Role of endoplasmic reticulum quality control system in unconventional secretion of transmembrane proteins

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2019년에 이민구 교수님 연구실에 들어오게 되어 많은 것을 배우고 얻게 되었습니다. 학사 과정 이후 저의 학위를 받아주시고 무사히 마치게 해주신 이민구 교수님께 감사드립니다. 교수님께 많은 것을 배웠고 데이터 해석하는 능력이나 실험 디자인하는데 정말 많은 도움을 받았습니다. 교수님의 인사이트에 많이 놀랐고 저도 많이 발전하게 된 것 같습니다. 처음 이 연구실에 아무것도 모른 채 들어왔을 때 많은 연구실원 분들의 도움 덕분에 여기까지 왔습니다. 처음 셀 컬쳐, 기본적인 웻 실험을 가르쳐 준 Le, 실험의 정석 중의 정석을 강조하고 가르쳐주신 노신혜 선생님, 여러 웻 실험과 다양한 것들을 가르쳐주시고 얘기를 나눴던 김지윤 선생님, 연구실에서 좋은 말씀 해주신 김연정 선생님, 연구 활동에 도움을 주신 엄소원 선생님, 커피숍에서 다양한 주제에서 얘기를 나눈 은성호 선생님, 홀로 고군분투하시고 성격 좋은 유정우 선생님, 연구실에 들어와서 많은 도움 주시고 특히 펜드린 과제에서 항상 도와주신 심주리 선생님, 동갑내기면서 학위 과정 중에 편하게 그리고 영문교정까지 도와준 민재, 힘든 과정에서도 열심히 하는 예진이, 최근에 많은 토론으로 즐거움을 주시는 송민석 선생님, 항상 친근하게 대해주시는 신동훈 선생님, 연구실에 도움 주시는 신선미 선생님, 학위과정 중에서 가장 많이 물어보고 어떤 것이든 자기 일처럼 도와준 수경이, 모든 분들께 감사의 말씀드립니다. 이외에도 지헌영 교수님, 지헌영 교수님 연구실원 분들께도 많은 도움 받았고 좋은 의견 많이 주셔서 감사했습니다. 석사 과정을 지내면서 2년이 넘게 학위 과정을 지내면서 힘든 점도 많았지만 정말 많이 배우고 부족한 점 많이 채운 것 같습니다. 실험실이지만 실험 외적인 것에서도 저의 부족한 점은 많았고 저를 알아가는데 도움이 되는 시간들이었습니다. 실험실 생활하면서 지원해주시고 제가 하는 일에 대해 긍정적이게 봐주신 부모님께도 감사의 말씀 전합니다.

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ABSTRACT

Role of endoplasmic reticulum quality control system in unconventional secretion of transmembrane proteins

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Secretory proteins that have folding defects are mostly degraded by the protein quality control systems in the endoplasmic reticulum (ER). The most common disease-causing mutation in the plasma membrane protein CFTR (ΔF508-CFTR) evokes a protein folding defect, which consequently causes a trafficking defect of the protein to the cell surface. However, this mutant protein can be rescued to the plasma membrane by GORASP2/GRASP55-dependent unconventional trafficking route. Unconventional trafficking pathway is activated by ER stress or blocking ER to Golgi transport. It is predicted that many proteins, in addition to ΔF508-CFTR, can be secreted by the unconventional trafficking pathway under ER stress condition. In this study, we aim to identify the properties and characteristics of the unconventionally secreted proteins. To induce unconventional protein secretion by blocking ER-to-Golgi transport, HEK293 cells were transfected with dominant-inhibitory ADP-ribosylation factor 1 (ARF1), GTP-binding protein SAR1 (SAR1) plasmids. Then, plasma membrane proteins were prepared by surface biotinylation and analyzed by mass spectrometry. Through the graph for unconventional trafficking rate and protein sequence length in ER lumenal part, we discovered that their relationship is inversely proportional to each other. If the protein has a long peptide sequence in the ER lumen, it is hard to reach the

plasma membrane. We hypothesize that Endoplasmic Reticulum Associated Degradation (ERAD) and Endoplasmic Reticulum Quality Control (ERQC) are inversely proportional to unconventional trafficking because the proteins in the ER lumen may interact with many chaperones and co-chaperones for modifying their properties or moving to other organelles. We used VSV-G proteins as trafficking cargo since it has 2 glycosylation sites, 6 disulfide bonds, and some domains which are properties considered as proper cargo proteins. When blocking the calnexin cycle (CNX cycle), VSV-G can be rescued under the blockade of ER to Golgi. We discovered that VSV-G can reach the plasma membrane by deteriorating ERQC. Also, the Depletion of co-chaperones associated with ERAD using small-interfering RNA (siRNA) makes VSV-G rescued in the plasma membrane. It implies that proteins can be rescued when they are not degraded in the ER and transported by the unconventional trafficking route. these findings will provide insight into unconventional trafficking.

Key words : Unconventional trafficking, membrane proteome, ARF1, ERQC, ERAD

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I. INTRODUCTION

In general, most of the secretory proteins in cells are synthesized in the ER. After translation, they are transported to the Golgi for additional modifications and secreted or located to other organelles. It is also known as conventional trafficking. However, cells sometimes face nutrient deficiency, imbalance of ion concentration, and ER stress which can block the conventional trafficking pathway. Cystic Fibrosis Transmembrane Conductance regulator (CFTR) is located on the plasma membrane and transported by conventional trafficking. CFTR is an important anion channel that transports chloride and bicarbonate and its mutations cause Cystic Fibrosis. Among many mutations, CFTR which has a deletion of phenylalanine at position 508 (ΔF508-CFTR) is the most common that causes the folding mutation limiting the transport of the protein to the plasma membrane. Most unfolded proteins are degraded by ERAD system and so does ΔF508-CFTR[1]. There are many efforts to rescue of ΔF508-CFTR. Even though ΔF508-CFTR has the folding mutation, it can be secreted by GRASP55-dependent unconventional trafficking[2]. Due to folding mutation, ΔF508-CFTR almost remains in ER. However, through the unconventional trafficking routes, they can be rescued by bypassing the Golgi[3]. If the membrane protein bypasses the Golgi, it has only high mannose glycan forms and its molecular size appears in a small size than the original in western blot. ΔF508-CFTR has a fewer function of anion transport than normal CFTR, but if mutants are secreted by unconventional trafficking, the function of anion transport is recovered partly[4]. It is expected to be treated if even ΔF508-CFTR reach the plasma membrane and have a 50% ability compared to normal

because homozygote of CFTR does not show CF disease. In addition to ΔF508-CFTR, many diseases are occurred by mutations of proteins. Another mutant pendrin, an anion channel, causes pendred syndrome. It can be also rescued by unconventional trafficking and its transportation needs DNAJC14 and hsc70[5]. There are many diseases due to mutant membrane proteins. By using the unconventional trafficking route, we expect many diseases caused by protein mutations can be treated. In ER to Golgi blockade condition, most proteins remain in ER and are degraded to reduce ER stress. ER to Golgi blockade can be induced using ARF1 and SAR1 mutant. ARF1 and SAR1 are essential for coating vesicles[6,7]. ARF1 mutant and SAR1 mutant are dominant-negative mutant forms. To make coat complex, ARF1 and SAR1 have to hydrolysis of GTP to GDP but their mutant forms lose hydrolysis ability. Therefore, transfecting the ARF1 and SAR1 mutant in the cells induce a blockade of ER to Golgi. Blockade of ER to Golgi makes ER stressed. To lessen ER stress, unfolded protein response (UPR) is activated in ER[8]. Activating transcription factor 6 (ATF6), inositol-requiring protein 1 (IRE1), and PKR-like ER localized eIF2α kinase(PERK) are ER stress signal. Their downstream factors cause the degradation of proteins in ER and maintaining proteostasis in ER. UPR makes cells recover by degrading proteins or releasing proteins like ΔF508-CFTR. In the present study, we report common characteristics of proteins that can be transported under ER to Golgi blockade. It is associated with the stability of proteins. Also, we aim to elucidate why most proteins should be degraded in ER stress conditions and methods to evade the degradation system.

II. MATERIALS AND METHODS

1. Cell culture and Transfection

HEK293 cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. HEK293 cells were grown at 37℃ in a 5% CO_2 incubator. Plasmids were transfected into HEK293 cells using Lipofectamine Plus Reagent (Invitrogen, Carlsbad, CA, USA) and Opti-Minimal Essential Medium (MEM). siRNAs were transfected into HEK293 cells using JetPrime (Polyplus, Illkirch-Graffenstaden, France)

2. Chemicals, plamids, siRNAs, and antibodies

Kifunensine (KIF), 1-deoxynojirimicin (DNJ), HA15 were purchased from Sigma-Aldrich (Burlington, MA, USA). MG132 was purchased from Selleckchem (Houston, TX, USA). FLAG-VSV-G, SAR1, and ARF1 constructs were synthesized from Bionics (Seoul, South Korea). ERdj3, ERdj4, DNAJC12 construct were purchased from Origene. ERdj5 (accession number NM_018981.4), ERdj6 (accession number NM_006260.5), DNAJB14 (accession number NM_001031723.4), SEC61A1 (accession number NM_013336.4) coding regions of human were amplified from the complementary DNA of HEK293 cells and transferred PCMV-myc using NEB HIFI DNA Assembly. Information on consensus DNA sequences is provided by the National Center for Biotechnology Information (NCBI). ERdj3, ERdj4, ERdj5, ERdj6, DNAJB14, DNAJC12, DNAJC14-specific siRNAs were purchased from Bioneer (Daejeon, South Korea). DYKDDDK (FLAG), HA, Myc, Bip, CHOP antibodies were purchased from Cell signaling Technology (Danvers, MA, USA). Aldolase A antibody was purchased from Santacruz (Dallas, TX, USA).

3. Preparing Proteomics sample

HEK293 cells were used for plasma membrane proteome. To induce ER stress in cells, mutant ARF1 and SAR1 were transfected. Mutant ARF1 was transfected for 24hr and mutant SAR1 for 48hr. A difference in transfection time comes from ER stress-inducing time. To collect only Plasma membrane proteins, surface biotinylation is used. After collecting proteome, SDS-PAGE is performed using 10% polyacrylamide gel. 1-DE gel analyzed in Korea Basic Science Institute (KBSI). The gel was performed by in-gel digestion and analyzed by mass spectrometry.

4. Immunoblotting

To immunoblot, HEK293 cells were grown at 37℃ in a 5% CO_2 incubator. After 48~72hr, cells were lysed with lysis buffer(NaCl 150mM, Tris-HCl pH7.4 50mM, EDTA 0.1mM, sodium deoxycholate 0.25%(m/v), NP40 1% (v/v) and 1 complete protease inhibitor tablet (Roche, Basel, Switzerland)). After lysis, sonicate the sample and centrifuged at 13000rpm, 4℃, 20mins. Collect supernatant and samples were mixed 2X sample buffer (Komabiotech). Samples were separated by SDS-PAGE. Separated proteins were transferred to nitrocellulose membranes. Nitrocellulose membranes were incubated with appropriate primary antibodies. Primary antibodies were amplified from secondary antibodies which have HRP and target proteins were detected chemiluminescence (Cytiva lifesciences, Marlborough, MA, USA)

III. RESULTS

1. Proteomics sampling and analyzed data

To find out which proteins of the cells reach the plasma membrane under normal state or ER stress state, the samples using surface biotinylation were prepared 24hr after transfecting ARF1 mutant and 48hr after transfecting SAR1 mutant. Prepared samples were lysed and incubated with streptavidin beads overnight at 4℃. Samples were eluted by using tricine sample buffer added 1,4-Dithiothreitol (DTT). Samples were considered plasma membrane proteins and it is called membrane proteome. This proteome was separated by SDS-PAGE and stained by Coomassie blue. After staining, these samples were analyzed at KBSI. Analyzed data showed that 875 proteins were detected in the control group, 1194 proteins, and 1142 proteins in the ARF1 mutant group, SAR1 group respectively. Among them, 324, 124, 131 membrane proteins were detected in the control, ARF1 mutant and SAR1 mutant group respectively. The ratios of membrane proteins to the total proteins were 37.03%, 10.39%, 11.47% in each group and its quantitative ratios of membrane proteins to the total proteins were 24.84%, 5.27%, 3.75% in each group. Through quantitative ratios, we discovered that most of the proteins located on the plasma membrane in normal condition disappeared in the plasma membrane. It is shown in **Table 1**. ARF1 mutant group was mainly analyzed because it was clearly contrasted with the control group. In the ARF1 mutant group, it could be divided into proteins with increased and decreased groups. Increased group shows that the ratio of the protein in ARF1 mutant treatment to the protein in normal conditions is increased. In other words, the increased group means that they can reach the plasma membrane under ER to Golgi blockade conditions. The decreased group is the opposite.

In the ARF1 mutant group, increasing proteins are 72, and decreasing proteins are 314. It is shown in **Table 2**. As expected, most of the membrane proteins cannot reach the plasma membrane because of blocking the conventional trafficking route and ER stress when ARF1 was transfected.

Membrane proteins consist of 4 types. In our proteomic data, most of the proteins are type 1 which has a long peptide in the ER lumen. Information about proteins,

such as protein sequence length, glycosylation, disulfide bonds, domains, and motifs are not well illuminated yet. Most of the parameters are not significant with the ratio of unconventional trafficking to conventional trafficking. Among these parameters, as shown in **Figure 1**, the most significant parameter is protein sequence length in ER lumenal part. Proteins are translated in ER and separated in ER lumenal part and cytosolic part. This parameter and the ratio of unconventional trafficking to conventional trafficking had an inversely proportional relationship. Thus, it can be deduced that because of ER chaperones the longer the protein sequence length of ER lumen, the higher the probability of getting Endoplasmic Reticulum Quality Control (ERQC). On the other hand, the shorter the length of ER lumen, the lower the chance of getting ERQC. While many different conditions enable unconventional trafficking under ER stress, the results reveal that it is important to avoid the proteins from undergoing either ERAD or ERQC.

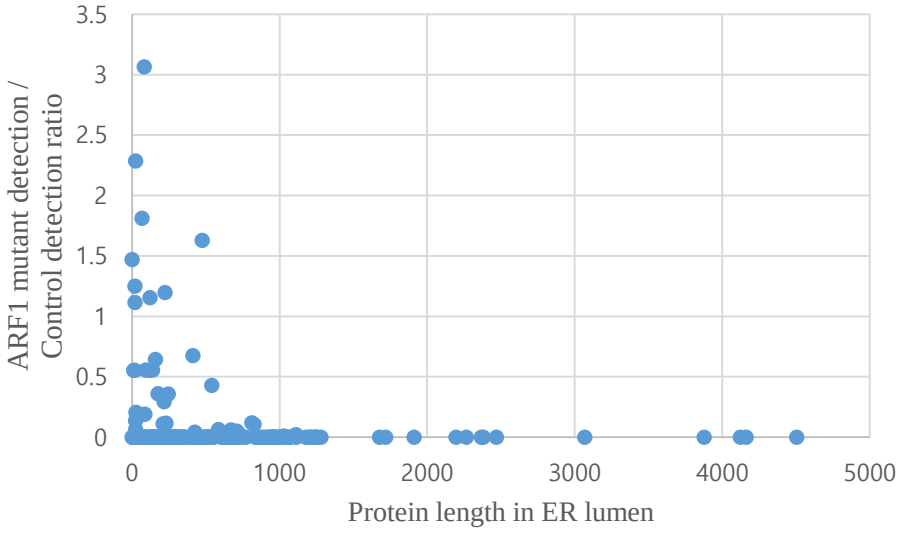

Figure 1. Graphs of protein length in ER lumen and ratio of ARF1 mutant detection to control detection for each protein A graph of protein length of ER lumenal part and ratio of unconventional trafficking to conventional trafficking. Its relationship is inversely proportional. Proteins that have short ER lumen sequences tend to reach the cell surface rather than other proteins that have long ER lumen sequences.

Table 1. Detected plasma membrane proteome

	Control	ARF1-Q71L	SAR1-T39N
Total proteins	875	1194	1142
Transmembrane protein (TM protein)	324	124	131
TM protein / Total (%)	37.03	10.39	11.47
Quantitation of TM protein/Total (%)	24.84	5.27	5.75

Table 2. The number of increasing and decreasing proteins in proteomics data

	Increase		Decrease	
Control detection	X	O	O	O
ARF1-Q71L detection	O	O	O	X
Total detection	64	6	50	264
Plasma membrane	16	2	12	183
Endoplasmic reticulum	17	4	16	27
mitochondrion	16	0	16	18
other organells	15	0	6	36

2. Glycosylation of proteins is important not only for strengthen ERQC but also for unconventional trafficking

Most of the experiments are performed by using VSV-G as cargo because VSV-G cannot be rescued under ER stress. When ARF1 mutant is treated in cells, ER to Golgi transportation is blocked and proteins in ER are degraded because of unfolded protein response (UPR). IRE1 and ATF6, UPR major components, express ERAD factors. Therefore, VSV-G is meant to be broken down because of ERQC and ERAD. In addition, there is no helper for VSV-G to go unconventional trafficking like GRASP55. We anticipated that blocking ERQC or ERAD will help keep VSV-G from degrading. ERQC is performed by 2 cycles in ER. One is the calnexin(CNX) cycle and another is the Bip cycle. CNX cycle recognizes the glycosylations of proteins and performs quality control. In this cycle, ER mannosidase and ER glucosidase are quite important components. To block the function of ER glucosidase and ER mannosidase, we used their inhibitor, 1-deoxynojirimicin (DNJ) and Kifunensine (KIF)[9,10] respectively. It was confirmed that rescue of VSV-G under ER stress increased when KIF was treated in cells. It is shown in **Figure 2A**. It means that ER mannosidase is the crucial factor in CNX cycle and ERQC. To confirm precisely whether ER mannosidase affects the rescue of VSV-G, we performed knockdown of ER mannosidase which is coded MAN1B1 gene using siRNA. According to expectation, knockdown of ER mannosidase increases the rescue of VSV-G. It is shown in **Figure 2C**. We can consider glycosylations of proteins as an important part of ERQC. In other words, glycosylations interfere with unconventional trafficking. So, we assumed that VSV-G with mutants of glycosylation sites can be rescued under ER stress. VSV-G has 2 N-glycosylation sites at 179, 336 asparagine[10]. We changed 179, 336 asparagine to aspartic acid. That mutation makes VSV-G not glycosylated. We made 3 mutation types, N179D, N336D, N179D + N336D. By using these mutation VSV-G constructs, we performed experiments. As shown in **Figure 2E**, we obtained unexpected results. As shown in lanes 4, 6, 8, VSV-G that has mutated glycosylation were not rescued under ER stress. It probably shows that unconventional trafficking requests glycosylations. We discovered that

glycosylation is important for both conventional and unconventional trafficking.

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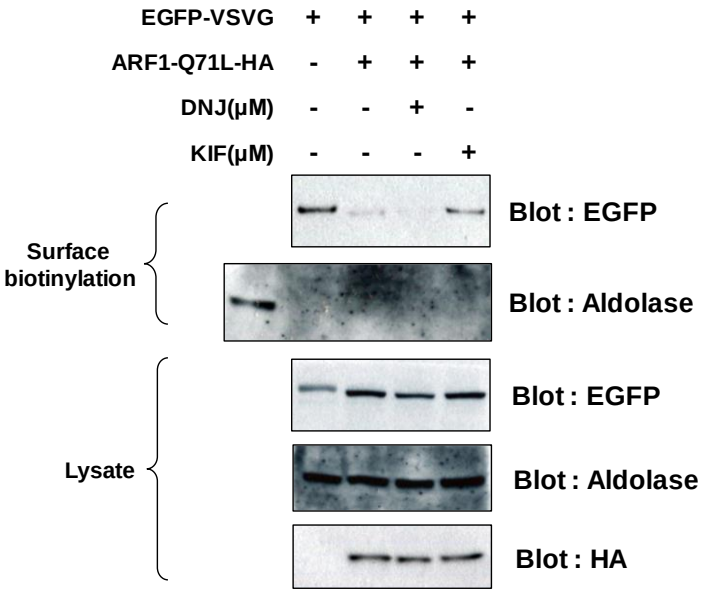

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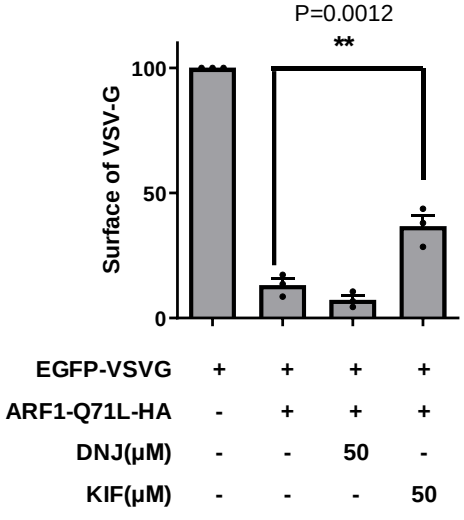

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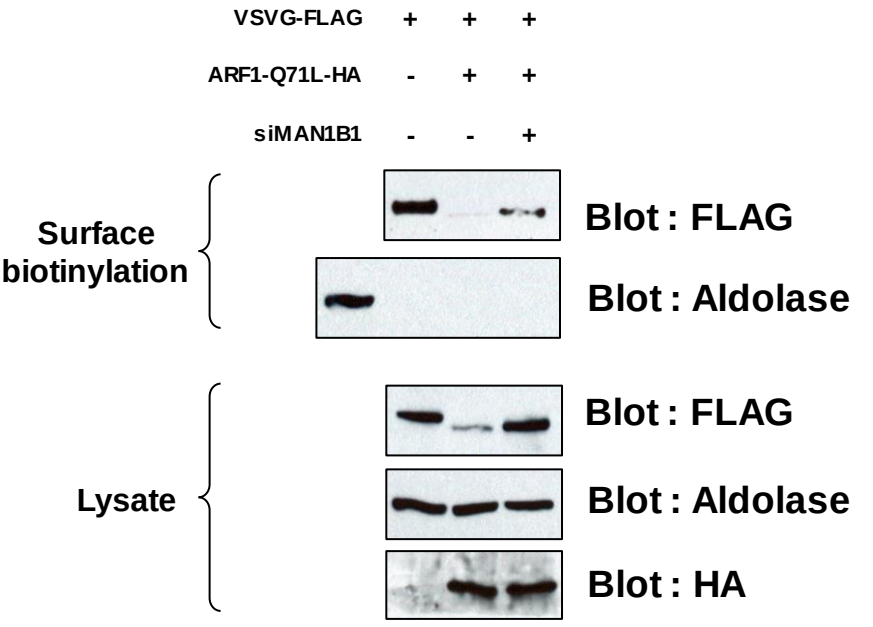

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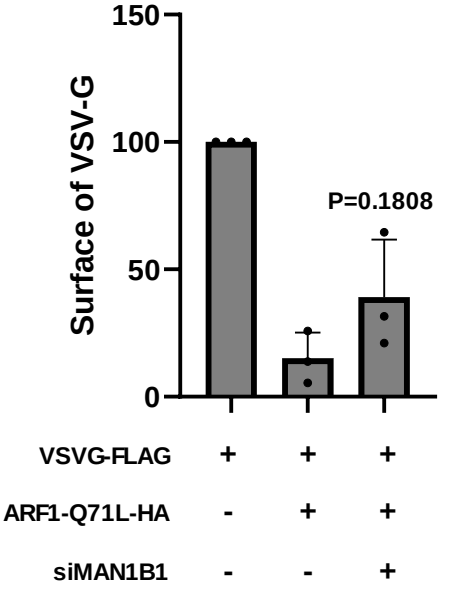

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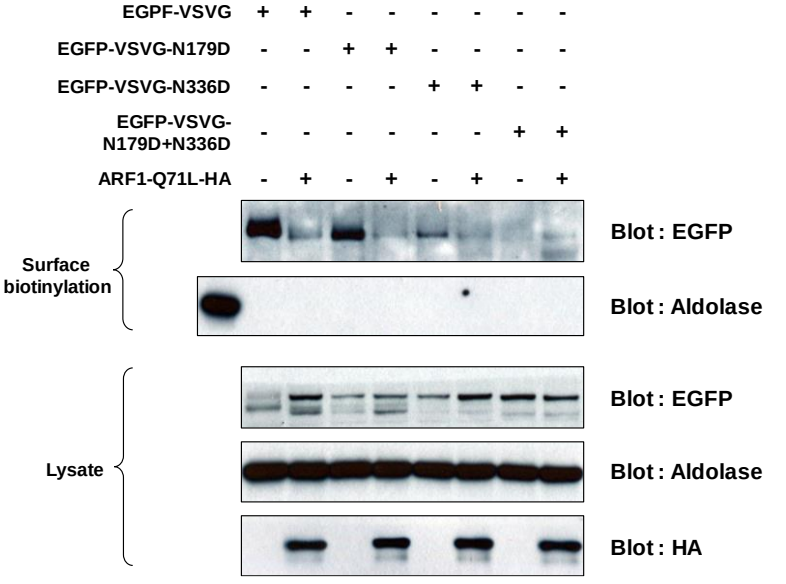

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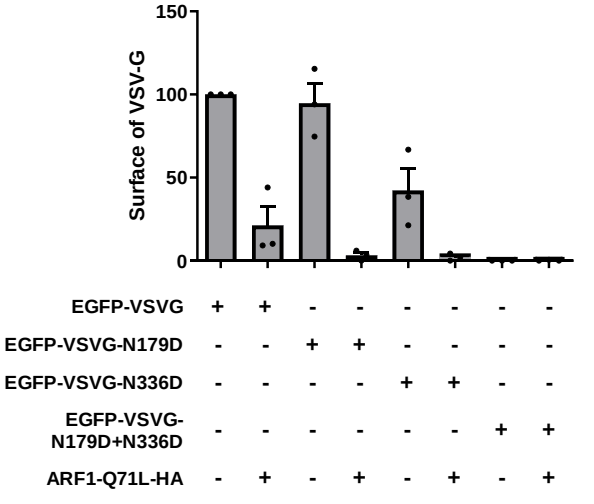
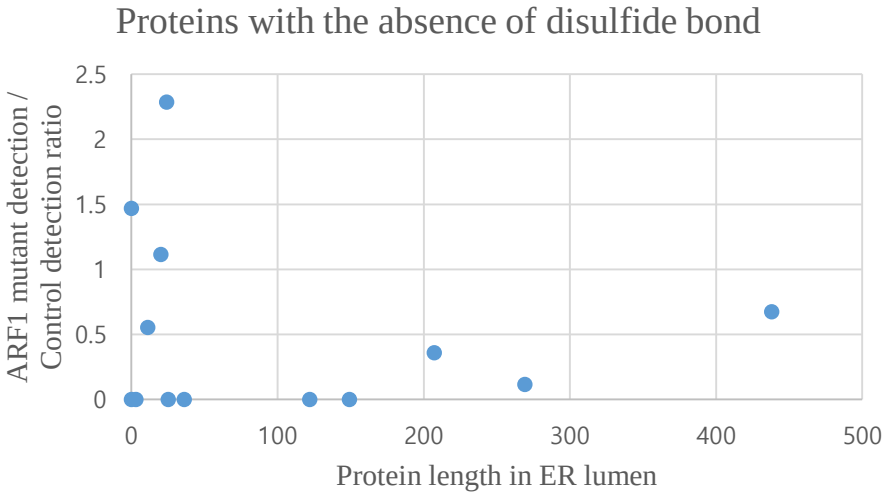


Figure 2. Gycosylation of proteins needs for ERQC and unconventional trafficking. (A) The group treated KIF, Lane 4, shows that inhibition of ER mannosidase makes VSV-G be rescued. (B) Quantification of figure 2A. Analyzed by one-way ANOVA, Tukey's multiple comparisons, n=3. (C) Knockdown of MAN1B1 shows increase VSV-G rescue. (D) Quantification of figure 4C. Analyzed by one-way ANOVA, Tukey's multiple comparisons, n=3. (E) The deletions of glycosylations has no influence of unconventional trafficking of VSV-G. It is shown in lanes 4, 6, 8. (F) Quantification of figure 4E, n=3.

3. Disulfide bonds do not affect unconventional trafficking

We investigated the number of cysteine residues in the ER lumen and find the significance of the ratio of unconventional trafficking to conventional trafficking. As shown in **Figure 3**, most of the proteins have cysteines and disulfide bonds and there is no significance between disulfide bonds and UPS through this graph. Through proteomics data, we determine that disulfide bonds are not an unconventional trafficking factor. Through this data, Proteins rescued in ER stress has only one common feature and it is that protein length in ER lumen is shorter than others.



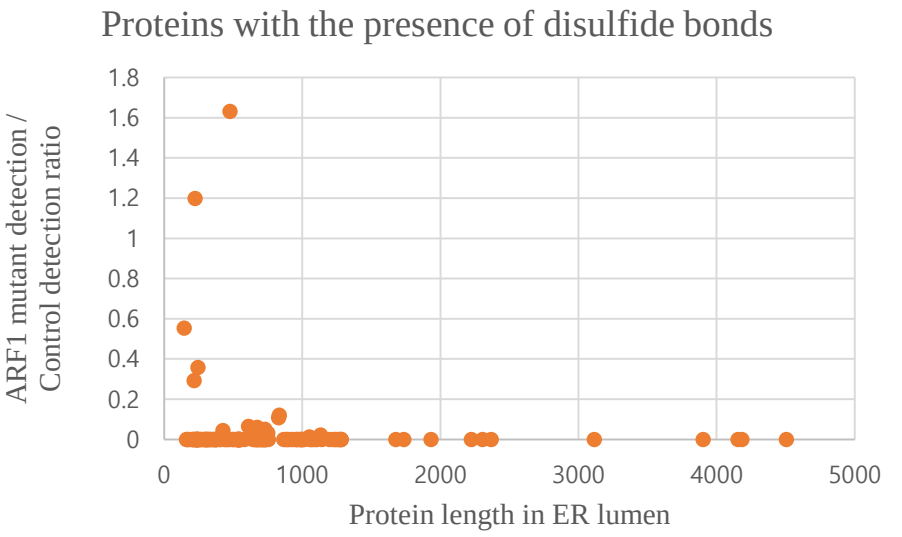


Figure 3. Disulfide bonds are not significant in unconventional trafficking. Disulfide bondsare not related to the ratio of unconventional trafficking to conventional trafficking. Only the protein length in ER lumen is considered an unconventional trafficking factor.

4. Degradation and protein accumulation in the ER are necessary for unconventional trafficking

If ARF1 mutant is transfected, ER stress will increase and ERQC and ERAD molecules will be expressed causing proteins to degrade. We assumed that if we block ERAD not to degrade VSV-G in ER or force VSV-G expression to increase in ER, VSV-G will be accumulated in ER and it will result in the rescue of VSV-G by unconventional trafficking pathway. MG132 is the molecule that inhibits 26S proteasome[12]. As shown in **Figure 4A**, when MG132 was treated 12hr, VSV-G can be rescued under ER stress. What's unique is that when MG132 was treated 24hr, VSV-G was rescued dramatically. It is shown in **Figure 4C**. Treating MG132 24hr made many cells died and cell states were quite bad. When the cells almost died, it seemed that most VSV-G could reach the plasma membrane by a

specific pathway. It's just speculation, but it seems to be related to autophagy. As a result of simply increasing the overexpression of VSV-G by concentration dependence, it was found that unconventional trafficking was possible even if we didn't block ERAD. It is shown in **Figure 4D**. We assumed that it was because the VSV-G amount has exceeded ERQC and ERAD limitation levels in cells. It is predicted that VSV-Gs escaped ERAD can be rescued. Next, we investigated whether strengthened ERAD decreases unconventional trafficking of proteins. If our hypothesis is correct, strengthened ERAD decreases the unconventional trafficking of proteins. ΔF508-CFTR can be rescued under ER stress by GRASP55. It is representative of unconventional trafficking cargo. There are some degradation factors of ΔF508-CFTR because it has folding defects in domain NBD1 localized in the cytosol. It is known that degradation of ΔF508-CFTR can be accelerated by the ERAD-C component, DNAJB14. Also, SEC61A1 is known as the retrotranslocation channel of CFTR[13,14,15]. We overexpressed DNAJB14 and Sec61A1 to strengthen ERAD. As shown in **Figures 4F and 4H**, when we overexpressed DNAJB14 and SEC61A1, ΔF508-CFTR was not detected in the plasma membrane. It means that if ΔF508-CFTR eliminate quickly through strengthened ERAD , it cannot be rescued under ER stress. Especially, the decrease of lysate of CFTR shows that ERAD is strengthened by DNAJB14 and SEC61A1. Based on these data, degradation of proteins in ER is an obstacle for unconventional trafficking. Overall, it shows that remaining in ER is a prerequisite for unconventional trafficking.

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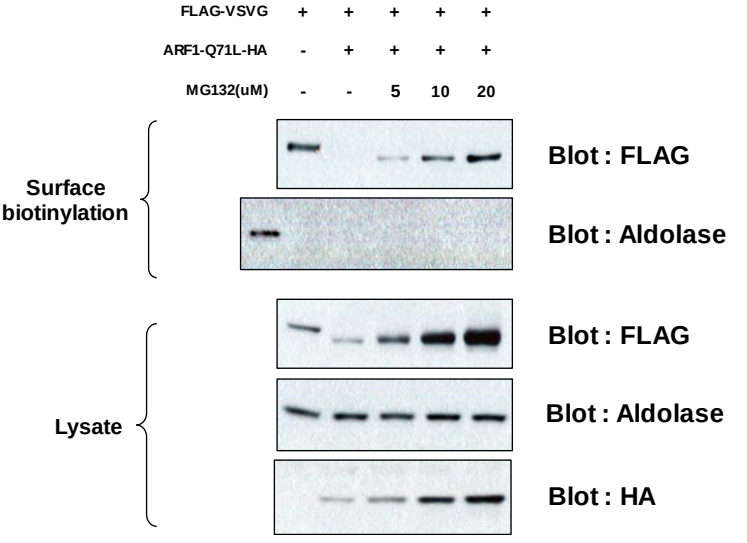

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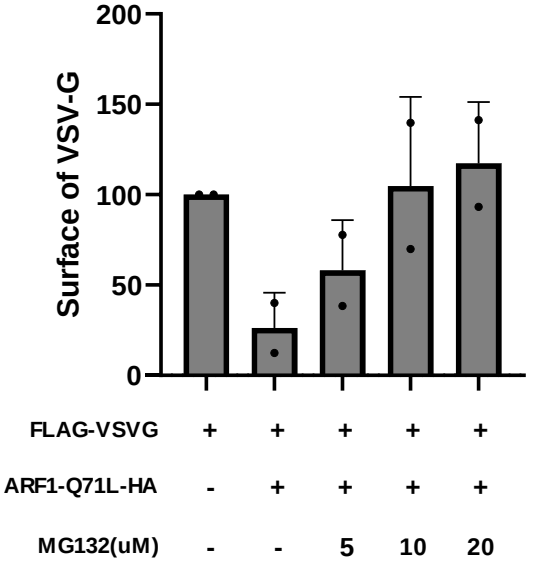

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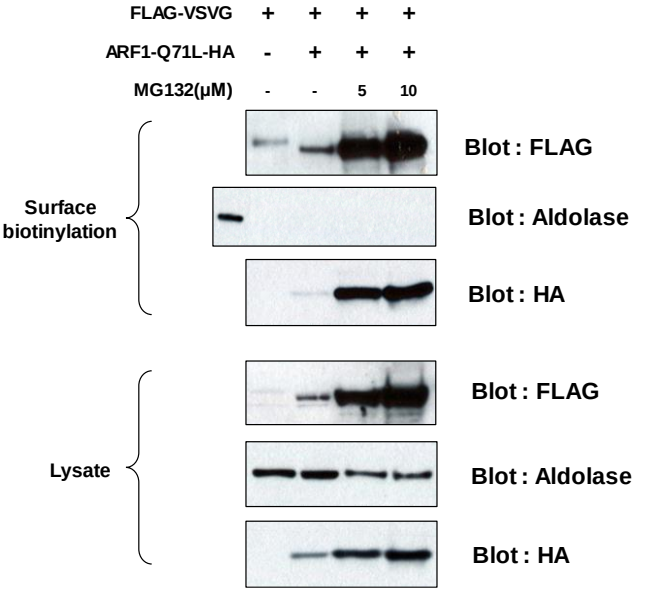

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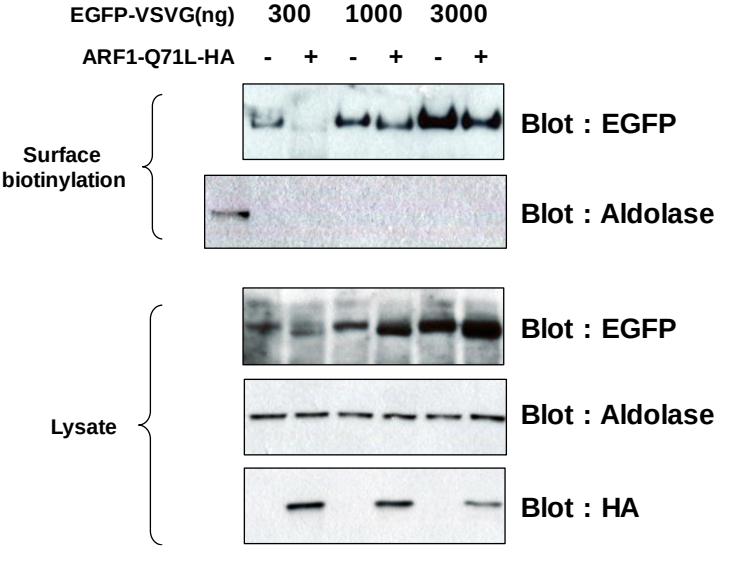

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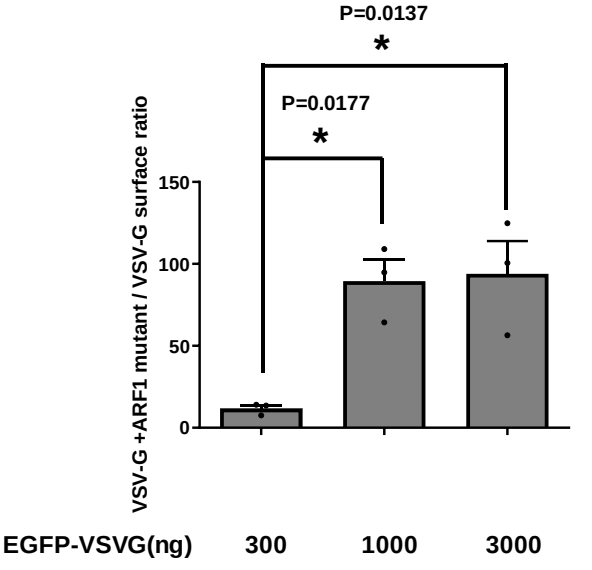

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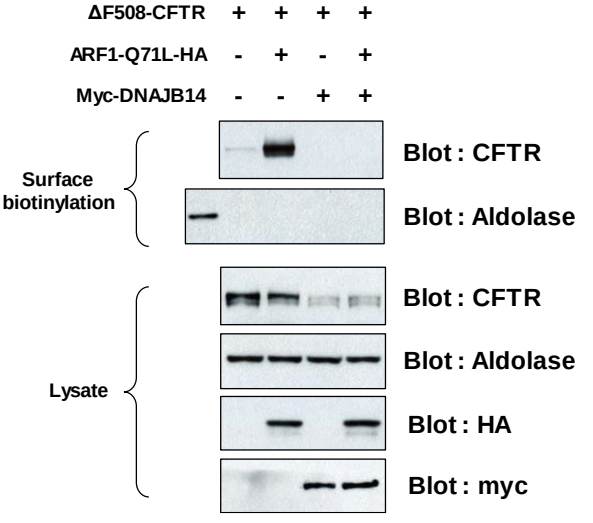

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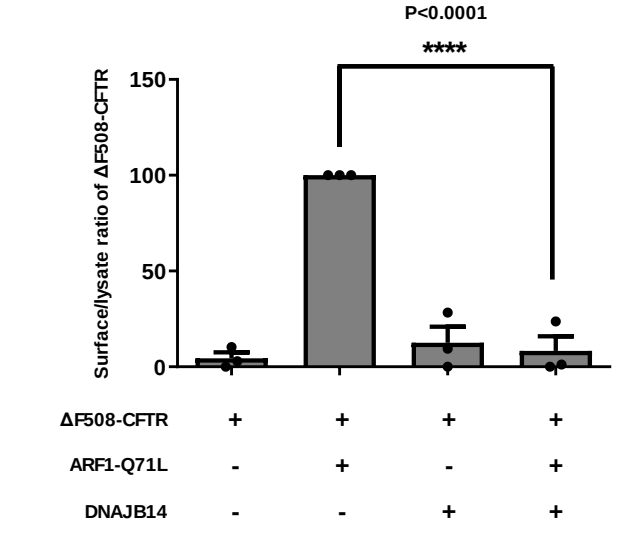

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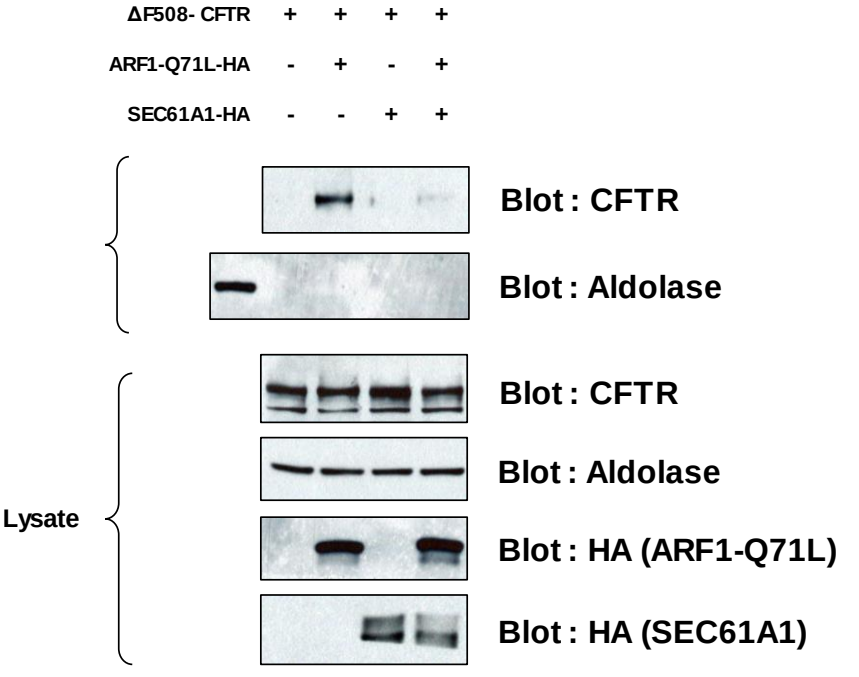

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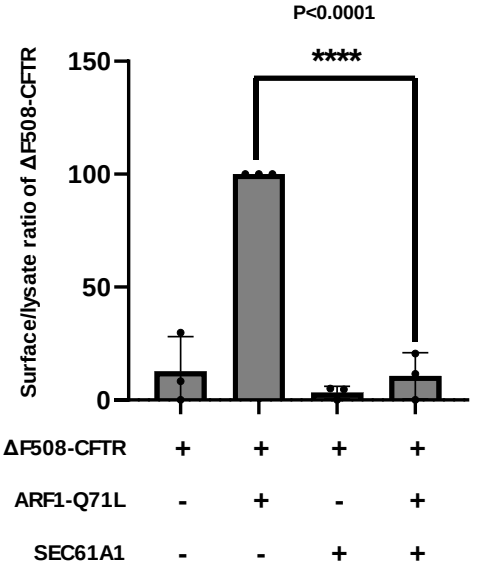

Figure 4. Accumulating proteins in ER causes unconventional trafficking and degrading proteins in ER blocks unconventional trafficking. (A) MG132 is treated 12hr. Inhibition of 26S proteasome blocks ERAD and it causes VSV-G to reach plasma membrane. (B) Quantification of Figure 4A, n=2. (C) VSV-G unconventional trafficking occurs dramatically because of treatment MG132 24hr. (D) The increased overexpression of VSV-G causes accumulation of VSV-G in the ER and makes it possible VSVG to reach plasma membrane. (E) Quantification of figure 4C, n=3. As expressions of VSV-G increased, the surface and lysate ratio also increases. (F) DNAJB14 accelerates the degradation of ΔF508-CFTR and the result leads to the failure of unconventional trafficking. (G) Quantification of figure 4F. lane 3,4 are almost equal to negative control (lane 1). Analyzed by one-way ANOVA, Tukey's multiple comparisons, n=3. (H) Overexpressing SEC61A1 leads to degradation of ΔF508-CFTR. (I) Quantification of Figure 4H, . Analyzed by one-way ANOVA, Tukey's multiple comparisons, n=3.

5. Co-chapeones are important to degradation of proteins in ER

Bip cycle in ERQC is also crucial. Bip is a chaperone and binds to the substrate directly. It seems that Bip is important to ERAD, however, as shown in **Figure 5A**, when HA15, an inhibitor of Bip[16], was treated, the cell surface of VSV-G fell further than the negative control. It is predicted that Bip is associated with unconventional trafficking positively. To confirm accurately, we use siRNA which decreases Bip expression and verify the same phenotype of HA15. It is shown in **Figure 5C**. The main function of Bip is to fold or degrade substrate. To bind to substrate tightly, Bip needs co-chaperones. J-domain in co-chaperones makes bip bind to substrate tightly. There are 7 co-chaperones that have J-domain in ER[17]. They are called ERdj proteins. ERdj1 and ERdj 2 are known to be associated with translation of proteins. ERdj3, 4, 5, and 6 are predicted to play roles in protein folding and ERAD-L and ERdj7 is not widely known. To determine which ERdj protein is critical to ERAD, siRNA was transfected in the cells to lower ERdj expressions. As shown in **Figure 5E**, when the Erdj5 expression was reduced by siRNA, the cell surface of VSV-G increased. So, It is considered that ERdj5 is a key component of ERAD-L. In addition to ERAD-L, ERAD-C seems to participate in ER degradation. As shown in **Figure 5G**, knockdown of ERAD-C component showed increasing the cell surface of VSV-G. ERAD-C components, DNAJB14, DNAJC12, and DNAJC14 are also important to degrade VSV-G under ER stress. In the case of VSV-G, considering that the C-terminal of VSV-G has 29 amino acids, so ERAD-C components maybe not interact with VSV-G's C-terminal sequences directly. We deduce that ERAD-L and ERAD-C are not separate areas but have a cooperative relationship with each other. To find out whether ERdj5 pratically accelerate the VSV-G protein, stability experiments were performed by using cycloheximide(CHX). After overexpression of ERdj and ARF1 mutant, CHX was treated to confirm VSV-G stability, and as a result, it was confirmed that VSV-G disappeared fast rate in ERdj5 overexpression group. It is shown in **Figure 5I~K**. Based on this result, ERdj5 can accelerate the degradation of VSV-G directly under blockade ER to Golgi.

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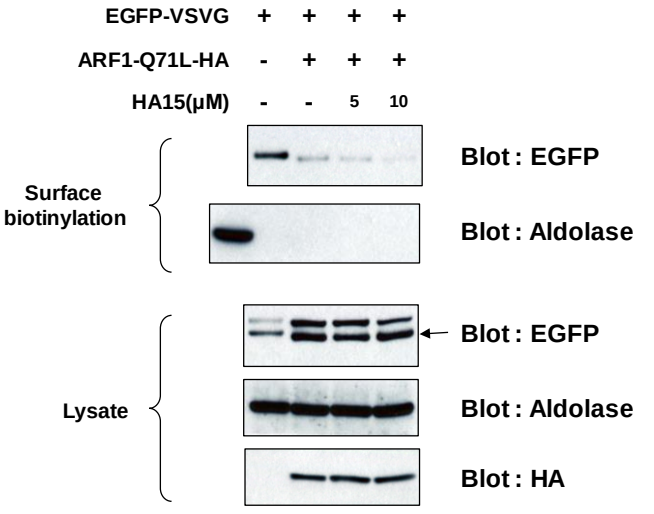

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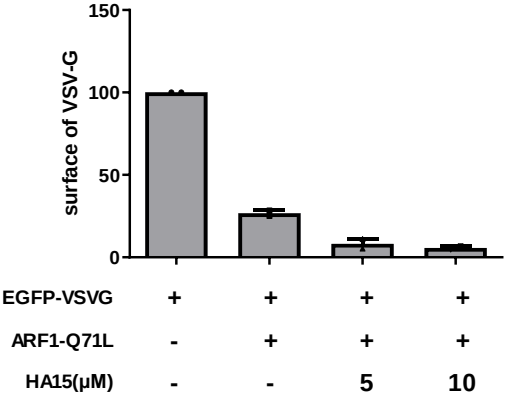

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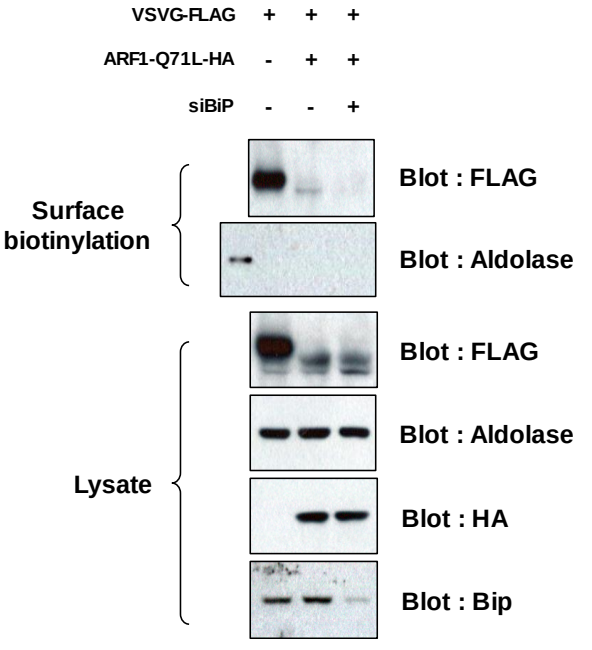

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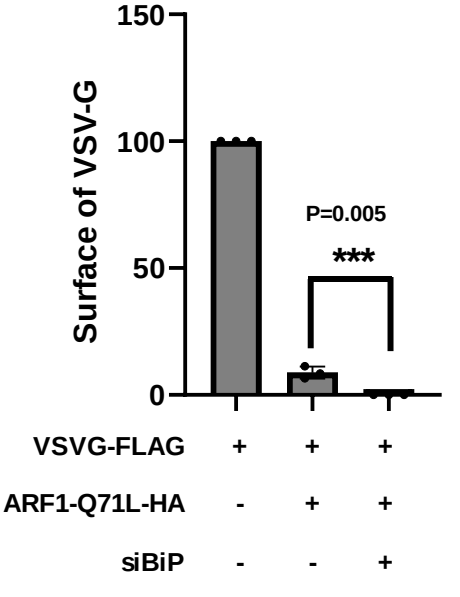

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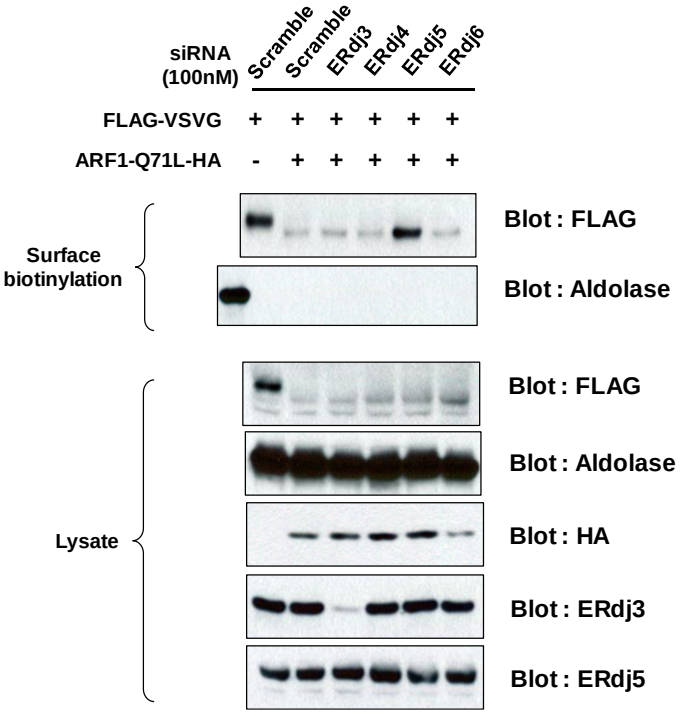

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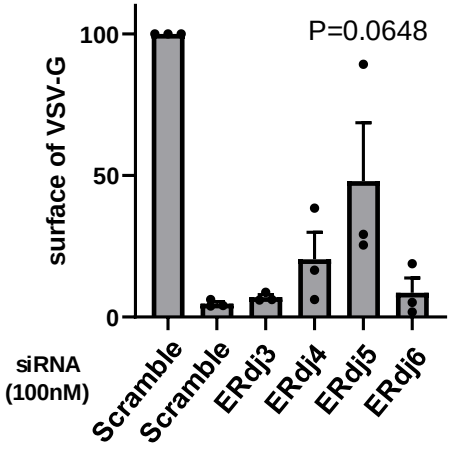

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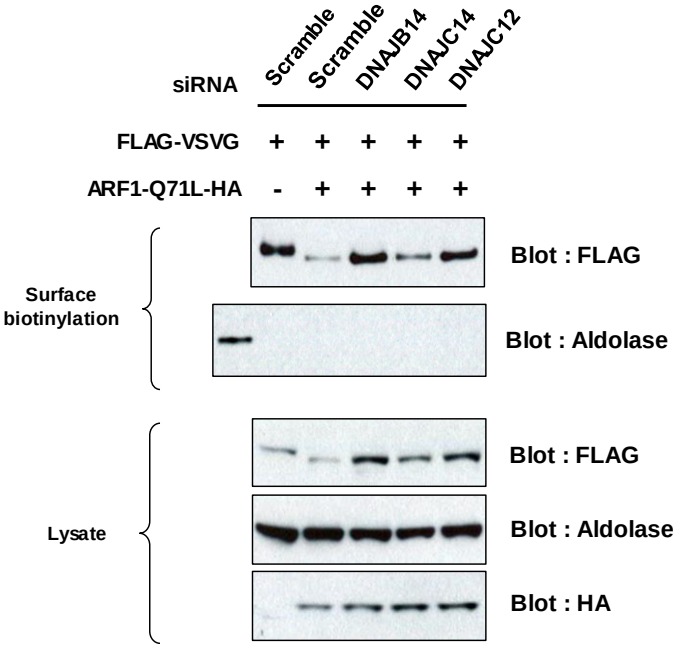

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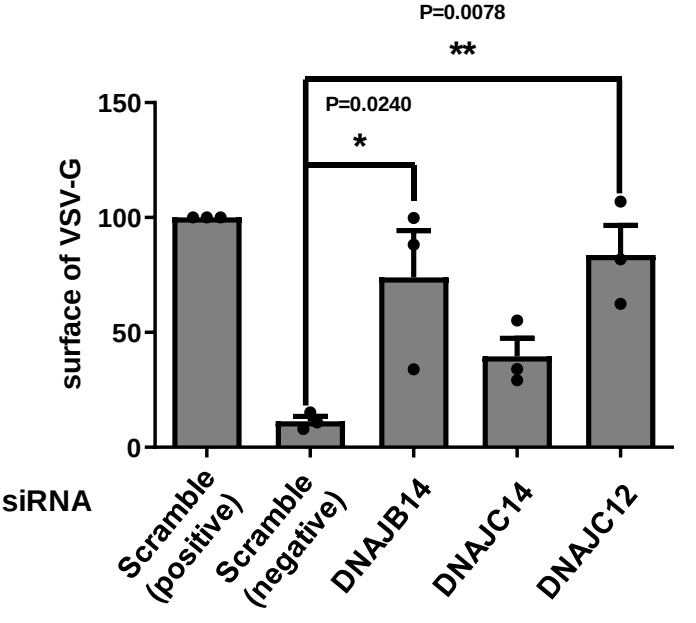

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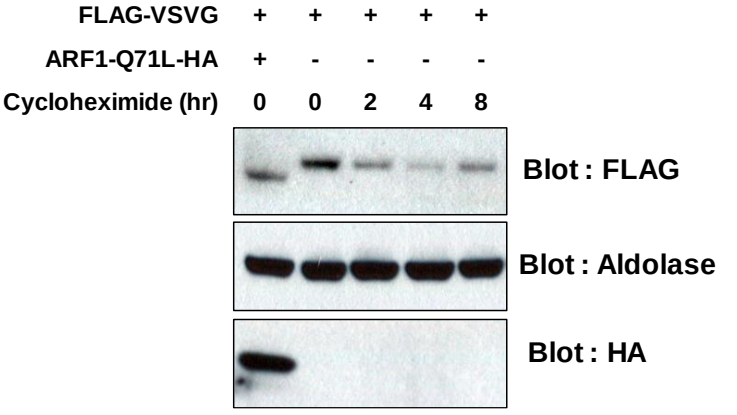

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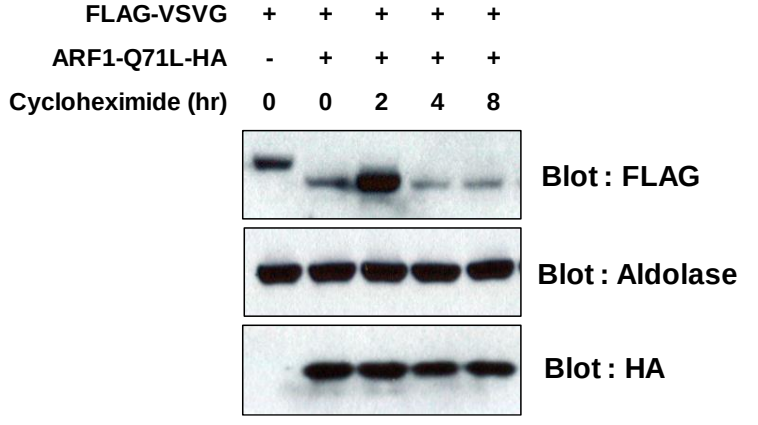

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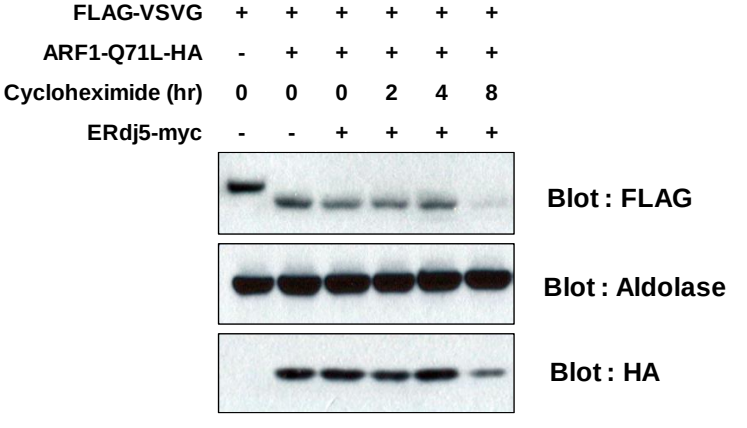

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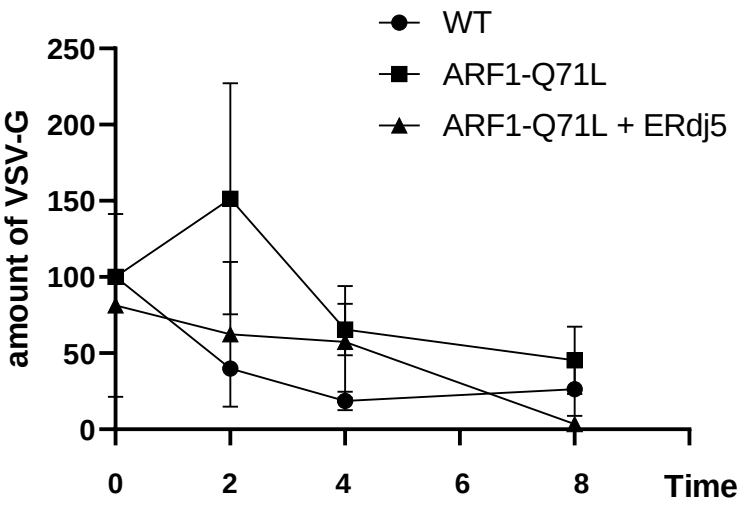

Figure 5. Co-chaperones are quite important as ERAD component.
(A) Inhibition of Bip is not affected on unconventional trafficking. (B) Quantification of figure 5A. n=3 (C) Knockdown of Bip shows the same phenotype of HA15. (D) Quantification of figure 4C. Analyzed by one-way ANOVA, Tukey's multiple comparisons, n=3. (E) Erdj5 is a key component of ERAD-L. when ERdj5 expression is lowered by siRNA, VSV-G can evade ERAD. (F) Quantification of figure 5E. Analyzed by one-way ANOVA, Tukey's multiple comparisons, n=3**.** (G) In addition to Erdj5, DNAJB14, DNAJC14 and DNAJC12 are associated with the degradation of VSV-G. (H) Quantification of figure 5E. Analyzed by one-way ANOVA, Tukey's multiple comparisons, n=3**.** (I)~(K) Co-transfected VSVG and Erdj5 and treated CHX. ERdj5 can accelerate the rate of degrading VSV-G.

IV. DISCUSSION

In this present study, some common characteristics of unconventional trafficking were discovered. We identified plasma membrane proteome in both normal and ER to Golgi blockade conditions in HEK293 cells. Based on the proteomic data, many membrane proteins were detected in the plasma membrane in normal conditions, but under the ER to Golgi blockade conditions, most proteins disappeared in the plasma membrane. We analyzed proteomic data and find a characteristic. Proteins secreted by unconventional trafficking have short peptides in the ER lumenal part. Not only that, most of the detected membrane proteins are type 1 membrane proteins. So, we use VSV-G which is a type 1 membrane protein and is considered as the representative model of type 1 membrane proteins. We assume that tendency of VSV-G in the ER stress condition represents common proteins. By using VSV-G, we can understand parts of the UPS mechanism and discover some characteristics.

To reach the plasma membrane, membrane proteins have to evade the degradation system in ER. It is indicated that the proteins detected in the plasma membrane must evade the ERAD or ERQC and bypass the Golgi through the unconventional trafficking route. The evidence that proteins rescued have to bypass the Golgi is that they have a smaller molecular size than the original size since proteins bypassing Golgi have only high mannose glycan. They appear distinct in size in western blot. By inhibiting ERAD or ERQC factors, it is possible to confirm that the stability of proteins in ER is important for unconventional trafficking. Even though VSV-G cannot be rescued under ER to Golgi blockade, it is detected in the plasma membrane by avoiding ERAD or ERQC. It is comprehended that ERAD and ERQC are the main components of protein stability under ER stress conditions. ER mannosidase and ER glucosidase are the factors in CNX cycle. Interestingly, VSV-G was not detected in the plasma membrane when ER glucosidase was suppressed by DNJ but was detected when ER mannosidase was blocked by KIF. This reveals that ER mannosidase is the main factor in the CNX cycle and rate-determining step. ER mannosidase is known to trim the mannose-9 glycan to mannose-5 or -8 glycans[18]. To degrade the proteins with

glycans, glycans have to be cleaved to under mannose-7. We speculate many proteins with mannose-9 can evade ERQC and ERAD systems when ER mannosidase is blocked. Exiting the ER by conventional trafficking needs the mannose-8 glycan, but it is unknown which glycan is needed when proteins are transported by the unconventional trafficking route. We speculate that the proteins with mannose-9 glycan can exit the ER because VSV-G can be rescued when ER mannosidase loses mannose-9 trimming function. It is predicted that releasing the proteins in the ER under blockade ER to Golgi is different from the conventional trafficking route.

ERAD can be divided into 3 parts ERAD-L, ERAD-M, ERAD-C[19]. ERAD-L & M components are in ER lumen or ER membrane, but ERAD-C factors are in ER membrane or cytosol. Their localization is different but their role is the same in that they degrade proteins. Most proteins cannot be rescued under blockade ER to Golgi and VSV-G is used as their representative cargo. VSV-G is a type 1 membrane protein which has 462 amino acids in ER lumenal part and 29 amino acids in the cytosolic part. During translation or staying in ER, many chaperones and co-chaperones modify VSV-G. Due to its long peptide in ER, ERAD-L components most likely interact with ER lumenal peptide of VSV-G. Among ERdj proteins, ERdj5 showed the greatest effect of VSV-G rescue in surface biotinylation assay (Figure 5C). ERdj5 has another alias, PDIA19. Protein Disulfide Isomerase (PDI) is an enzyme that plays a role in reducing disulfide bonds. ERdj5 has 4 thioredoxin domains and reduces disulfide bonds to fold substrates accurately or to degrade substrates[20,21]. In conditions that blocked ER to Golgi transportation, ERdj5 probably functions to degrade proteins in the ER. To retro-translocate membrane proteins to the cytosol, disulfide bonds have to be cleaved because the pore of retro-translocation channel is quite narrow. Therefore, membrane proteins can only be released to cytosol if all of their disulfide bonds are broken. ERdj5 also participates in Bip cycle. Depleting the ERdj5 using siRNA causes this cycle to deteriorate. Decreased ERQC functions provide a chance for VSV-G to evade QC cycles. Based on the protein stability experiment using CHX, it seems that ERdj5 can accelerate the degradation of VSV-G under

ER stress conditions. ERdj3 and ERdj4 are also known as ERAD factors but we couldn't rescue the VSV-G by siRNA knockdown. We speculate that the depletion of only one ERdj protein using siRNA may have no significant effect on the ERAD function because there are many proteins involved in ERQC and ERAD and there are some crosstalks between proteins. Among the ERdj proteins, only ERdj5 shows the rescue of VSV-G and it means that ERdj5 is the critical factor in ERAD-L. ERAD-L & ERAD-C may have a cooperative relationship because depletion of ERAD-C components causes VSV-G to be rescued. VSV-G has a very short peptide which is consisted of 29 amino acids, so we considered that it is hard for ERAD-C components to bind directly to the short peptide of VSV-G. However, decreased expression of co-chaperones associated with ERAD-C shows the rescue of VSV-G. It seems that ERAD-C participates in the degradation of VSV-G. We expect that ERAD-L components have roles that sense and eliminate proteins in the ER to recover homeostasis. ERAD-C components are probably a cooperative system that helps ERAD-L to degrade membrane proteins.

In ER stress conditions, UPR factors including ATF6, PERK, IRE1 are activated. Activated UPR factors result in increased ERQC and ERAD function, the recovery systems of cells. ERQC and ERAD always activate in ER and in stress conditions, their ability is strengthened by UPR factors. UPR system blocks conventional trafficking and removes most proteins in the ER to decrease ER burden including VSV-G. Even strengthened ERAD by overexpression of DNAJB14 and SEC61A1 shows a blockade of ΔF508-CFTR unconventional trafficking.

Only a few proteins are known to reach the plasma membrane through the unconventional trafficking route. Even proteins that reach the plasma membrane require factors that help induce the unconventional trafficking like ΔF508-CFTR and GRASP55. However through the proteomic data, we discovered more comprehensive unconventional transportation characteristics which do not require specific factors like GRASP55 or DNAJC14 and allow the induction of unconventional trafficking. These finding are considered as general principles of unconventional membrane protein secretion.

In the present study, we discovered the general UPS principles that ERQC and

ERAD are critical parts of unconventional trafficking. These findings provide insights into understanding the UPS system and allow future studies to identify methods of blocking ERQC and ERAD factors specifically under normal conditions, to treat many diseases caused by unfolded membrane proteins.

V. CONCLUSION

Based on the proteomics data, we can find the factors which affect the unconventional secretion of membrane proteins.

1. The length of the proteins in ER lumenal part is significant to unconventional trafficking and its shorter length help evade ERQC.
2. Glycosylations of proteins are important to ERQC and may be essential to unconventional trafficking.
3. Rescue of membrane proteins can be possible to overexpress excessively.
4. UPS is possible only when proteins remain in ER.
5. Co-chaperone components in ERAD-L and ERAD-C are important to degrade proteins in ER. MAN1B1, Erdj5, DNAJB14, DNAJC12 and DNAJC14 are the main factors of ERAD under ER stress condition.

These data demonstrate that unconventional secretion of membrane proteins depends on the stability of membrane proteins. It is contingent on ERAD and ERQC, the critical factors in the UPS system.

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ABSTRACT(IN KOREAN)

막 단백의 비전형적 수송에 관한 소포체 Quality Control 시스템의 역할

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장동건

단백의 구조적 접힘에 문제가 생길 경우 대부분의 단백들은 소포체 내에서 Quality Control을 받고 분해된다. 세포막 단백 중 CFTR은 여러 돌연변이 단백을 가지지만 그 중 가장 흔한 것은 페닐알라닌이 제거된 ΔF508-CFTR이다. 이 단백은 구조적 접힘에 문제가 생겨서 세포막에 도달하지 못한다. 하지만 이 단백은 GRASP55-의존적인 비전형적 수송통로를 통해 세포막에 도달할 수 있다. 비전형적 수송통로는 소포체 스트레스나 소포체-골지체 간 막힘에 의해 발생한다. ΔF508-CFTR 이외에도 많은 단백들은 비전형적 수송통로를 통해 세포막에 도달할 수 있을 것으로 예상한다. 이번 연구에서 우리는 비전형적 수송통로를 통해 세포막에 도달하는 단백들의 특징들을 밝혀내는 것에 목적을 두고 있다. 소포체-골지체 간의 막힘으로 비전형적인 단백의 분비를 유도하기 위해 HEK293 세포에 ADP-ribosylation factor 1 (ARF1) mutant, GTP-binding protein SAR1 (SAR1) mutant 플라스미드를 접종하였다. ARF1, SAR1 mutant는 우성 음성(dominant negative)이기 때문에 단순 접종으로 소포체-골지체 간의 이동을 막을 수 있다. 세포막 단백들은 surface biotinylation을 통해 얻었고 질량분석을 통해 단백들을 분석하였다. 비전형적 수송 비율과 소포체 내의 단백 길이의 관계는 반비례 관계임을 확인하였다. 만약 소포체 내의 단백 길이가 길다면 세포막으로 도달하기 어려울 것으로 예측된다. 이를 기반으로 Endoplasmic Reticulum Associated Degradation (ERAD) and Endoplasmic Reticulum Quality Control (ERQC)와 비전

형적 수송은 반비례하는 관계일 것으로 가정하였다. 왜냐하면 소포체 내의 단백들은 단백들을 변형키거나 분해시키는 많은 샤페론들과 상호작용하기 때문이다. VSV-G 단백은 2개의 당화, 6개의 이황화결합 그리고 몇 가지의 도메인을 가지기 때문에 세포내 수송 관찰에 적절하다고 판단하였다. Calnexin cycle을 막았을 때 소포체-골지체가 막힌 상황에서 VSV-G가 세포막에 도달한 것을 확인하였다. ERQC의 기능을 억제하면 VSV-G의 비전형적 수송이 가능함을 발견하였다. 또한 ERAD와 관련된 샤페론들을 siRNA를 이용하여 knockdown하였을 때 VSV-G가 세포막에 도달할 수 있음을 발견하였다. 이러한 발견은 ER 내에서 단백이 분해되지 않고 비전형적 수송경로를 통해 이동할 때 세포막에 도달할 수 있음을 의미한다. 이러한 발견은 비전형적 수송경로에 대한 이해를 높여줄 것으로 기대한다.

핵심되는 말 : 비전형적 단백 수송, 막단백 집합체, ARF1, ERQC, ERAD