% glycerol) at 65^{o}C for 15 min. Each sample was subjected to SDS-PAGE, and the indicated proteins were detected with the anti-Rsp5 rabbit polyclonal antibody, the anti-α-syn mouse antibody, or the anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit antibody (Nordic Immunological Laboratories) as a protein-loading control.

II.2.4. *In vivo* Ubiquitination assay

For the detection of ubiquitin conjugates of α-syn-GFP, yeast cells harboring pYES2-α-syn-GFP and pUbi or pMyc-Ubi (provided by Dr. Kitabatake) were grown to the logarithmic growth phase in SC-His-Ura medium at 25 °C. As a negative

control, strain *rsp5-1* (FW1808), a temperature sensitive mutant of Rsp5, was grown at the restrictive temperature (35^{o}C) during the overexpression of α-syn. After induction of α-syn and Myc-tagged or untagged ubiquitin for 15 h, cells were harvested, suspended in SDS buffer (1% SDS, 45 mM Na-HEPES [pH 7.5], 50 mM NEM, protease inhibitors cocktail (Sigma), and 0.1mM PMSF), and then lysed with glass beads. Lysates were diluted in Triton buffer (1% Triton X-100, 150 mM NaCl, 50 mM Na-HEPES [pH 7.5], 5 mM Na-EDTA, and 10 mM NEM with protease inhibitors and 0.1mM PMSF). After centrifugation (15 min at 14,000 × *g*), lysates were solubilized by incubation with the five-fold concentration of sample buffer at 65°C for 15 min. Samples were loaded on a 12% SDS-polyacrylamide gel. The α-syn protein was detected by anti-α-syn mouse antibody (BD Transduction Laboratory) at 1:4,000 dilutions. The ubiquitin-conjugated protein was detected using an anti-ubiquitin mouse antibody [P4D1] (Santa Cruz Biotechnology) at 1:2,000 dilutions.

II.3. Results

II.3.1. Reactive oxygen species levels in yeast cells overexpressing α-synuclein

Overexpression of α-syn was shown to induce intracellular reactive oxygen species (ROS) generation and growth inhibition in both mammalian (Zang et al., 2005) and yeast cells (Flower et al., 2005). In order to assess the functions of the novel Rsp5 variants, which could detoxify α-syn, I first measured the intracellular ROS levels of the *RSP5* mutants overexpressing α-syn (Fig. 18). The cells after exposure to H_2O_2 were used as a positive control for ROS accumulation, and the cells without α-syn overexpression were used as a negative control (data not shown). It was confirmed that wild-type cells accumulated intracellular ROS as a consequence of the α-syn overexpression. After 6-h induction of α-syn, wild-type cells showed approximately 155% increase in the ROS levels.

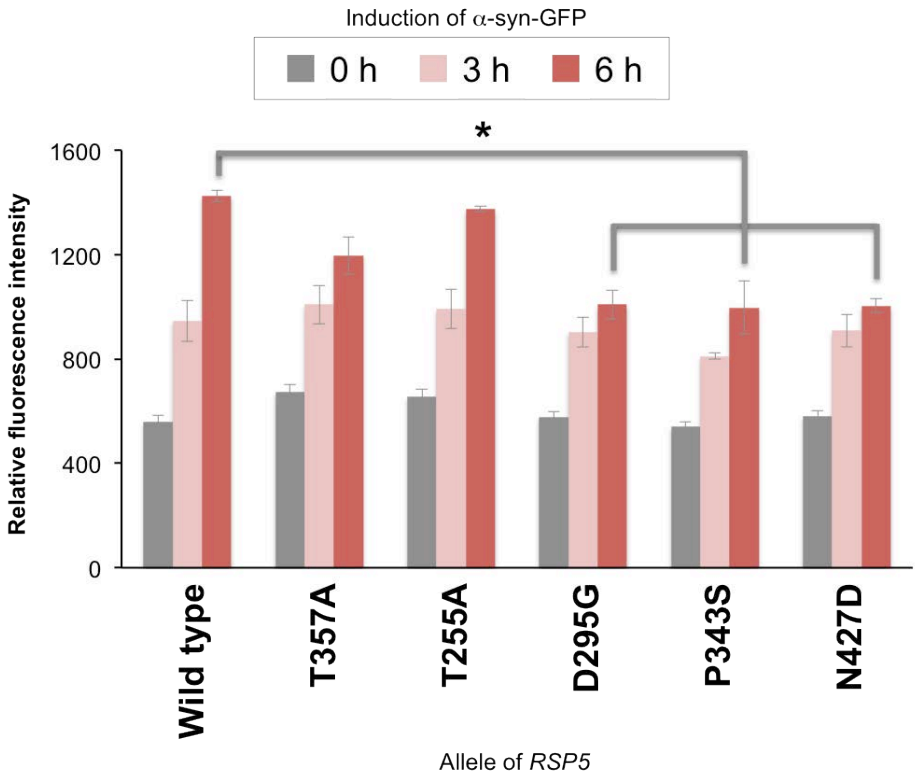


Figure 18. Intracellular ROS levels in Rsp5 variants during the overexpression of α-synuclein. ROS level was measured by flow cytometric analyses. Yeast cells grown to the log phase were collected (≈10^7 cells or OD_{600}=1.0) and treated with 20 μM DCFDA in PBS buffer for 30 min. Cells diluted in 1:20 were inoculated in the galactose containing medium to induce overexpress α-synuclein. Flow cytometry analysis was performed by DCFDA green fluorescence in FL-1 at 0, 3, and 6 h.

Three of the novel Rsp5 variants (D295G, P343S, and N427D) significantly reduced intracellular ROS levels caused by overexpression of α-syn (Fig. 18). The D295G, P343S, and N427D mutations increased the ROS levels by 74%, 84%, and 73%, respectively. These results indicate that the novel Rsp5 variants suppressed one of the cellular defects caused by the α-syn stress. In contrast, the ROS levels were clearly increased by the other variants T357A and T255A, as shown in wild-type cells. This is consistent with the fact that the T357A hyperactive mutation toward Gap1 did not alleviate the α-syn toxicity (Fig. 7). Although it was shown that the T255A variant was tolerant to α-syn (Figs. 13, 15, and 16), the T255A variant might detoxify α-syn via an unknown mechanism different from the D295G, P343S, and N427D variants.

II.3.2. Degradation of α-synuclein mediated by Rsp5 variants

Next, I wondered whether the Rsp5 D295G, P343S, and N427D variants reduced the ROS level directly by enhancing the degradation of α-syn. For this purpose, I examined the levels of galactose-induced α-syn after shut-off of its gene expression in the presence of glucose (Fig. 19). The wild-type or each variant Rsp5 was expressed from a centromeric plasmid pRS415 in yeast cells with *rsp5*^{A401E} background. Also, I quantified the signal intensity of α-syn using the Image J software and displayed as relative values in comparison to that of a loading control protein (Fig. 20). As a result, about 70% decrease in the α-syn levels was observed in the wild-type Rsp5. In contrast, three of the Rsp5 variants, D295G, P343S, and N427D, significantly reduced the α-syn levels by approxomately 80-90% during 6-h shut-off of the gene expression. It is thus suggested that these Rsp5 variants enhanced detoxification of α-syn overexpressed in yeast cells by promoting the degradation rates. However, the T357A and T255A variants decreased the α-syn levels similarly or less effectively than wild-type Rsp5. This result is consistent with the facts that *RSP5*^{T357A} did not alleviate the α-syn toxicity (Fig. 7) and that *RSP5*^{T255A} was phenotypically distinct from the three other variants (Figs. 17 and 18). A similar conclusion was obtained by analyzing the cells in which the Rsp5 variants were integrated into the authentic locus in the genome (Figs. 21 and 22). In this case, the α-syn levels were reduced by more than 60% after 6-h shut-off, although the wild-type Rsp5 decreased only about 40% of the α-syn levels.

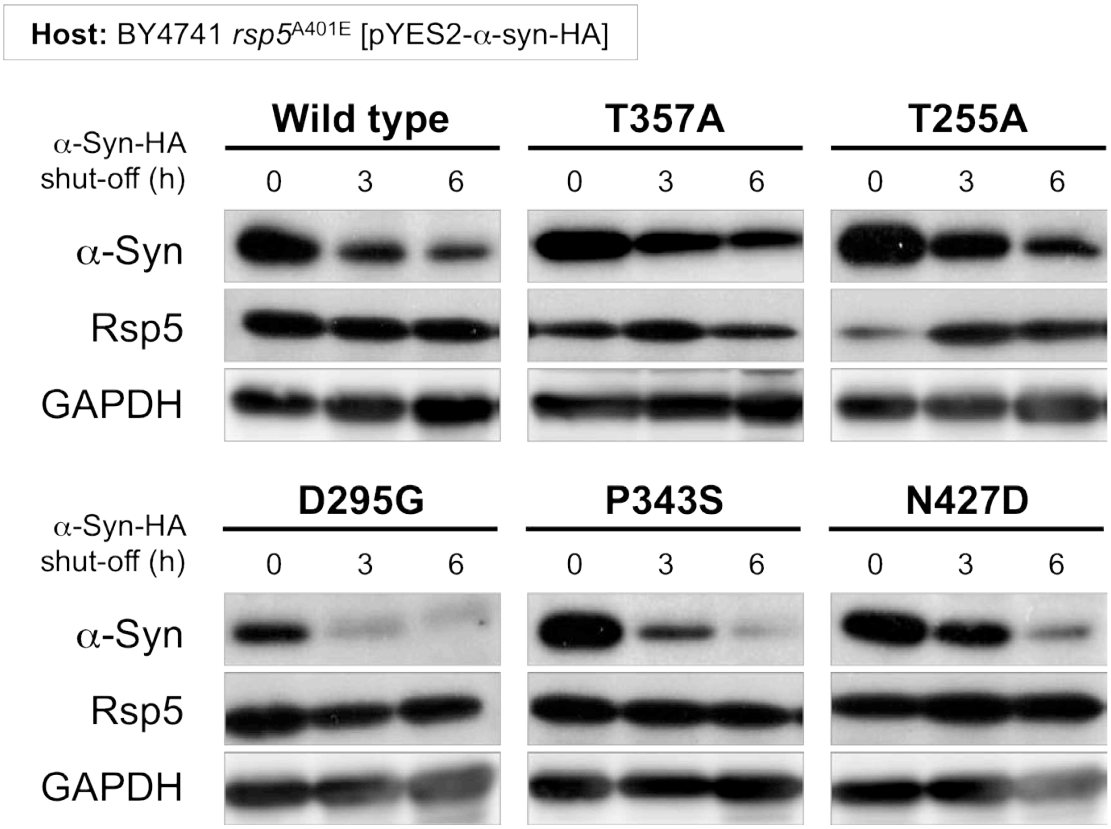


Figure 19. Degradation of α-synuclein mediated by Rsp5. Three of Rsp5 variants (D295G, P343S, and N427D) accelerated the degradation of α-synuclein after shut-off of α-synuclein expression. GAPDH was used as a protein-loading control.

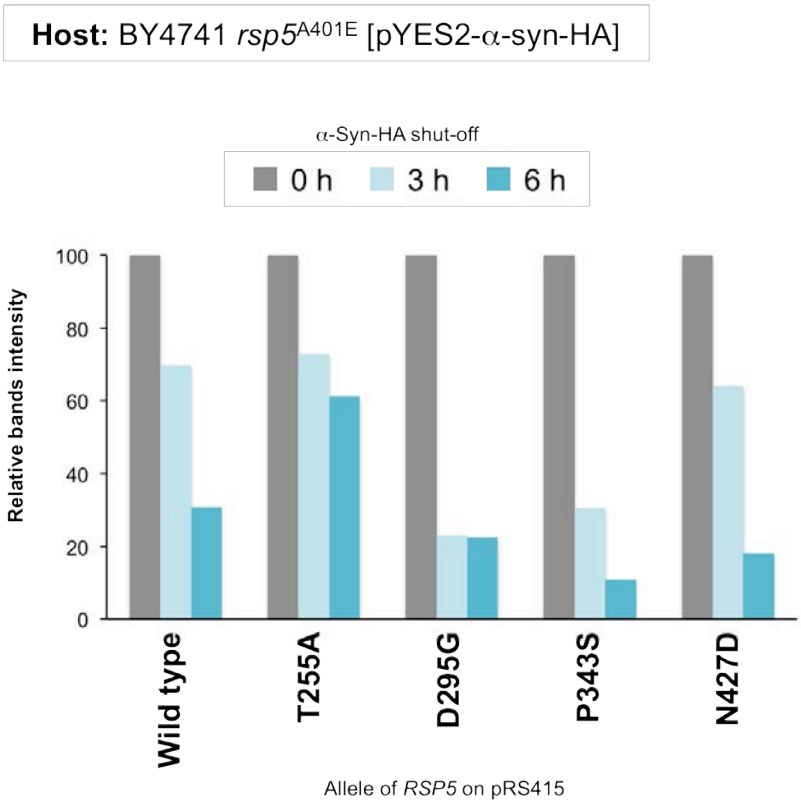


Figure 20. Quantification of the α-synuclein levels from Western blot analysis.

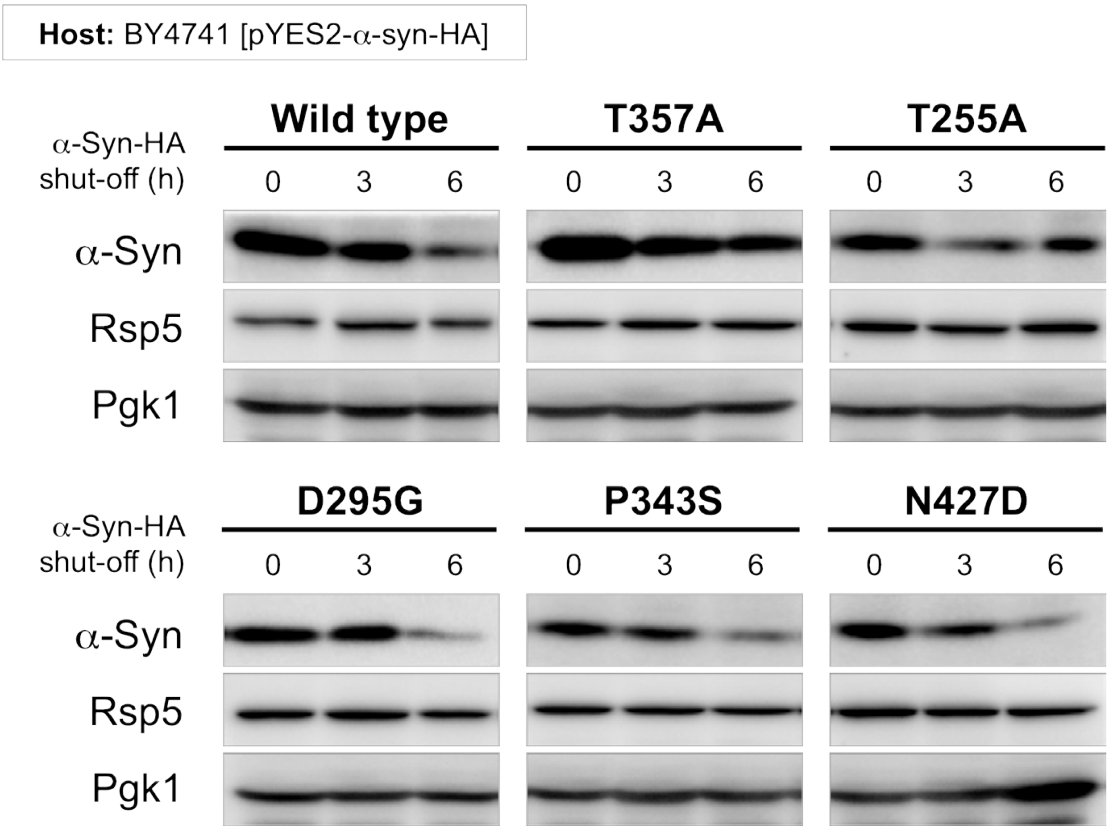


Figure 21. Degradation of α-synuclein mediated by Rsp5. Three of Rsp5 variants (D295G, P343S, and N427D) accelerated the degradation of α-synuclein after shut-off of α-synuclein expression. Pgk1 was used as a protein-loading control.

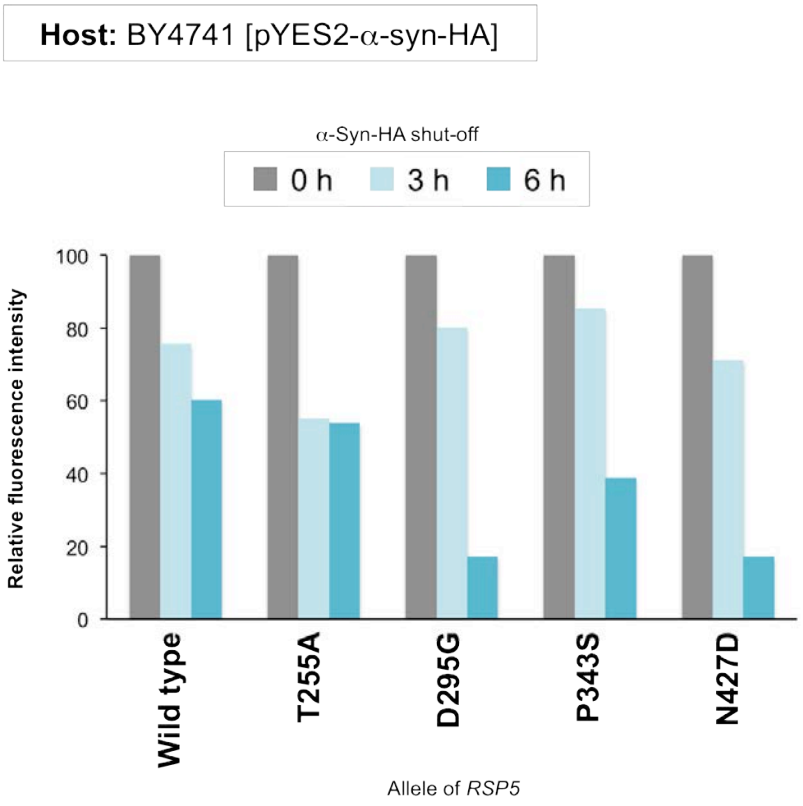


Figure 22. Quantification of the α-synuclein levels from Western blot analysis.

II.3.3. Recognition of α-synuclein by Rsp5 variants: co-IP experiment

As described above, I demonstrated that three mutations in the WW domains of Rsp5 (D295G, P343S, and N427D) significantly accelerated the degradation of α-syn. Considering that the WW domains have the key role in physical interactions with the substrate proteins, these variants were presumed to increase the binding affinity toward α-syn. In most cases, the WW domains directly bind to substrates containing PXY, LPXY, and PPXY sequences. α-Syn has a proline-rich region in the C-terminal domain, and deletion of this region abolishes ubiquitination by Nedd4 *in vitro* (Tofaris et al., 2011). Thus, I performed a co-immunoprecipitation (co-IP) assay to evaluate the binding affinity *in vivo* of Rsp5 variants to α-syn (Fig. 23).

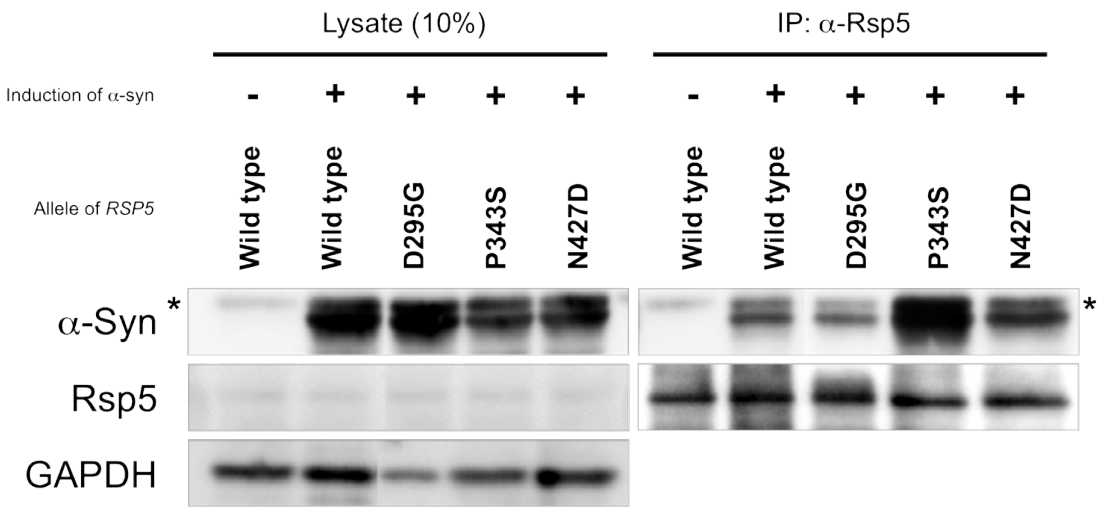


Figure 23. Physical interaction of Rsp5 with α-synuclein in yeast cells. Yeast cells were cultivated to mid-log growth phase. α-Synuclein expression was induced with galactose for 3 h. Approximately 10×10^7 cells were harvested and lysed. The lysate was then subjected to co-immunoprecipitation with anti-Rsp5 polyclonal antibody for 90 min and by protein G sepharose for another 1 h. Interaction of proteins was detected by Western blot analysis. GAPDH was used as a protein-loading control.

The result raised convincing evidence of the physical interaction of Rsp5 with α-syn overexpressed in yeast cells. Furthermore, the P343S and N427D Rsp5 variants enhanced this interaction. The α-syn level in the co-immunoprecipitants with an anti-Rsp5 antibody in D295G was almost the same as that in the wild-type Rsp5, suggesting that the WW domains has a novel role in regulating α-syn degradation without increasing the binding affinity with α-syn.

II.3.4. Ubiquitination of α-synuclein mediated by Rsp5 variants

Finally, I addressed the ubiquitination of α-syn in *RSP5*^{P343S} cells, in which the binding affinity toward α-syn was most prominently enhanced (Fig. 23). Rsp5-mediated ubiquitination of α-syn was clearly observed only when both α-syn and ubiquitin were overexpressed (Fig. 24). Since Tofaris et al. (2011) analyzed the ubiquitination of α-syn by purified Rsp5 or Nedd4 only *in vitro*, this is the first result on ubiquitination of α-syn by Rsp5 *in vivo*. Mono- and poly-ubiquitination occurred in the wild-type and were remarkably enhanced in the P343S variant. It is also noteworthy that overexpression of untagged ubiquitin increased the ubiquitinated forms of α-syn in the P343S variant, compared to myc-tagged ubiquitin.

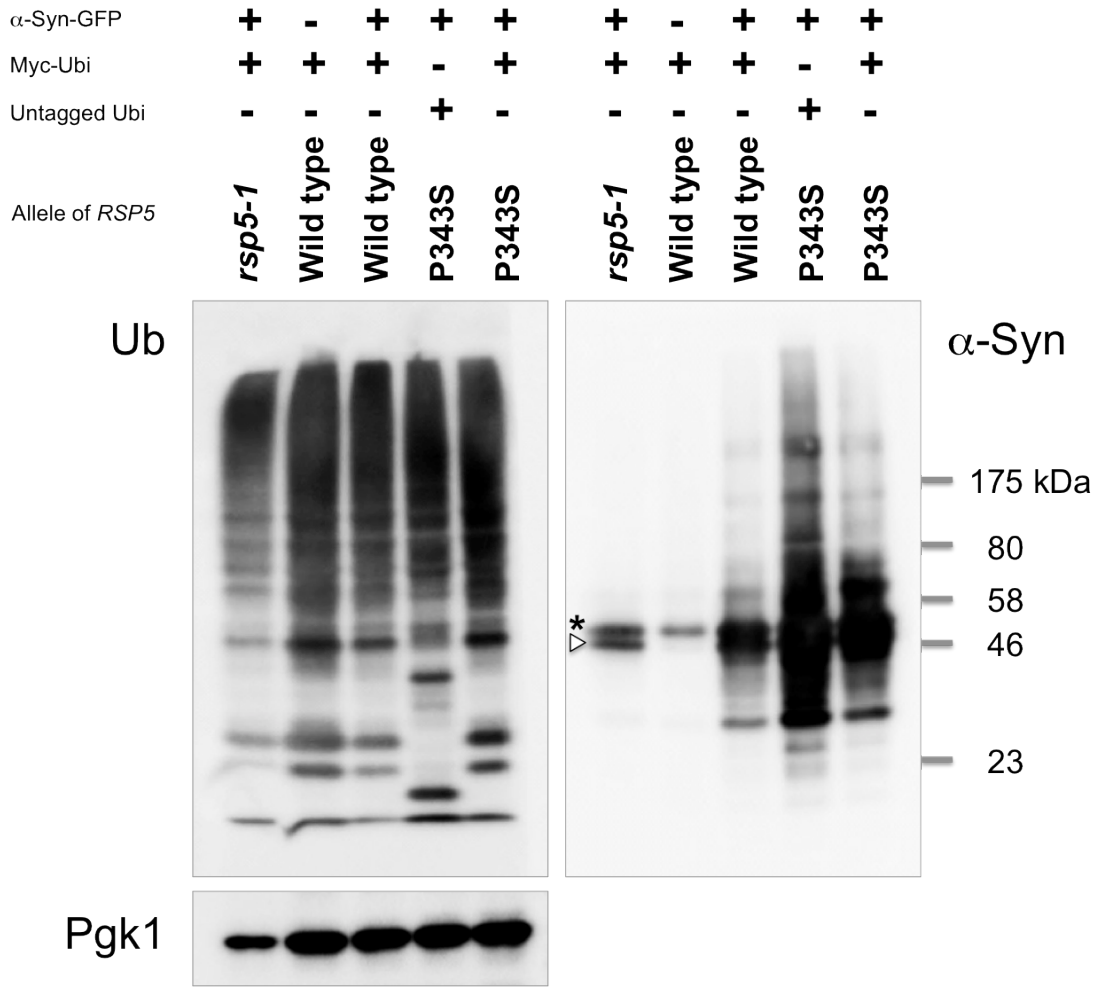


Figure 24. *In vivo* ubiquitination assay. Yeast cells were cultivated for overnight. α-Synuclein was overexpressed by induction of galactose for 15 h. Ubiqitin and ubiquitin-myc were simultaneously overexpressed from the *CUP1* promoter by addition of 0.1 mM $CuSO_4$. Approximately 2.5×10^8 cells were collected and treated with SDS buffer, NEM, PMSF, and the protease inhibitors. Immunoblotting was performed with indicated antibody. Pgk1 was used as a protein-loading control.

II.4. Discussion

In this chapter, I focused on how the novel Rsp5 variants alleviate the α-syn toxicity, and revealed that the three variants, D295G, P343S, and N427D, significantly suppressed the generation of ROS by overexpression of α-syn (Fig. 18). So far, the mechanism of ROS production under the condition of α-syn accumulation is not really clear. When expressed in yeast, human wild-type α-syn (WT-syn) is delivered to the plasma membrane through the secretory pathway (Dixon et al., 2005). The accumulation of these proteins at the plasma membrane leads to the formation of inclusion bodies. This process starts with the appearance of small nuclei, which grow and further transform into larger cytoplasmic aggregates, as shown in Figure 4B. As α-syn transits the secretory pathway to reach the plasma membrane in yeast, the first compartment encountered is the ER. Consistently, it causes ER stress, and leads to proteasome defect (Franssen et al., 2010). Defect in ubiquitin-proteasome system will induce accumulation of misfolded proteins and increase ROS levels. The mechanistic details of α-syn-induced ROS formation have not been analyzed in detail.

However, a study on the induction of apoptotic cell death by accumulation of misfolded carboxypeptidase Y within the lumen of the yeast ER showed that increased ROS levels result from a prolonged unfolded protein response (UPR) activation. This activation triggers ROS production in the ER during the formation of disulfide bonds, which indirectly also stimulates ROS production in the mitochondria (Haynes et al., 2002). Given that α-syn impairs ERAD through blockage of ER-to-Golgi vesicular transport in yeast, it is more than likely that this UPR-dependent mechanism is one of the ROS-generating scenarios deployed by α-syn.

Among the three novel Rsp5 variants obtained (D295G, P343S, and N427D), it is interesting that the P343S variant clearly enhanced the degradation of α-syn (Figs. 19-22), the interaction with α-syn (Fig. 23), and the ubiquitination of α-syn (Fig. 24). Altogether, throughout this study, I achieved to isolate novel hyperactive forms of Rsp5, which can selectively target α-syn (Fig. 25). Our previously found T357A variant specifically recognizes and degrades the general amino acids permease Gap1 (Sasaki and Takagi, 2013). In contrast, the P343S variant, as well as N427D and D295G, was able to effectively enhance the degradation of α-syn (Figs. 19-22). I hypothesize that the conformational changes caused by these three mutations might bring similar effects on the recognition of α-syn. Since their specific phenotypes

under physiological conditions have not been found yet (Fig. 17), their preferred substrate proteins are still unknown. The T255A variant did not enhance the degradation of α-syn (Figs. 19-22), but exhibited higher tolerance to the α-syn toxicity (Figs. 11, 13, and 14), suggesting that this mutation might suppress some secondary effects caused by α-syn overexpression. It should be noted that the T255A variant were more tolerant to acetic acid than the wild-type Rsp5 and other variants (Fig. 17). The specific target for the T255A variant might be associated with acetic acid stress tolerance. Therefore, this study has proven the importance of the WW domains of Rsp5 in specific recognition of various substrate proteins.

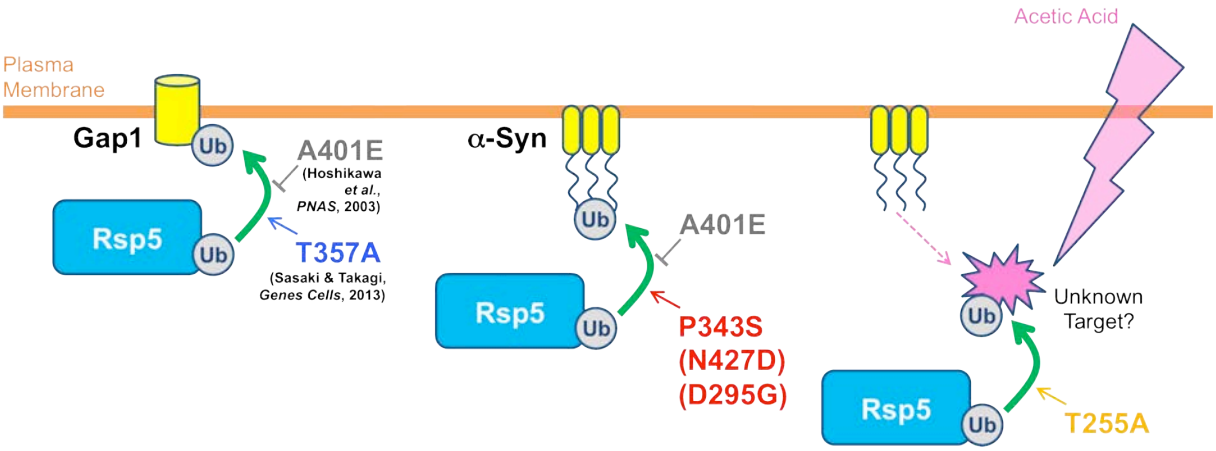


Figure 25. Novel Rsp5 variants found in this study and their presumed substrate specificity.

How does α-syn undergo the modification before its degradation? The *in vivo* ubiquitination assay (Fig. 24) revealed that not only monoubiquitination but also multi-monoubiquitination and/or polyubiquitination of α-syn occurred, and these processes were enhanced by the P343S mutation. In a previous report, monoubiquitination of α-syn in dopaminergic cells promoted by Siah ubiquitin ligase increases the aggregation of α-syn *in vitro* and *in vivo* as a signal toward the autophagy-proteolytic pathway (Engelender, 2008). In general, Lys48-linked polyubiquitin tagging is mostly used to target proteins for degradation via the proteasome, whereas Lys63-linked polyubiquitination has been linked to numerous cellular events, such as DNA repair, kinase activation, and endocytosis (Pickart, and Fushman, 2004; Kulathu and Komander, 2012). Therefore, ubiquitination patterns of α-syn should be investigated to clarify the molecular mechanism and the physiological importance of the ubiquitination of α-syn by Rsp5. A recent study also uncovered that inhibition of α-syn phosphorylation compromises its degradation. The

inclusion bodies formed by the S129A α-syn variant were more slowly degraded than those formed by the wild-type α-syn (Tanreiro et al., 2014), suggesting that phosphorylation occurs prior to the ubiquitination of α-syn. Thus, the molecular analyses of α-syn phosphorylation including identification of the related protein kinase(s) will be also required to understand the signaling pathway engaged in the detoxification of α-syn.

One of the most important achievements in this study is identification of the P343S mutation, which hyperactivates Rsp5 specifically toward α-syn. The WW domains of Rsp5 are presumably located at cellular membranes and fold into three antiparallel β-sheets (Macias et al., 1996). Based on the tertiary structure composed of the Rsp5 WW3-HECT domains, a substrate (Sna3 adaptor protein), and an ubiquitin molecule, Asn399 in the WW3 domain, which corresponds to Pro343 in the WW2 domain (Fig. 26A), is located in the loop region between two β-sheets (Fig. 26B). Ala401 in the WW3 domain, which substitution to Glu conferred hypersensitivity to AZC or various stresses (Hoshikawa et al., 2003), is also resided in the same loop region. Although the sequences are not highly conserved, this loop structure might play important roles in the substrate specificity of Rsp5. In contrast, the conserved Thr413 in the WW3 domain, which corresponds to Thr357 in the WW2 domain, is located in the β sheet close to the substrate protein. Substitution of Thr357 into Ala led to the constitutive hyperactivity of Rsp5 specifically toward Gap1 (Sasaki and Takagi, 2013). These data suggest that, in addition to two conserved tryptphan residues, the WW domains contain at least two important regions responsible for recognition of various substrate proteins by Rsp5. To reveal the molecular mechanism that determines the specificity toward α-syn, the conformational changes of the WW domain caused by the P343S mutation should be elucidated.

α-Syn is a small lipid-binding protein associated with several neurodegenerative diseases, including Parkinson's disease (PD) (Taylor et al., 2002, Takalo et al., 2013). A recent screening for drugs to develop therapeutics identified *N*-aryl benzimidazole (NAB) that can revert the core pathology of α-syn (Fig. 27) (Tardiff et al., 2014). All of the effects brought by NAB hit the principal roles of Rsp5 in stress responses and protein homeostasis. This pharmacological knowledge corroborates our findings concerning the role of Rsp5 in detoxification of α-syn.

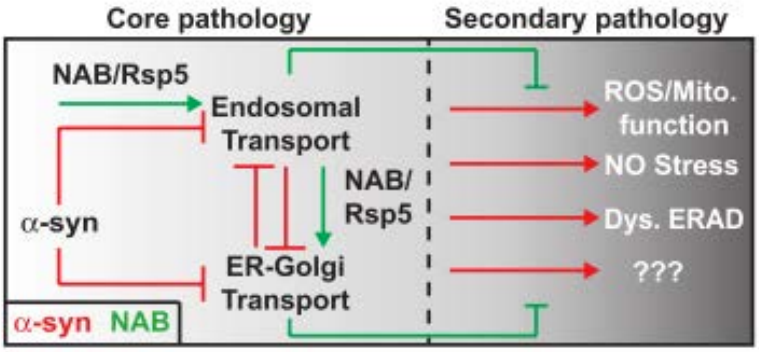


Figure 27. Schematic mechanism of NAB in antagonizing core and secondary α-syn pathologies (Tardiff et al., 2014).

In conclusion, I identified the P343S variant of the ubiquitin ligase Rsp5, a novel hyperactive mutation specifically toward α-syn, by introducing random mutagenesis into the DNA regions encoding the WW domains. If each substrate protein is sophisticatedly recognized by the conformational changes of the WW domains, the specific hyperactive form toward each target substrate might exist. To comprehensively elucidate the physical interactions between the WW domains and the substrates, isolation of hyperactive variants from the WW domain-mutagenized library adopted in this study is highly effective. By making use of this method, novel mechanisms related to selective protein degradation in response to the stress signaling in yeast cells will be revealed in eukaryotic cells. To have more information on how the WW domains play such important roles, it must be an advantage to determine the crystal structure of full-length Rsp5.

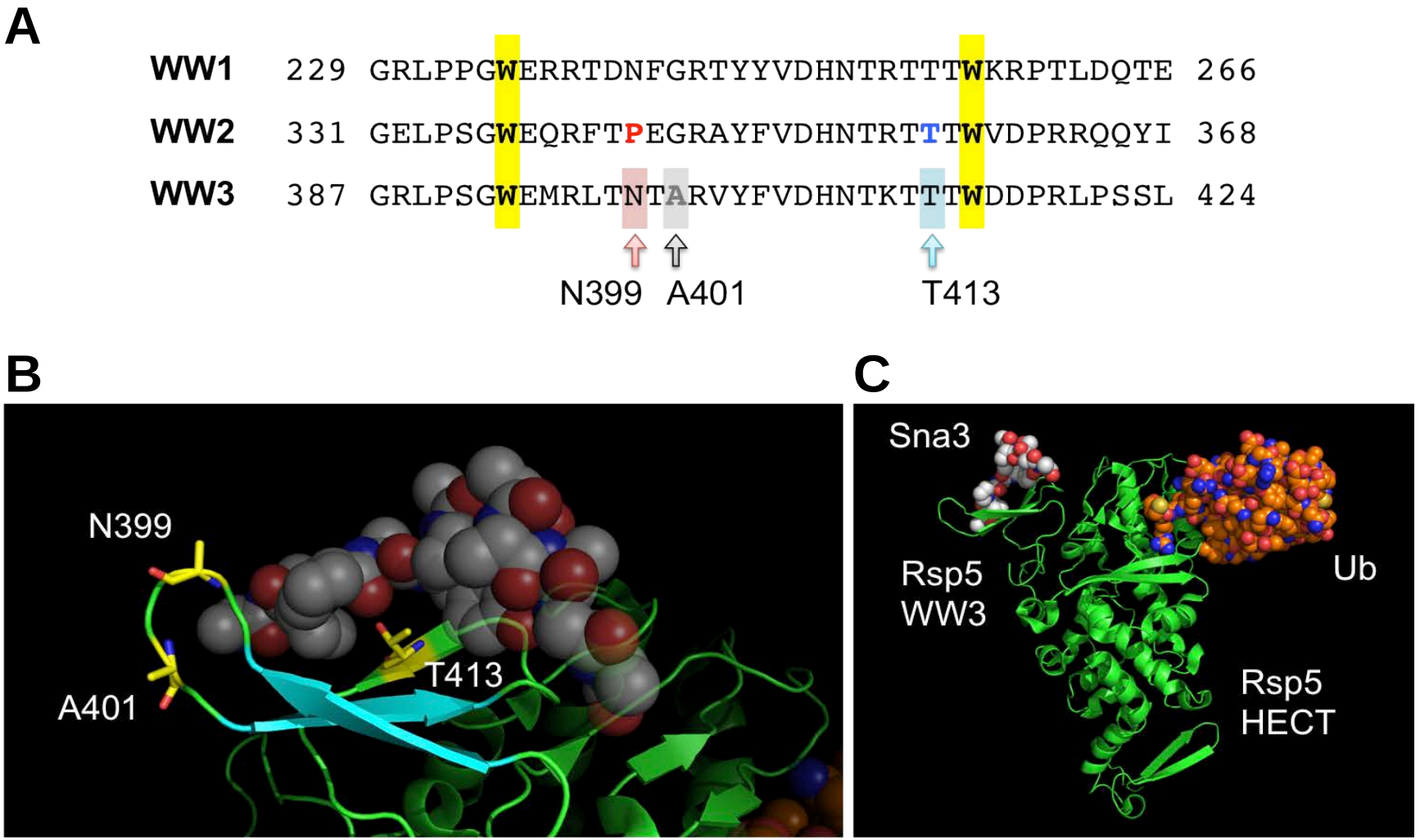


Figure 26. Structure of Rsp5. (A) Amino acid sequence alignment of each WW domain in Rsp5. Each arrow indicates the position of amino acid substitution. (B)(C) Crystal structure of Rsp5 complexed with its substrate Sna3 and ubiquitin (PDB ID code; 4LCD) (Kamadurai et al., 2013). Rsp5 is shown by cartoon model and Sna3 and ubiquitine are shown by sphere model. Some residues in Rsp5 are shown by stick model. The closeup of the region around WW3 domain (B) and whole structure (C) are exhibited.

ACKNOWLEDGEMENTS

In the name of Allah, the Most Gracious and the Most Merciful.

I wish to express my great gratitude thanks to Prof. Hiroshi Takagi for his continuous guidance, valuable discussions, and encouragement throughout this study. Together with Assistant Prof. Daisuke Watanabe who was very supportive, helpful and always motivated me. Both of you spent so much time to make correction and proofreading on my manuscripts and also very patience on my slow progress in research and writing manuscript. Of course, my Ph.D. thesis will not be completed without both of you. I wish you all the best with fully healthy and happy life and I hope we will have the opportunity to make collaboration research in the future.

I would like to thanks to Prof. Hisaji Maki and Prof. Kazuhiro Shiozaki, for their continuous advices and invaluable discussions as the Doctoral Supervising Committee. Sincerely thanks also goes to Prof. Hideki Mochizuki (Osaka University) for providing me the cDNA for human α-synuclein and its antibody, Dr. Jon Huibregste (The University of Texas at Austin) for supplying the Rsp5 antibody, Dr. Toshiya Sasaki for valuable discussion, guiding and teaching me experimental techniques and providing me some plasmids and yeast strains, and Dr. Satoshi Oshiro for valuable discussion, teaching me experimental techniques and help me to install my life in Japan smoothly, and also constructing some plasmids used in my study.

I also thank to the members of Laboratory of Applied Stress Microbiology-NAIST; Assistant Prof. Iwao Ohtsu, Dr. Ryo Nasuno, Hiromi Yamada-san and all the students in this laboratory, all members of Department of Animal Science – Bogor Agricultural University, Indonesia, all members of Indonesian Students Association in Kansai Regions Japan for their supports, collaborations and friendships.

I would also like to thank to Rector of Bogor Agriculture University for educational permission in Japan and Ministry of Education, Indonesia, and Ministry of Education, Culture and Science, Japan, for education opportunity and financial support through the NAIST Global COE Program.

I would like to thank to Ida-san who kindly gave me an opportunity of housing with free of charge.

Last but not least, I would like to express my deepest gratitude and highest compliments to my beloved husband, Sri Wilarso Budi, and my daughter, Shabrina Firdaus Fivinda, and my parents and families in Indonesia, for their never ending love, pray, and supports so that I can finish my study in Japan.

REFERENCES

Auluck PK, Caraveo G, Lindquist S. 2010. α-Synuclein: Membrane interaction and toxicity in Parkinson's disease. *Annu Rev Cell Dev Biol*. **26**:211-233.

Bedford MT, Richard S. 2005. Arginine methylation an emerging regulator of protein function. *Mol Cell*. **18**:263-272.

Belgareh-Touzé N, Léon S, Erpapazoglou Z, Stawiecka-Mirota M, Urban-Grimal D, Haguenauer-Tsapis R. 2008. Versatile role of the yeast ubiquitin ligase Rsp5p in intracellular trafficking. *Biochem Soc Transact*. **36**:791-796.

Butler EK, Aaron Voigt A, Lutz AK, Toegel JP, Ellen Gerhardt E, Karsten P, Falkenburger B, Andrea Reinartz A, Winklhofer KF, Schulz JB. 2012. The mitochondrial chaperone protein TRAP1 mitigates α-synuclein toxicity. *PLoS Genet.* **8**:1-15.

Campello L, Esteve-Rudd J, Cuenca N, Martín-Nieto J. 2013. The ubiquitin-proteasome system in retinal health and disease. *Mol Neurobiol*. **47**:790-810.

Cebollero E, Reggiori F. 2009. Regulation of autophagy in yeast *Saccharomyces cerevisiae*. *Biochim Biophys Acta*. **1793**:1413-421.

Chang A, Cheang S, Espanel X, Sudol M. 2000. Rsp5 WW domains interact directly with the carboxyl-terminal domain of RNA polymerase II. *J Biol Chem*. **275**:20562-20571.

Ciechanover A. 1998. The ubiquitin proteasome pathway: on protein death and cell life. *EMBO J*. **17**:7151-7160.

Cookson MR. 2005 The biochemistry of Parkinson's disease. *Ann Rev Biochem.* **74**:29-52.

Cookson MR. 2009. α-Synuclein and neuronal cell death. *Mol Neurodegener*. **4**:9 doi:10.1186/1750-1326-4-9.

Conway KA, Lee SJ, Rochet JC, Ding TT, Williamson RE, Lansbury PT. Jr. 2000. Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy. *Proc Natl Acad Sci USA.* **97**:571-576.

Clayton DF, George JM. 1998. The synucleins: a family of proteins involved in synaptic function, plasticity, neurodegeneration and disease. *Trends neurosci.* **21**:249-254.

Crews L, Spencer B, Desplats P, Patrick C, Paulino A, Roc-kenstein E, Hansen L, Adame A, Galasko D, Masliah E. 2010. Selective molecular alterations in the autophagy pathway in patients with Lewy body disease and in models of alpha-synucleinopathy. *PLoS One.* **5**(2):e9313.

Cuervo AM, Stefanis L, Fredenburg R, Lansbury PT, Sulzer D. 2004. Impaired

degradation of mutant alpha-synuclein by chaperone-mediated autophagy. *Science.* **305**:1292-1295.

Davidson JF, Whyte B, Bissinger PH, and Schiestl RH. 1996. Oxidative stress is involved in heat-induced cell death in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA.* **93**:5116-512.

De Craene JO, Soetens O, Andre B. 2001. The Npr1 kinase controls biosynthetic and endocytic sorting of the yeast Gap1 permease. *J Biol Chem*. **276**:43939-43948.

Diaz M, Rodriguez S, Fernandez-Abalos JM, De Las Rivas J, Ruiz-Arribas A, Shnyrov V, Santamaria R. 2004. Single mutations of residues outside the active center of the xylanase Xys1Δ from *Streptomyces halstedii* JM8 affect its activity. *FEMS Microbiol Lett.* **240**:237-243.

Dixon C, Mathias N, Zweig RM, Davis DA, Gross DS. 2005. Synuclein targets the plasma membrane via the secretory pathway and induces toxicity in yeast. *Genetics.* **170**:47-59.

Dunn R, Hicke, L. 2001. Multiple roles for Rsp5p-dependent ubiquitination at the internalization step of endocytosis. *J Biol Chem.* **276**:25974-25981.

Engelender, S. 2008. Ubiquitination of α-synuclein and autophagy in Parkinson's disease. *Autophagy*. **4**:372-374.

Ebrahimi-fakhari D, Mclean PJ, Unni VK. 2012. Alpha-synuclein's degradation in vivo. *Autophagy*. **8**:281-283.

Erdinez N, Rothstein R. 2000. Rsp5, a Ubiquitin-protein ligase, is involved in degradation of the single-stranded-DNA binding protein Rfa1 in *Saccharomyces cerevisiae. Mol Cell Biol.* **20**:224-232.

Feng LR, Federoff HJ, Vicini S, Maguire-Zeiss KA. 2010. Alpha- synuclein mediates alterations in membrane conductance: a potential role for alpha-synuclein oligomers in cell vulnerability. *Eur J Neurosci*. **32**:19366-19375.

Flower TR, Chesnokova LS, Froelich CA, Dixon C, Witt SN. 2005. Heat shock prevents α-synuclein-induced apoptosis in a yeast model of Parkinson's disease. *J Mol Biol.* **351**:1081-1100.

Flower TR, Clark-Dixon C, Metoyer C, Yang H, Shi R, Zhang Z. 2007. YGR198w (YPP1) targets A30P α-synuclein to the vacuole for degradation. *J Cell Biol.* **177**:1091-1104.

Franssens V, Boelen E, Anandhakumar J, Vanhelmont T, Büttner S, Winderickx J. 2010. Yeast unfolds the road map toward α-synuclein-induced cell death. *Cell Death Diff.* **17**:746-753.

Gajewska B, Kamińska J, Jesionowska A, Martin NC, Hopper AK, Zoładek T. 2001. WW domains of Rsp5p define different functions: determination of roles in fluid phase and uracil permease endocytosis in *Saccharomyces cerevisiae*. *Genetics*. **157**:91-101.

Gasser B, Saloheimo M, Rinas U. 2008. Protein folding and conformational stress in microbial cells producing recombinant proteins: a host comparative overview. *Microb Cell Fact.* **7**:11. doi: 10.1186/1475-2859-7-11.

Gosavi N, Lee HJ, Lee JS, Patel S, Lee SJ. 2002. Golgi fragmentation occurs in the cells with prefibrillar alpha-synuclein aggregates and precedes the formation of fibrillar inclusion. *J Biol Chem*. **277**:48984-48992.

Gwizdek C, Hobeika M, Kus B, Ossareh-Nazari B, Dargemont C, Rodriguez MS. 2005. The mRNA nuclear export factor Hpr1 is regulated by Rsp5-mediated ubiquitylation. *J Biol Chem*. **280**:13401-13405.

Haitani Y, Takagi H. 2008. Rsp5 is required for the nuclear export of mRNA of HSF1 and MSN2/4 under stress conditions in *Saccharomyces cerevisiae. Genes Cells.* **13**:105-116.

Haitani Y, Shimoi H, Takagi H. 2006. Rsp5 regulates expression of stress proteins via post-translational modification of Hsf1 and Msn4 in Saccharomyces cerevisiae. *FEBS Lett.* **580**:3433-3438.

Haitani Y, Nakata M, Sasaki T, Uchida A, Takagi H. 2009. Engineering of the yeast ubiquitin ligase Rsp5: isolation of a new variant that induces constitutive inactivation of the general amino acid permease Gap1. *FEMS Yeast Res.* **9**:73-86.

Hamilton AM, Zito K. 2013. Breaking It Down: The ubiquitin proteasome system in neuronal morphogenesis. *Hindawi Publishing Corporation Neuronal Plasticity*. Article ID 196848.

Haynes CM, Caldwell S, Cooper AA. 2002. An HRD/DER-independent ER quality control mechanism involves Rsp5p-dependent ubiquitination and ER-Golgi transport. *J Cell Biol*. **158**:91-101.

Hershko A, Ciechanover A. 1998. The ubiquitin system. *Annu Rev Biochem.* **67**:425-479.

Hiraishi H, Okada M, Ohtsu I, Takagi H. 2009. A Functional analysis of the yeast ubiquitin ligase Rsp5: The involvement of the ubiquitin-conjugating enzyme Ubc4 and poly-ubiquitination in ethanol-induced down-regulation of targeted protein. *Biosci Biotechnol Biochem*. **73**:2268-2273.

Hoshikawa C, Shichiri M, Nakamori S, Takagi H. 2003. A nonconserved Ala401 in the yeast Rsp5 ubiquitin ligase is involved in degradation of Gap1 permease and stress-induced abnormal proteins. *Proc Natl Acad Sci USA*. **100**:11505-11510.

Huibregtse, JM, Yang JC, Beaudenon SL. 1997. The large subunit of RNA polymerase II is a substrate of the Rsp5 ubiquitin-protein ligase. *Proc Natl Acad Sci USA*. **94**:3656-3661.

Jakes R, Spillantini MG, Goedert M. 1994. Identification of two distinct synucleins from human brain. *FEBS Lett.* **354**:27-32.

Kamadurai HB, Qiu Y, Deng A, Harrison JS, Macdonald C, Actis M, Schulman BA. 2013. Mechanism of ubiquitin ligation and lysine prioritization by a HECT E3. *eLife*. **2**: e00828.

Kim DW, Cameron RA, Drake JJ, Evans NR, Freeman P, Gaetz TJ, Grimes JP. 2004. ChaMP. I. First X-ray source catalog (Kim+; 2004).

Kloetzel PM. 2004. The proteasome and MHC class I antigen processing. *Biochim Biophys Act.* **1695**:217-225.

Kuchner O, Arnold FH. 1997. Directed evolution of enzyme catalysts. *Trends Biotechnol*. **15**:523-530.

Kulathu Y, Komander D. 2012. Atypical ubiquitylation the unexplored world of polyubiquitin beyond Lys48 and Lys63 linkages. *Mol Cell Biol*. **13**:508-523.

Labrou NE. 2010. Random mutagenesis methods for in vitro directed enzyme evolution. *Curr Prot Pep Sc*. **11**:91-100.

Lauwers E, Erpapazoglou Z, Haguenauer-Tsapis R, Andre B. 2010. The ubiquitin code of yeast permease trafficking. *Trends Cell Biol.* **20**:196-204.

Lecker SH, Alfred L, Goldberg AL, Mitch WE. 2006. Protein degradation by the ubiquitin-proteasome pathway in normal and disease states. *J Am Soc Nephrol.* **17**:1807-1819.

Lipp J, Korandab M, Piskacek M. 2009. Rsp5 promotes gene activation mediated by 9aaTAD transcription factors Oaf1 and Gal4. Available from *Nature Precedings* <http://hdl.handle.net/10101/npre.2009.3968.1>.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. **25**:402-408.

Lu K, Yin X, Weng T, Xi S, Li L, Xing G, He F. 2008. Targeting WW domains linker of HECT-type ubiquitin ligase Smurf1 for activation by CKIP-1. *Nature Cell Biol*. **10**: 994-1002.

MacDonald C, Stringer DK, Piper RC. 2012. Sna3 is an Rsp5 adaptor protein that relies on ubiquitination for its MVB sorting. *Traffic*. **13**:586-598.

Macias MJ, Hyvonen M, Baraldi E, Schultz J, Sudol M, Saraste M, Oschkinat H. 1996. Structure of the WW domain of a kinase-associated protein complexed with a proline-rich peptide. *Nature*. **382**:646-649.

Martinez-Vicente M, Talloczy Z, Kaushik S, Massey AC, Mazzulli J, Mosharov EV, Hodara R, Fredenburg R, Wu DC, Follenzi A, Dauer W, Przedborski S, Ischiropoulos H, Lansbury PT, Sulzer D, Cuervo AM. 2008. Dopamine-modified alpha- synuclein blocks chaperone-mediated autophagy. *J Clin Invest.* **118**:777-788.

Moraitis C, Curran BP. 2004. Reactive oxygen species may influence the heat shock

response and stress tolerance in the yeast *Saccharomyces cerevisiae*. *Yeast.* **21**:313-323.

Moon J, Parry G, Estelle M. 2004. The ubiquitin-proteasome pathway and plant development. *The Plant Cell*. **16**:3181-3195.

Morano KA, Grant CM, Moye-Rowley WS. 2012. The response to heat shock and oxidative stress in *Saccharomyces cerevisiae, Genetics*. **190**:1157-1195.

Outeiro TF, Lindquist S. 2003. Yeast cells provide insight into α-synuclein biology pathobiology. *Science*. **302**:1772-1775.

Petroi D, Popova B, Taheri-Talesh N, Irniger S, Shahpasandzadeh H, Zweckstetter M, Braus GH. 2012. Aggregate clearance of α-synuclein in Saccharomyces cerevisiae depends more on autophagosome and vacuole function than on the proteasome. *J Biol Chem*. **287**:27567-27779.

Pickart CM. 2001. Mechanisms underlying ubiquitination. *Ann Rev Biochem*. **70**:503-533.

Pickart CM, Fushman D. 2004. Polyubiquitin chains: Polymeric protein signals. *Curr Op Chem Biol*. **8**:610-616.

Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A. 1997. Mutation in the α-synuclein gene identified in families with Parkinson's disease. *Science.* **276**:2045-2047.

Rahimi N. 2012. The Ubiquitin-Proteasome System Meets Angiogenesis. *Mol Cancer Ther*. **11**:538-548.

Rappley I, Gitler AD, Selvy PE, LaVoie MJ, Levy BD, Brown HA. 2009. Evidence that α-synuclein does not inhibit phospholipase D. *Biochemistry*. **48**:1077-1083.

Rotin D, Kumar S. 2009. Physiological functions of the HECT family of ubiquitin ligases. *Mol Cell Biol*. **10**:398-409.

Rotin D, Staub O, Haguenauer-Tsapis R. 2000. Ubiquitination and endocytosis of plasma membrane proteins: role of Nedd4/Rsp5p family of ubiquitin-protein ligases. *J Membrane Biol*. **176**:1-17.

Sambrook J, Russell DW. 2001. Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Lab. Press, Plainview, NY), 3rd Ed. 24.

Sasaki T, Takagi H. 2013. Phosphorylation of a conserved Thr 357 in yeast Nedd4-like ubiquitin ligase Rsp5 is involved in down-regulation of the general amino acid permease Gap1. *Genes Cells*. **18**:459-475.

Sharma N, Brandis KA, Herrera SK. 2006. α-Synuclein budding yeast model toxicity enhanced by impaired proteasome and oxidative stress. *J Mol Neurosci*. **28**:161-178.

Shearwin-Whyatt L, Dalton HE, Foot N, Kumar S. 2006. Regulation of functional diversity within the Nedd4 family by accessory and adaptor proteins. *BioEssays*. **28**:617-628.

Shcherbik N, Kumar S, Haines DS. 2002. Substrate proteolysis is inhibited by dominant-negative Nedd4 and Rsp5 mutants harboring alterations in WW domain 1. *J Cell Sci*. **115**:1041-1048.

Smith WW, Jiang H, Pei Z, Tanaka Y, Morita H, Sawa A, Dawson VL, Dawson TM, Ross CA. 2005. Endoplasmic reticulum stress and mitochondrial cell death pathways mediate A53T mutant alpha-synuclein-induced toxicity. *Hum Mol Genet*. **14**:3801-3811.

Soper JH, Roy S, Stieber A, Lee E, Wilson RB, Trojanowski JQ. 2008. Synuclein-induced aggregation of cytoplasmic vesicles in *Saccharomyces cerevisiae*. *Mol Biol Cell*. **19**:1093-1103.

Spencer B, Potkar R, Trejo M, Rockenstein E, Patrick C, Gindi R, Adame A, Wyss-Coray T, Masliah E. 2009. Beclin1 gene transfer activates autophagy and ameliorates the neurodegenerative pathology in alpha-synuclein models of Parkinson's and Lewy body diseases. *J Neurosci.* **29**:13578-13588.

Springael JY, and André B. 1998. Nitrogen-regulated ubiquitination of the Gap1 permease of *Saccharomyces cerevisiae*. *Mol Biol Cell*. **9**:1253-1263.

Stamenova SD, Dunn R, Adler AS, Hicke L. 2004. The Rsp5 ubiquitin ligase binds to and ubiquitinates members of the yeast CIN85-endophilin complex, Sla1-Rvs167. *J Biol Chem*. **279**:16017-16025.

Stemmer, WP. 1994. Rapid evolution of a protein in vitro by DNA shuffling. *Nature*. **370**:389-391.

Sullivan JA, Shirasu K, Deng XW. 2003. The diverse roles of ubiquitin and 26S proteasome in life of plants. *Nature Rev*. **4**:948-958.

Sudol M, Hunter T. 2000. New wrinkles for an old domain. *Cell.* **103**:1001-1004.

Stawiecka-Mirota M, Pokrzywa W, Morvan J, Zoladek T, Haguenauer-Tsapis R, Urban-Grimal D, Morsomme P. 2007. Targeting of Sna3p to the endosomal pathway depends on its interaction with Rsp5p and multivesicular body sorting on its ubiquitylation. *Traffic*. **8**:1280-1296.

Takalo M, Salminen A, Soininen H, Hiltunen M, Haapasalo A. 2013. Protein aggregation and degradation mechanisms in neurodegenerative diseases. *Am J Neurodegener Dis.* **2**:1-14.

Tardiff DF, Jui NT, Khurana V, Mitali A. Tambe AT. 2013. Yeast reveal a "druggable" Rsp5/Nedd4 network that ameliorates α-synuclein toxicity in neurons. *Science.* **342**:979-983.

Taylor JP, Hardy J, aFishbeck KH. 2002. Toxic proteins in neurodegenerative disease. *Science*. 296:1991-1995.

Tenreiro S, Reimão-Pinto MM, Antas P, Rino J, Wawrzycka D, Macedo D, Outeiro TF. 2014. Phosphorylation modulates clearance of alpha-synuclein inclusions in a yeast model of Parkinson's disease. *PLoS Genet*. **10**:e1004302.

Tetzlaff JE, Putcha P, Outeiro TF, Ivanov A, Berezovska O, Hyman BT, McLean PJ. 2008. CHIP targets toxic alpha-synuclein oligomers for degradation. *J Biol Chem*. **283**:17962-17968.

Tofaris GK, Kim HT, Hourez R, Jung JW, Kim KP, Goldberg AL. 2011. Ubiquitin ligase Nedd4 promotes α-synuclein degradation by the endosomal-lysosomal pathway. *Proc Natl Acad Sci USA*. **108**:1774-1779.

Vamvaca K, Volles MJ, Lansbury PT Jr. 2009. The first N-terminal amino acids of α-synuclein are essential for a-helical structure formation in vitro and membrane binding in yeast. *J Mol Biol*. **389**:413-424.

Vlachostergios PJ, Voutsadakis IA, Christos N, Papandreou CN. 2012. The ubiquitin-proteasome system in glioma cell cycle control. *Cell Div*. **7**:18. doi: 10.1186/1747-1028-7-18.

Vogiatzi T, Xilouri M, Vekrellis K, Stefanis L .2008. Wild type alpha-synuclein is degraded by chaperone-mediated autophagy and macroautophagy in neuronal cells. *J Biol Chem.* **283**:23542-23556.

Volles MJ, Lansbury PT. 2007. Relationships between the sequence of alpha-synuclein and its membrane affinity, fibrillization propensity, and yeast toxicity. *J Mol Biol*. **366**:1510-1522.

Wang G, McCaffery JM, Wendland B, Dupré S, Haguenauer-Tsapis R, Huibregtse JM. 2001. Localization of the Rsp5p ubiquitin-protein ligase at multiple sites within the endocytic pathway. *Mol Cell Biol.* **21**:3564-3575.

Webb JL, Ravikumar B, Atkins J, Skepper JN, Rubinsztein DC. 2003. Alpha-synuclein is degraded by both autophagy and the proteasome. *J Biol Chem*. **278**:25009-25013.

Willingham S, Outeiro TF, DeVit MJ, Lindquist SL, Muchowski PJ. 2003. Yeast genes that enhance the toxicity of a mutant huntingtin fragment or alpha-synuclein. *Science.* **302**:1769-1772.

Yamada S, Niwa J, Ishigaki S, Takahashi M, Ito T, Sone J, Doyu M, Sobue G. 2006. Archaeal proteasomes effectively degrade aggregation-prone proteins and reduce cellular toxicities in mammalian cells. *J Biol Chem.* **281**:23842-23851.

Yu WH, Dorado B, Figueroa HY, Wang L, Planel E, Cookson MR, Clark LN, Duff KE. 2009. Metabolic activity determines efficacy of macroautophagic clearance of pathological oligomeric alpha-synuclein. *Am J Pathol.* **175**:736-747.

Zabrocki P, Bastiaens I, Delay C, Bammens T, Ghillebert R, Pellens K. 2008.

Phosphorylation, lipid raft interaction and traffic of α-synuclein in a yeast model for Parkinson. *Biochim Biophys Acta*. **1783**:1767-1780.