The Role of Motor Protein in Unconventional Protein Secretion

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The Role of Motor Protein in Unconventional Protein Secretion

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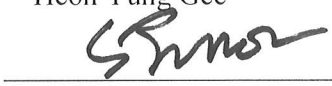

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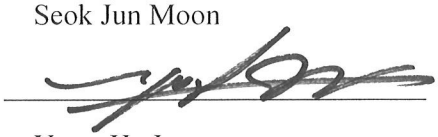

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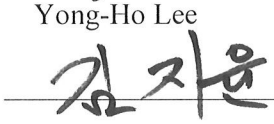

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ABSTRACT

The Role of Motor Protein in Unconventional Protein Secretion

Typically, secretory proteins are transported via the conventional pathway from the endoplasmic reticulum to the Golgi apparatus, before reaching their destination at the plasma membrane or being secreted out of the cell. Recent research has discovered that numerous proteins without a signal peptide are secreted via unconventional protein secretion (UPS), which bypasses the traditional ER-Golgi trafficking route. Several transmembrane proteins, such as folding defective cystic fibrosis transmembrane regulator (CFTR) protein and Spike of coronaviruses are secreted via UPS induced by ER stress. However, the UPS pathway has not been fully elucidated yet. In this study, we found that not only some specific kinesins (KIF1A, KIF5A, and KIF15), but also cytoplasmic dynein, and even specific adaptor proteins such as FYCO1 and SKIP, have an important role in the unconventional trafficking of CFTR. The results of gene silencing indicated that these motor proteins are involved in unconventional secretion of △F508-CFTR, but not in conventional secretion of wild type CFTR. Moreover, these motor proteins also participate in the unconventional transport of the SARS-CoV-2 Spike. These findings suggest that specific motor proteins, distinct from those involved in conventional trafficking, are implicated in the stress-induced UPS of transmembrane proteins.

Key words : CFTR, Spike, unconventional protein secretion (UPS), kinesin, dynein

1. INTRODUCTION

Secretory proteins typically undergo transportation via the endoplasmic reticulum and the Golgi apparatus, before being conveyed to the plasma membrane or released from the cell. Recent findings reveal that numerous substrates also utilize unconventional routes to reach their destinations. Unconventional protein secretion (UPS) is a complex mechanism that includes cargos lacking a signal peptide or transmembrane domain, which can translocate across the plasma membrane, as well as cargos that, despite entering the endoplasmic reticulum (ER), bypass the Golgi apparatus to reach the plasma membrane.[1]

Several transmembrane proteins, such as the misfolded cystic fibrosis transmembrane regulator (CFTR) protein and the Spike of coronaviruses, have been shown to be secreted via UPS induced under cell stress conditions.[2,3] △F508-CFTR protein is created by a deletion of three nucleotides that results in the loss of phenylalanine, the 508th amino acid of the cystic fibrosis transmembrane conductance regulator (CFTR) protein which maintains ion and water exchange on various surfaces in the body.[4] H723R-pendrin protein is created by a mutation of the *SLC26A4* gene which encodes pendrin, an iodide/chloride/bicarbonate transporter expressed in the inner ear.[5] It is also suggested that the assembly and cellular exit of coronaviruses (CoVs) share the unconventional secretion pathway with other budding viruses and lipoproteins.[6]

In conventional Golgi-mediated protein secretion, the CFTR protein is synthesized and undergoes core glycosylation in the ER and transported to the Golgi apparatus, where complex glycosylation occurs. However, △F508-CFTR protein is recognized as a misfolded protein via the ER quality control system and eliminated by ER-associated degradation (ERAD).[7] The unconventional protein secretion of the △F508-CFTR protein, which bypasses elimination by ERAD and goes to the cell surface, is reportedly induced by ER stress, mediated by GRASP-dependent rescue[8], and related to specific autophagy and ESCRT components.[2]

Kinesin and cytoplasmic dynein are microtubule-based motor proteins which transport materials within the cell.[9] Kinesin facilitates cargo transport moving along microtubules toward the plus ends, whereas dynein transports cargo toward the minus end of microtubules. The kinesin superfamily (KIF) is composed of 14 large families,[10] whereas the dynein family has two main

branches; cytoplasmic dynein and axonemal dynein[11]. The purpose of cytoplasmic dynein is to traffic cargo along vesicles and to localize the Golgi apparatus to the center of the cell.[12]

In unconventional protein secretion, the folding-defective △F508-CFTR protein reaches the plasma membrane through an unknown mechanism. However, the specific motor proteins facilitating this transport and the exact directionality of the pathway require further investigation. Motor proteins may interact with the △F508-CFTR protein to facilitate unconventional protein secretion. This study aims to elucidate the role of motor proteins in unconventional protein secretion.

2. MATERIALS AND METHODS

2.1. Cell culture, plasmids, siRNAs, and transfection

HeLa and HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, #11995-065, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gibco, #26140-079, Carlsbad, CA) and 1% penicillin/streptomycin (Gibco, #15140-122, Carlsbad, CA). The cells were incubated at 37°C in an environment containing 5% CO2.

pCMV-CFTR and pcDNA3.1-Spike have been described previously.[6,8] The coding region of ARF1-Q71L-HA was synthesized and inserted into pcDNA3. eGFP-KIF1A was a gift from Juan Bonifacino (Addgene plasmid # 172206 ; http://n2t.net/addgene:172206 ; RRID:Addgene_172206). pCMV-KIF5A-FLAG was commercially purchased (Sino biological, HG19049-CF, Beijing, China).

Scrambled siRNA and siRNAs targeting each gene were purchased from Bioneer, (AccuTarget™ Genome-wide Predesigned siRNAs; KIF1A gene ID:547, KIF5A gene ID:3798, KIF13A gene ID:63971, KIF13B gene ID:23303, KIF14 gene ID:9928, KIF15 gene ID:356992, DHC1 gene ID:1778, DIC1 gene ID:1780, DLIC1 gene ID:51143; Daejeon, Korea).

For transfections, plasmids were introduced into HeLa and HEK293 cells using Lipofectamine LTX Reagent (Invitrogen, #15338-100, Carlsbad, CA), while siRNAs were transfected into HEK293 cells utilizing the RNAiMAX transfection reagent (Invitrogen, #13778-150, Carlsbad, CA), following the manufacturer's instructions.

2.2. Chemicals and antibodies

Thapsigargin was purchased (Sigma Aldrich, T9033).

The following antibodies were procured commercially: anti-CFTR from Alomone Labs (ACL006, Jerusalem, Israel), anti-aldolase A from Santa Cruz Biotechnology (sc-390733, Dallas, TX), anti-HA from Cell Signaling Technology (#2367, Danvers, MA), anti-SARS-CoV-2 S2 from GeneTex (GTX632604), anti-DYKDDDDK from Cell Signaling Technology

(8146), and anti-α-tubulin from Invitrogen (MA1-80017).

2.3. RNA extraction and reverse transcription quantitative polymerase chain reaction (RT-qPCR)

RNA was isolated from the cells using an RNA extraction kit from Bioneer (#K-3140, Daejeon, Korea), following the manufacturer's instructions. The extracted RNA was then used to synthesize cDNA with the cDNA EcoDry Premix (Takara, #639549, San Jose, CA, USA), also according to the manufacturer's protocol. The primer sequences for qPCR for each gene are provided in Table 1.

Table 1. qPCR primers of each gene.

GAPDH	Forward	*5′- GTC TCC TCT GAC TTC AAC AGC G-3*
	Reverse	*5′- ACC ACC CTG TTG CTG TAG CCA A -3′*
KIF1A	Forward	*5’- GCC GAC ATC TTC TGC CAG TTC A -3’*
	Reverse	*5’- GAA GGA CTT GGT CAC CTC CAC T –3’*
KIF5A	Forward	*5’- AGG AGA AGA GCC AGC AGA ACC A -3’*
	Reverse	*5’- CGT TTT CGC TGG TGT CCA CTG A -3’*
KIF13A	Forward	*5′- ATC CAT CGG CTG TGT AAC TGC C-3′*
	Reverse	*5′- GCA TCT GAC CAC CTC TCC CTT A-3′*
KIF13B	Forward	*5′- CGG TGA TTG TCG CTC CTG AAG T-3′*
	Reverse	*5′- CCA ATG CTC CTT CGG AAA GAT GC-3′*
KIF14	Forward	*5′- GCA CTT TCG GAA CAA GCA AAC CA-3′*
	Reverse	*5′- ATG TTG CTG GCA GCG GGA CTA A-3′*

KIF15	Forward	*5′- TTG GAC CAA ACA GCA GGA AGA GC-3′*
	Reverse	*5′- GAC TAC TCG CAG GTC ATG TAC C-3′*
DHC1	Forward	*5′- GCC ATC AGC AAA GAC CAC CTC T-3′*
	Reverse	*5′- AAG ACG ATC CAC TGG CGC TTC T-3′*
DIC1	Forward	*5′- TCA GCC AAG TCT GGC AAG CAC T -3′*
	Reverse	*5′- GAG TCC AAG ACA CAA TCC TGC C-3′*
DLIC1	Forward	*5′- GCA AAG CAA CCA CCA ACT GCA G-3′*
	Reverse	*5′- ATG GGT GAC ACG CTG GCA ACA T -3′*
ARL8b	Forward	*5’- GAT ACC CAC AGT GGG CTT CAA C -3’*
	Reverse	*5’- TGA CTC CTC TGC AAT ACC GCT C -3´*
SKIP	Forward	*5´- CCG AGA AAA CGA AGA GCA GCT G-3´*
	Reverse	*5´- TCC ACC AGG TAT GGC TTC TCT G -3´*
FYCO1	Forward	*5´- CCA GAA GAT GCT TGC TGA CCT G-3´*
	Reverse	*5´- AGC CAT CTC TCC TCA GCA CTG A -3´*

2.4. Surface biotinylation and immunoblotting

To conduct the biotinylation assay, HEK293 cells were initially rinsed twice with cold PBS to eliminate any unbound proteins. The cell surface proteins were then biotinylated using Sulfo-NHS-SS-Biotin (Thermo Pierce, #21331, Waltham, MA, USA) in cold PBS (0.3 mg/ml biotin in PBS) for 30 minutes on ice in the dark. Following the biotinylation process, the cells were treated with a quenching buffer containing 1% BSA in cold PBS for 10 minutes in the dark. The cells were subsequently washed three times with cold PBS, harvested, and lysed in a buffer

containing 150 mM NaCl, 20 mM Tris (pH 7.4), 1 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, and a protease inhibitor (Roche, #04693159001). The cells were homogenized using a sonicator for 20 seconds and centrifuged at 13,200 rpm for 20 minutes at 4°C. The supernatant was collected, and 300 μg of the lysate was incubated overnight at 4°C with 300 μl of 10% streptavidin agarose resin (Thermo Pierce, #20349). The biotinylated protein-bound resin was centrifuged and washed four times with lysis buffer. Biotinylated proteins were eluted using 2x sodium dodecyl sulfate (SDS) sample buffer containing dithiothreitol (DTT, 0.02 g/ml) at 38°C for 40 minutes and subsequently separated by SDS-polyacrylamide gel electrophoresis (PAGE). The separated proteins were transferred onto a nitrocellulose membrane, which was then probed with the appropriate primary antibodies and HRP-conjugated secondary antibodies in 5% skim milk.

2.5. Immunofluorescence assay

HeLa cells were cultured on 18-mm round coverslips and transfected for 24 hours. The cells were then fixed with 4% paraformaldehyde in PBS for 7 minutes at room temperature and incubated in a PBS solution containing 2% BSA and 0.1% Triton X-100 for 20 minutes at room temperature for blocking and permeabilization. After this step, the cells were incubated with the appropriate primary antibodies for 1 hour at room temperature and then washed three times with PBS. Subsequently, the cells were stained with secondary antibodies conjugated to a fluorophore for 30 minutes at room temperature and washed three times with PBS. For nuclear staining, cells were treated with DAPI or Hoechst 33342 solution for 5 minutes at room temperature and washed twice with PBS. The samples were then mounted onto slide glasses using mounting medium (Agilent Dako, #S3025, Santa Clara, CA), and images were acquired using a confocal microscope (LSM 700, Carl Zeiss, Berlin, Germany).

2.6. Statistical analysis

Densitometric analyses for western blot quantification were conducted using Multi Gauge V3.0 software. The results from multiple experiments are expressed as the mean ± standard error of the mean (SEM). Statistical comparisons were performed using one-way analysis of

variance (ANOVA) followed by Tukey's multiple comparison test, utilizing GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA). A p-value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Specific kinesins are involved in UPS, but not in conventional protein secretion, of CFTR.

KIF5A and microtubule-dependent vesicular traffics have a role in unconventional secretion of ΔF508-CFTR[2]. However, it is not fully known which route the misfolded ΔF508-CFTR travels, and which vesicles and motor proteins are involved in the pathway. Kinesin-1 and kinesin-3 moves late endosomes and lysosomes towards the cell periphery[13]. From this background, we first assessed the knockdown effect of kinesin-1 motor KIF5A to reaffirm the previous result and kinesin-3 motor KIF1A on unconventional secretion of ΔF508-CFTR. The overexpression of dominant-negative ARF1 mutant ARF1-Q71L blocks ER-to-Golgi pathway and induces unconventional trafficking of ΔF508-CFTR[8].

To investigate how silencing KIF1A or KIF5A influences the unconventional secretion of ΔF508-CFTR induced by ARF1-Q71L, we employed a surface biotinylation assay. The results show that knockdown of KIF1A and KIF5A genes suppressed unconventional secretion of core-glycosylated ΔF508-CFTR (band B, Figure 1A, B). Next, a surface biotinylation assay was performed to assess the knockdown effect of KIF5A and KIF1A on conventional secretion of wild-type (WT) CFTR (Figure 1C, D). The silencing of the KIF1A and KIF5A did not affect conventional secretion of the complex glycosylated form (band C) of WT-CFTR. The RT-qPCR analyses of KIF1A and KIF5A mRNAs confirmed the gene silencing of siRNAs against these genes (Figure 1E).

Next, we investigated the effect of overexpression of KIF1A or KIF5A or both on unconventional transport of ΔF508-CFTR under ER stress induced by ARF1Q71L (Figure 2). Simple overexpression of one subunit of kinesin motor protein did not increase unconventional transport, or even decreased it. This may be due to the stoichiometric imbalance of the kinesin motor protein caused by the overexpression of the kinesin heavy chain. Alternatively, the GFP-tag or FLAG-tag on each kinesin motor may have inhibited the function of the motor protein itsef.

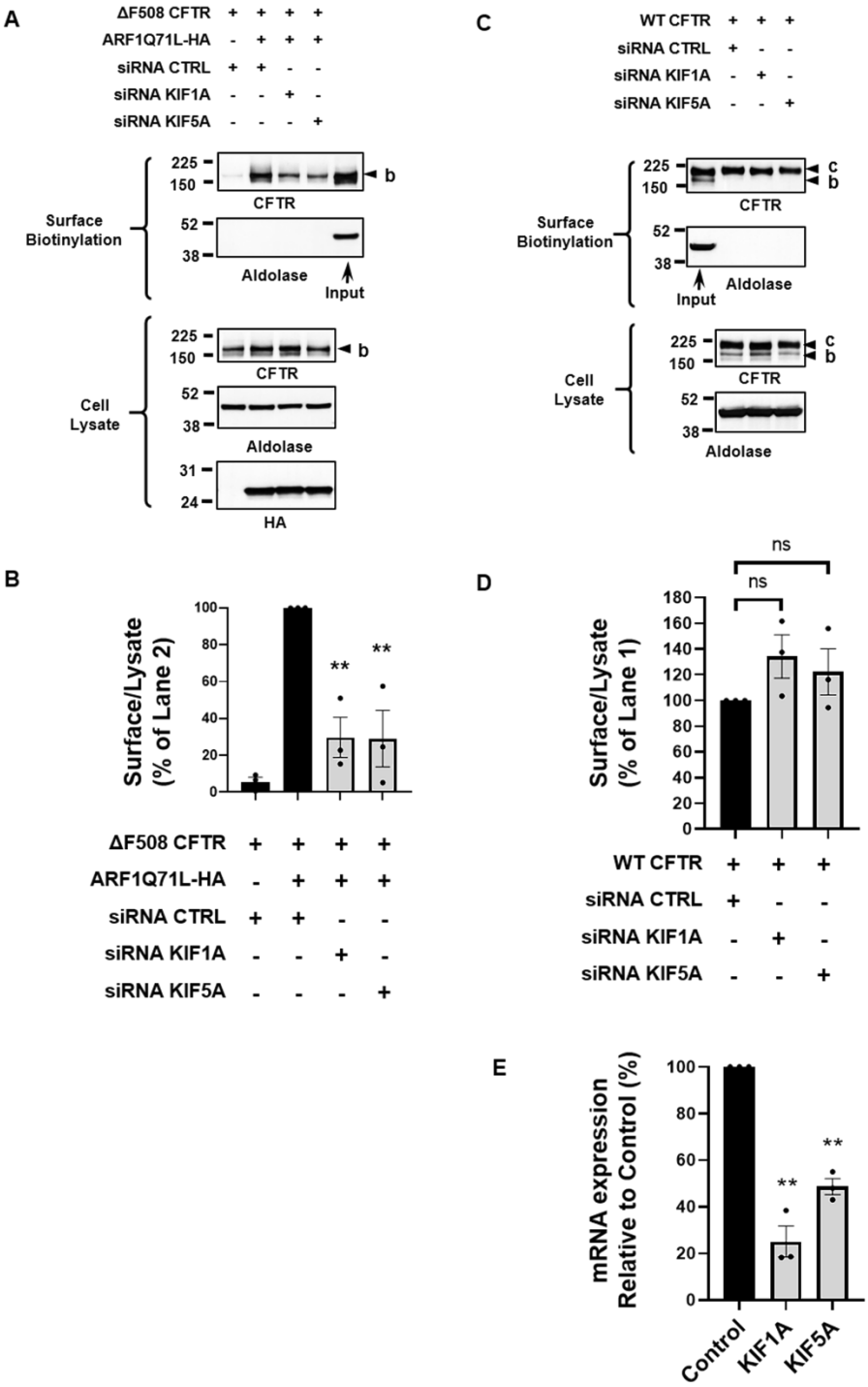


Figure 1. KIF1A and KIF5A are involved in unconventional trafficking of ΔF508-CFTR but not in conventional trafficking of WT-CFTR.

Effects of silencing KIF1A and KIF5A genes on the unconventional trafficking of ΔF508-CFTR (A) and the conventional trafficking of WT-CFTR (C). HEK293 cells were used to perform a cell surface biotinylation assay after being transfected with either control (scrambled) siRNA or specific target siRNAs (50nM each, 48h), along with plasmids encoding ΔF508/WT-CFTR (24h). Some cells were also co-transfected with ARFQ71L-HA to induce ER stress. Surface proteins and cell lysates were quantified respectively (B, D). (E) Shows the knockdown efficiency of each target gene. Bar graph data are expressed as mean ± SEM. *P<0.05, **P<0.01. Statistical analysis was conducted using one-way ANOVA, followed by Tukey's multiple comparison test. b represents band B (core-glycosylated) CFTR; c represents band C (complex-glycosylated) CFTR.

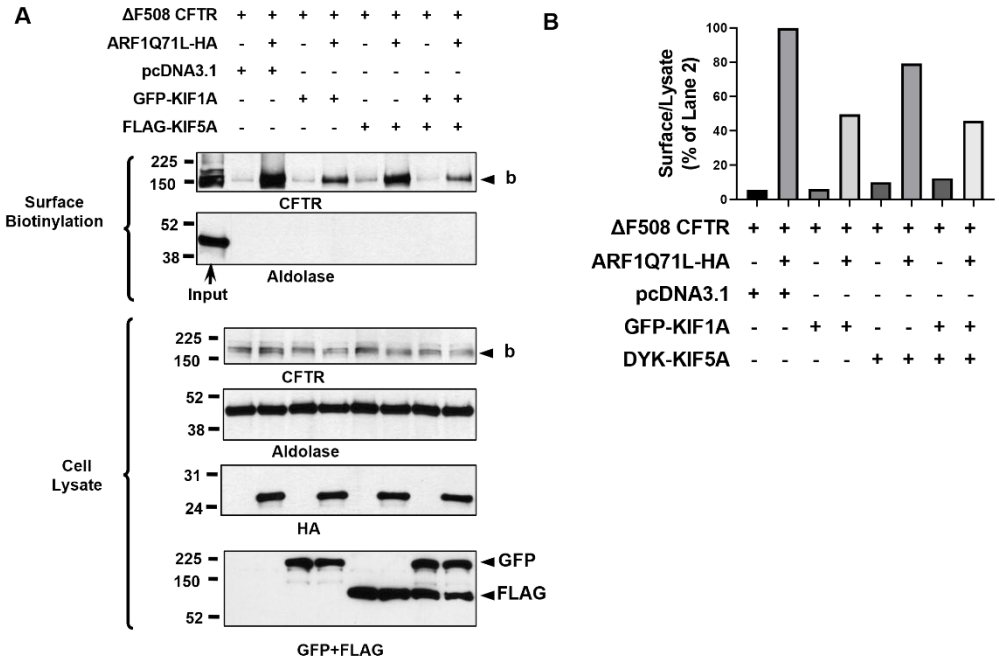


Figure 2. The overexpression of KIF1A or KIF5A or both did not enhance unconventional trafficking of ΔF508-CFTR.

Effect of overexpression of KIF1A or KIF5A or both on the unconventional transport of ΔF508-CFTR (A). HEK293 cells were used to perform a cell surface biotinylation assay after being transfected with annotated plasmids. Some cells were also co-transfected with ARFQ71L-HA to induce ER stress. Surface proteins and cell lysates were quantified respectively (B). b represents band B (core-glycosylated) CFTR.

From a separate screening study aimed at finding motor proteins related to UPS of CFTR, we got a set of motor proteins supposedly involved in UPS of CFTR. The screening of motor protein related to UPS utilized the CRISPR-Cas9 method [14]. Cas9 is a protein that recognizes target DNA based on guide RNA and creates a double-stranded break in the target DNA. Eventually, the gene that is targeted by Cas9 will be knocked out.

First, a cell line that simultaneously expresses Cas9 is created. After creating the Cas9 stable cell line that is necessary for screening, lentiviral transduction is performed with the guide RNA library to knockout the target genes. The guide RNA library is a custom-made set including 68 genes related to motor proteins and microtubule-associated proteins out of a total 1082 genes. After that, ΔF508-CFTR, which has an HA tag on extracellular loop so that only CFTR that has reached the plasma membrane can be detected by FACS, and ARF1-Q71L, which induces ER stress, are transfected to induce unconventional trafficking of ΔF508 CFTR. A simple schematic of this experiment is shown in Figure 3.

After sorting the cells that express CFTR on the membrane using FACS, deep sequencing of the sgRNA region that is inserted in the cell population is performed. Using the MaGeCK algorithm, essential genes are identified, and top 100 genes (Table 2) are selected in order of score. Among these, there are several motor proteins (Table 3).

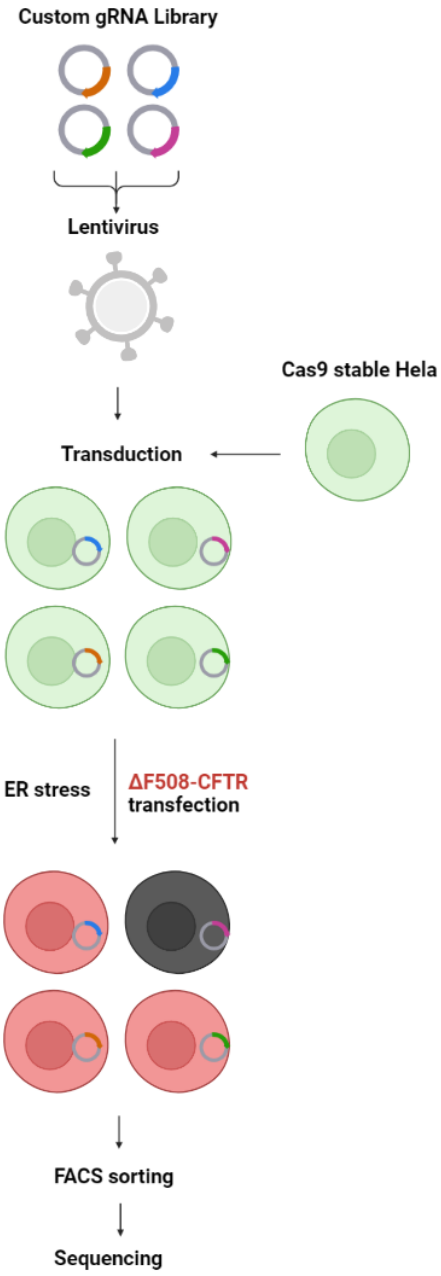


Figure 3. A schematic of CRISPR-Cas9 screening for motor proteins involved in unconventional transport using custom sgRNA library. This figure is created with BioRender.com.

Table 2. Top 100 genes of custom-library CRISPR/Cas9 screening.

VPS26A	VPS4B	IST1	PPP6C	SEC23B
CNIH1	VPS37B	FAM109B	SNAPIN	ACER2
PICALM	BET1L	BCL2L11	ATG2B	GJB2
RABGEF1	FKBP5	DNAJB11	IFT22	NAPG
TRIM8	NUCB2	VAMP8	KIAA0368	FKBP4
DNAJC15	TMEM165	ERN1	PFDN6	CHMP6
FCHSD2	DDX11	BBS12	UFM1	VPS13C
DNAJB6	VAMP4	ADRA1A	ARFGAP3	TBC1D5
ANXA7	CCDC132	PFDN2	MLC1	RAB1B
FOLR1	ALG8	ATG2A	ARPC5	COPG2
HFE	RAP2A	MT3	KIF6	SRPRB
PPID	GAK	UBA5	SYT17	RPN2
VPS37A	MGAT5	ACKR2	VPS35	KIF13B
MANF	TMED9	GOSR1	CHORDC1	FKBP1A
PSAP	PIP4K2A	BLOC1S6	TMED3	MEFV
TRIM9	SCYL1	LAMP3	FKBP14	KIF14
DNAH7	KIF13A	SNAP25	TARDBP	DYNC1LI1
YKT6	ATG14	P4HB	CSNK2A1	GAPVD1
TUSC3	DERL3	SYT2	IRF8	PRKCZ
KIF15	STX7	DNAJC30	HSPA2	VAPB

Table 3. Motor proteins in top 100 genes of custom-library CRISPR/Cas9 screening.

Rank	ID	neg|score	neg|p-value	Description
17	DNAH7	0.0022025	0.012145	Dynein heavy chain 7, axonemal
20	KIF15	0.0029747	0.015903	Kinesin-like protein KIF15
37	KIF13A	0.0057162	0.028647	Kinesin-like protein KIF13A
71	KIF6	0.014066	0.059255	Kinesin-like protein KIF6
93	KIF13B	0.021158	0.083893	Kinesin-like protein KIF13B
96	KIF14	0.022126	0.087047	Kinesin-like protein KIF14
97	DYNC1LI1	0.022267	0.087504	Cytoplasmic dynein 1 light intermediate chain 1

Due to the unavailability of commercial siRNA for KIF6 among kinesin motor proteins out of the top 100 genes, we focused on targeting KIF13A, KIF13B, KIF14, and KIF15. KIF13A has been observed to localize to various tubular endosomes, facilitating the recycling of cargo and transportation to developing melanosomes[15]. Furthermore, KIF13A is known to form either homodimers or heterodimers with KIF13B on early/sorting endosomes, leading to the creation of recycling endosomes[16]. In neurons, KIF13B has also been documented to interact with PIP_3 through centaurin-α1, transporting PIP_3-containing vesicles to the tips of axons, covering a long-distance journey[17]. KIF14 participates in chromosome congression/alignment and cytokinesis[12]. KIF15 is a tetrameric microtubule motor that can cross-link and transport parallel microtubules, leading to the formation of parallel bundles[18]. The surface biotinylation assays shows silencing of KIF14 and KIF15 gene reduced unconventional secretion of ΔF508-CFTR (Figure 4A, B). On the other hand, the knockdown of these kinesins has no effect on conventional secretion of WT-CFTR (Figure 4C, D). The efficacy of each siRNA treatment was validated by RT-qPCR (Figure 4E).

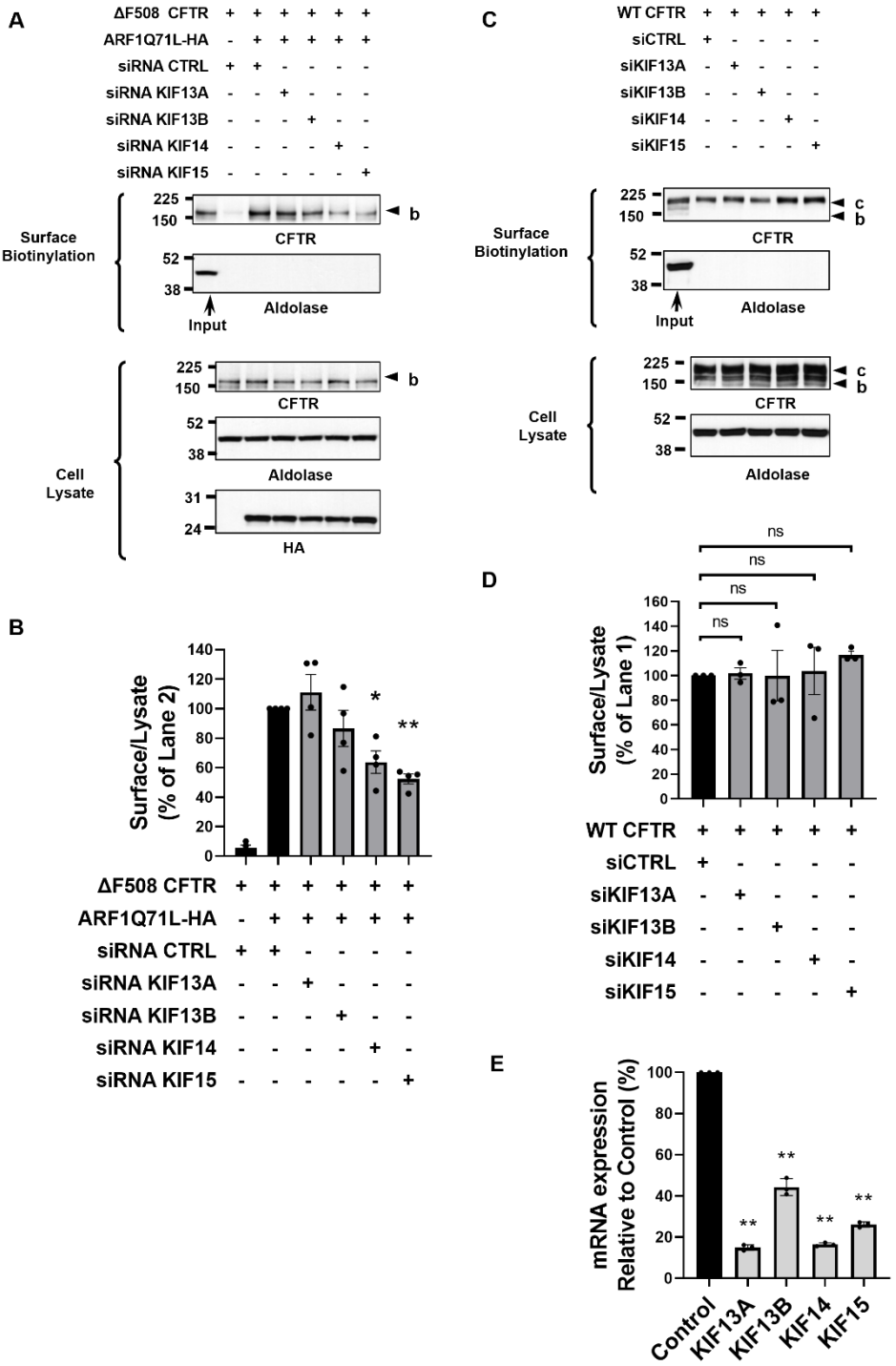


Figure 4. KIF14 and KIF15 are involved in unconventional trafficking of ΔF508-CFTR but not in conventional trafficking of WT-CFTR.

Effects of silencing KIF13A, KIF13B, KIF14 and KIF15 genes on the unconventional trafficking of ΔF508-CFTR (A) and conventional trafficking of WT-CFTR (C). HEK293 cells were used to perform a cell surface biotinylation assay after being transfected with either control (scrambled) siRNA or specific target siRNAs (500nM each, 48h), along with plasmids encoding ΔF508/WT-CFTR (24h). Some cells were also co-transfected with ARFQ71L-HA to induce ER stress. Surface proteins and cell lysates were quantified respectively (B, D). (E) hows the knockdown efficiency of each target gene. Bar graph data are expressed as mean ± SEM. *P<0.05, **P<0.01. Statistical analysis was conducted using one-way ANOVA, followed by Tukey's multiple comparison test. b represents band B (core-glycosylated) CFTR; c represents band C (complex-glycosylated) CFTR.

There are some adaptor proteins known to interact with kinesin-1or kinesin-3 to transport axonal endolysosomes; SKIP, Arl8, and FYCO1[19]. The BORC–Arl8–SKIP–kinesin-1 complex helps to couple lysosomes to kinesin-1, which allows lysosomes to be transported along microtubules.[20] The small GTPase Arl8, a member of Arf-like (Arl) family of proteins, has two paralogs, Arl8a and Arl8b in vertebrates. Arl8b has been more extensively studied than Arl8a, and it has been shown to recruits kinesin-1 to lysosomes, which helps drive the movement of lysosomes towards the cell periphery[21]. FYCO1 helps recruit kinesin-1, a motor protein, to endolysosomes, which allows endolysosomes to be transported along microtubules[22]. Based on this information, we examined the effect of knockdown of these adaptor proteins on unconventional transport of ΔF508-CFTR (Figure 5A). When ARL8b was silenced, the levels of cytosolic ΔF508-CFTR protein were significantly reduced, hindering the accurate determination of CFTR cell surface trafficking (Figure 5B). Therefore, it was excluded from the analysis of its effect on UPS. Notably, knockdowns of FYCO1 and SKIP significantly reduced the UPS of ΔF508-CFTR (Figure 5C), implying that these adaptor proteins may participate in the KIF5A- or KIF1A-mediated trafficking of CFTR-containing UPS vesicles. The RT-qPCR analyses of FYCO1, SKIP, and ARL8b mRNAs confirmed the gene silencing of siRNAs against these genes (Figure 5D).

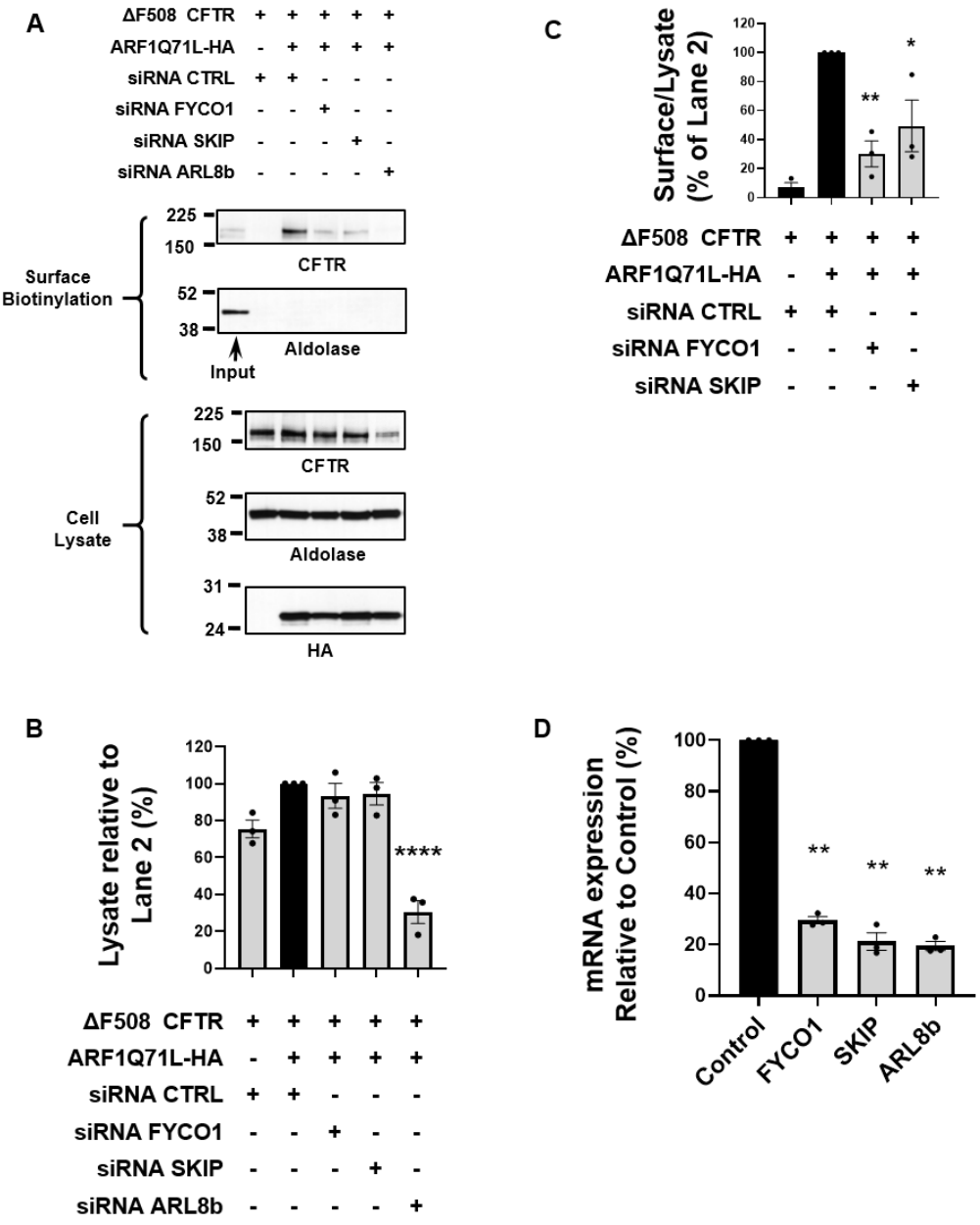
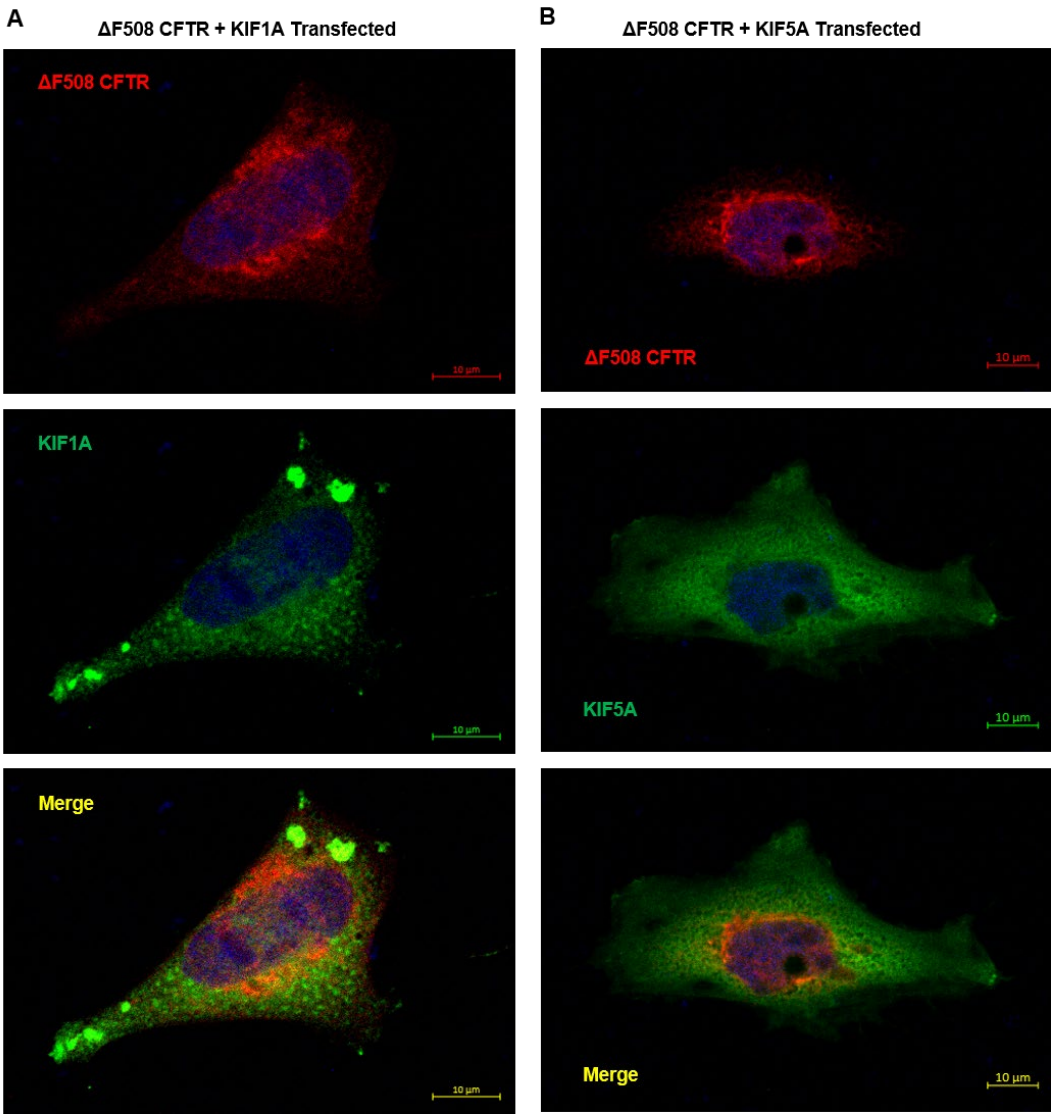


Figure 5. FYCO1 and SKIP, adaptor proteins, is involved in unconventional trafficking of ΔF508-CFTR.

Effects of silencing SKIP, ARL8b, and FYCO1 genes on the unconventional trafficking of ΔF508-CFTR (A). HEK293 cells were used to perform a cell surface biotinylation assay after being transfected with either control (scrambled) siRNA or specific target siRNAs (100nM each, 48h), along with plasmids encoding ΔF508/WT-CFTR (24h). Some cells were also co-transfected with ARFQ71L-HA to induce ER stress. (B) ΔF508-CFTR in lysate were quantified respectively. (C) Surface proteins and cell lysates were quantified respectively. (D) Shows the knockdown efficiency of each target gene. Bar graph data are expressed as mean ± SEM. *P<0.05, **P<0.01. Statistical analysis was conducted using one-way ANOVA, followed by Tukey's multiple comparison test.

An immunofluorescence assay was performed to assess the localization of KIF1A or KIF5A and ΔF508-CFTR cargo (Figure 6A, B). It was observed that ΔF508-CFTR were detected in perinuclear ER region and there was no specific colocalization between the motor protein and the cargo, ΔF508-CFTR. KIF1A is evenly distributed throughout the cell, but it has been observed that there are also concentrated areas near the cell membrane. On the other hand, KIF5A exhibits an overall dispersed pattern within the cell. Interestingly, we could observe intense red flourescent puncta scattered in cytosol where ΔF508-CFTR signals united together under ER stress conditions induced by ARF1Q71L co-expression (Figure 6C, D). The colocalization of ΔF508-CFTR (red) and KIF1A (green) was detected when unconventional trafficking of ΔF508-CFTR was induced by ARF1Q71L (Figure 6C, arrow) whereas no colocalization was observed between KIF5A and ΔF508-CFTR (Figure 6D).



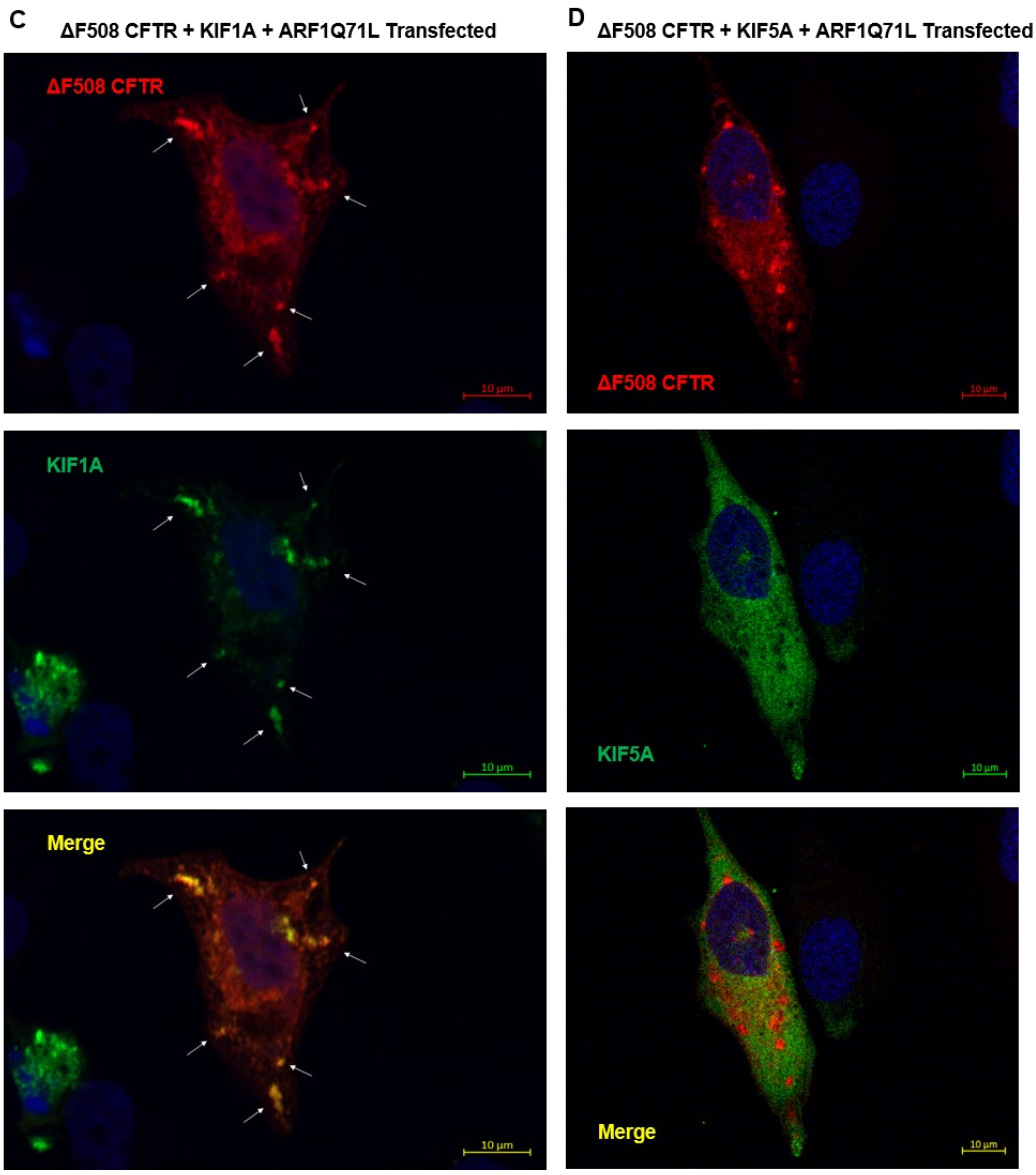


Figure 6. The ΔF508 CFTR puncta are formed by ER stress induced by ARF1Q71L expression and colocalized with KIF1A.

Immunofluorescence analysis of ΔF508 CFTR in cells. HeLa cells were transfected with plasmids encoding ΔF508, with some cells also co-expressing ARF1Q71L (C, D), GFP-KIF1A (A, C), and

FLAG-KIF5A. CFTR (B, D). ΔF508 CFTR was stained with anti-ACL006 antibody (red, Alexa Fluor 568) and KIF5A was stained with anti-FLAG antibody (green, Alexa Fluor 488). The colocalization was marked with a white arrow.

3.2. Cytoplasmic dyneins are involved in UPS of CFTR, and dynein heavy chain is the key subunit among dynein motor subunits.

After the screening of the kinesins, we also investigated the connection between dynein and secretion of UPS cargo. Unlike many different kinesins, there are two dyneins related to transportation of cargo; cytoplasmic dynein-1 and -2[11]. Dynein-1 is involved in mitosis, cell migration and intracellular transportation whereas dynein-2 is involved in cilia biogenesis and signaling[23]. Since this study is on intracellular cargo transportation, we conducted a knockdown experiment of the dynein-1. Dynein-1 is composed of a single dynein heavy chain (DHC) and two isomers of each intermediate chain (DIC) and light intermediate chain (DLIC) and three dynein light chain (DLC) [24]. Dynein architecture is shown in the Figure 7.

We evaluated the knockdown effect of each subunit of dynein-1; DHC1, DIC1 and DLIC1. The silencing of each three subunits of dynein reduced unconventional secretion of ΔF508-CFTR, and DHC1 has the biggest inhibitory effect among these (Figure 8A, B). However, the silencing of each dynein subunit did not affect conventional secretion of complex glycosylated form (band C) of WT-CFTR (Figure 8C, D). The efficacy of each siRNA treatment was validated by RT-q PCR (Figure 8E).

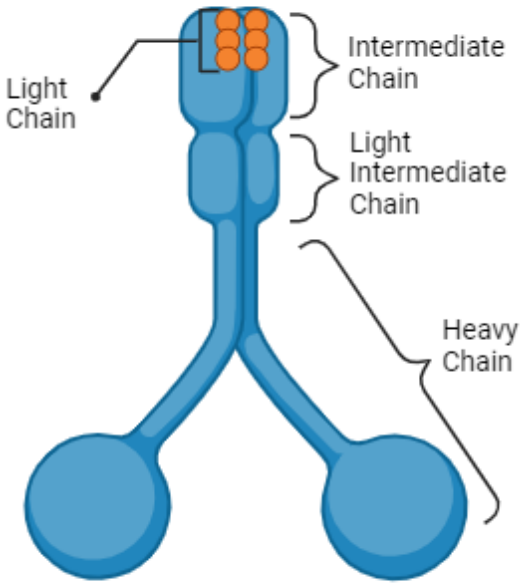
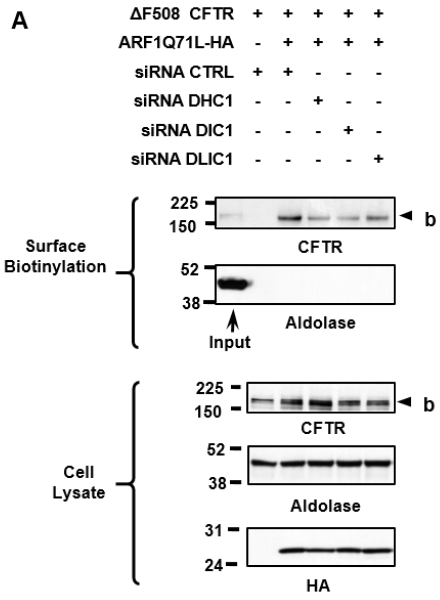
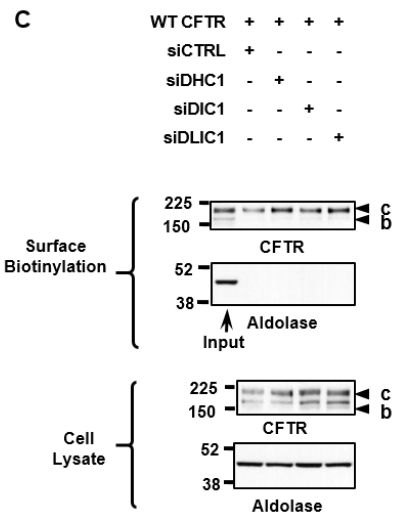
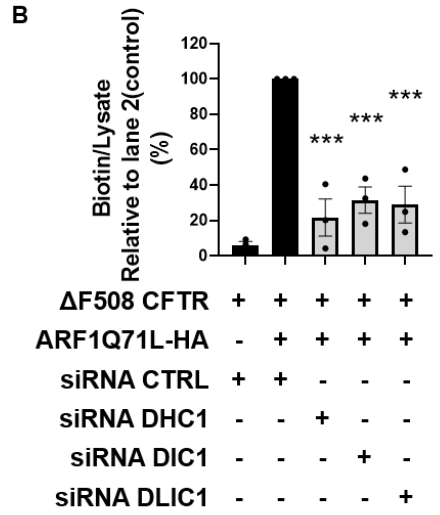


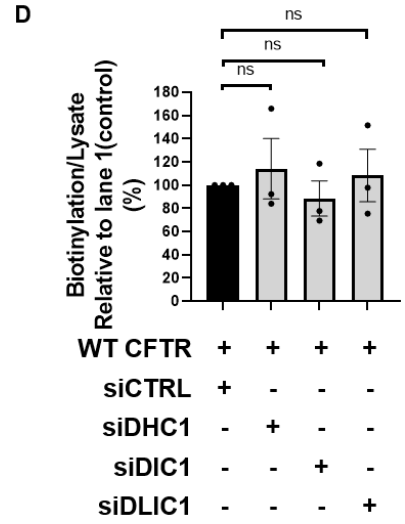
Figure 7. Dynein Architecture.

Dynein-1 is composed of a single dynein heavy chain (DHC) and two isomers of each intermediate chain (DIC) and light intermediate chain (DLIC) and three dynein light chain (DLC). This figure is created with BioRender.com.









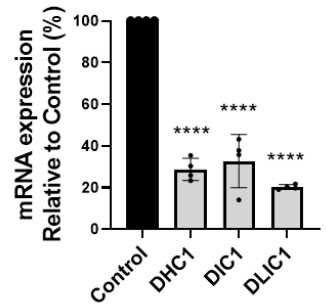
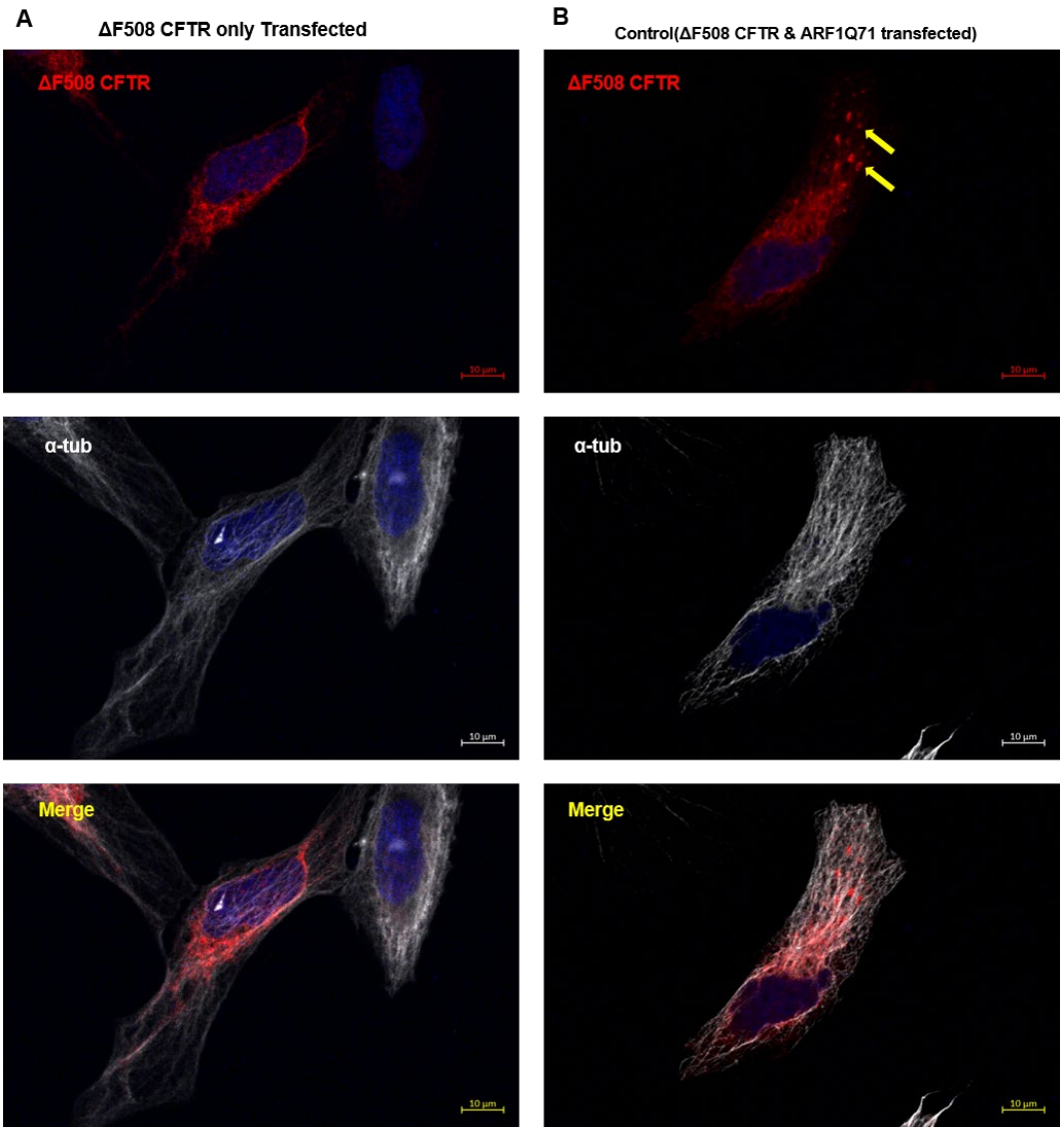


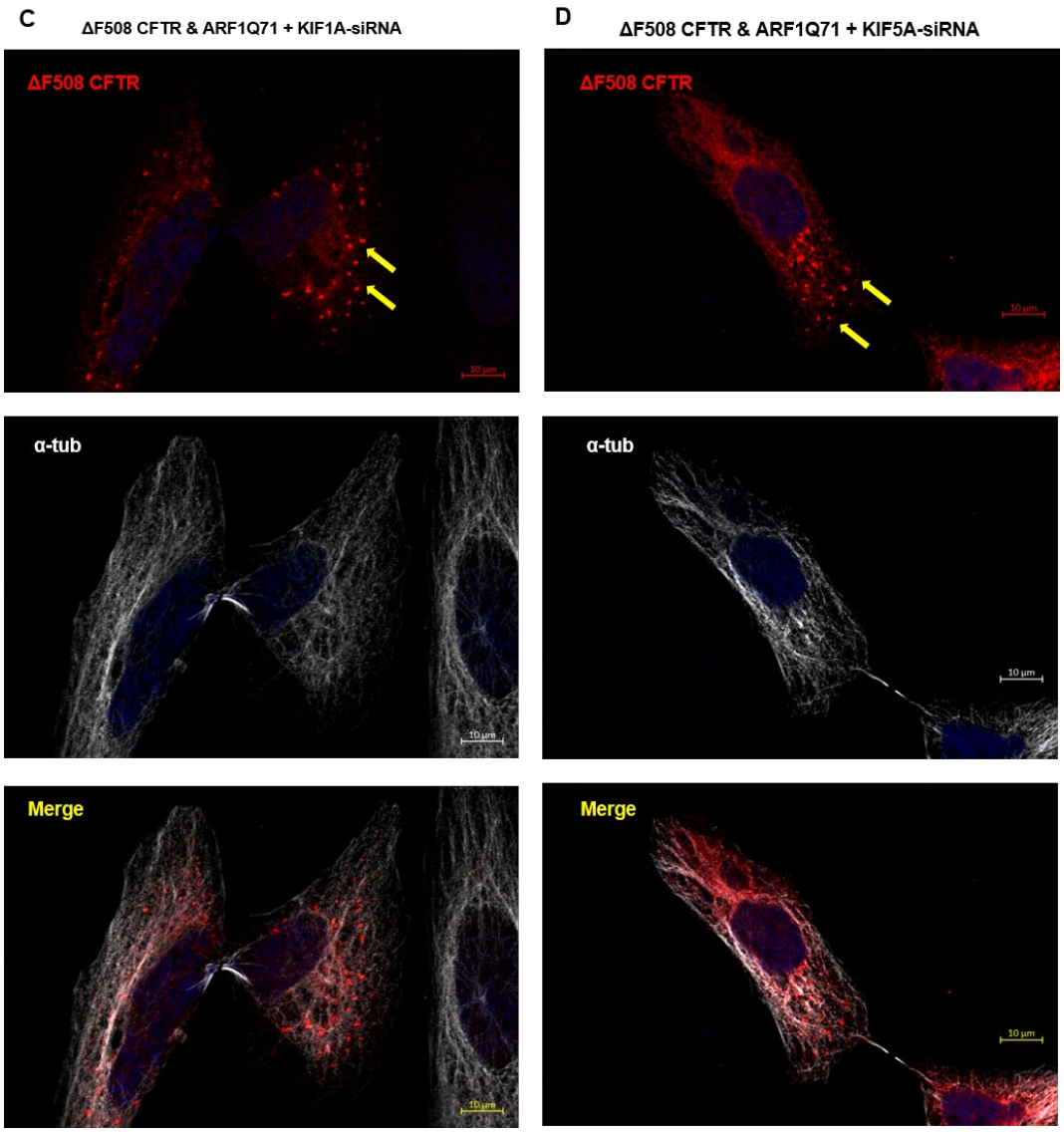
Figure 8. Dynein is involved in unconventional trafficking of ΔF508-CFTR but not in conventional trafficking of WT-CFTR.

Effects of silencing each dynein subunit; DHC1, DIC1 and DLIC1 genes on the unconventional trafficking of ΔF508-CFTR (A) and conventional trafficking of WT-CFTR (C). HEK293 cells were used to perform a cell surface biotinylation assay after being transfected with either control (scrambled) siRNA or specific target siRNAs (100nM each, 48h), along with plasmids encoding ΔF508/WT-CFTR (24h). Some cells were also co-transfected with ARFQ71L-HA to induce ER stress. Surface proteins and cell lysates were quantified respectively (B, D). (E) Shows the knockdown efficiency of each target gene. Bar graph data are expressed as mean ± SEM. *P<0.05, **P<0.01. Statistical analysis was conducted using one-way ANOVA, followed by Tukey's multiple comparison test. b represents band B (core-glycosylated) CFTR; c represents band C (complex-glycosylated) CFTR.

3.3. Dynein transfers ΔF508-CFTR to kinesin at “intermediate points”.

An immunofluorescence assay was conducted to investigate the localization of ΔF508-CFTR inside the cell with (Figure 9A) and without (Figure 9B) co-transfection of ARF1Q71L which induces UPS. As seen in Figure 6B, CFTR puncta formed by ARF1Q71L were also observed in this instance (Figure 9B, yellow arrow). Also, ΔF508-CFTR puncta were located along the microtubules, which supports ΔF508-CFTR move along the microtubules. Next, we assessed the change in the distribution of ΔF508-CFTR puncta when the previously identified motor proteins (KIF1A, KIF5A, DHC1) were under ER stress condition. When the expression of KIF1A (Figure 9C) or KIF5A (Figure 9D) was reduced, puncta formation occurred, and the number of puncta was not significantly different from the group treated with control siRNA (Figure 9F). On the contrary, when we downregulated DHC1, the puncta in cytosols diminished and red fluorescent signals clumped together in the perinuclear area (Figure 9E, F).





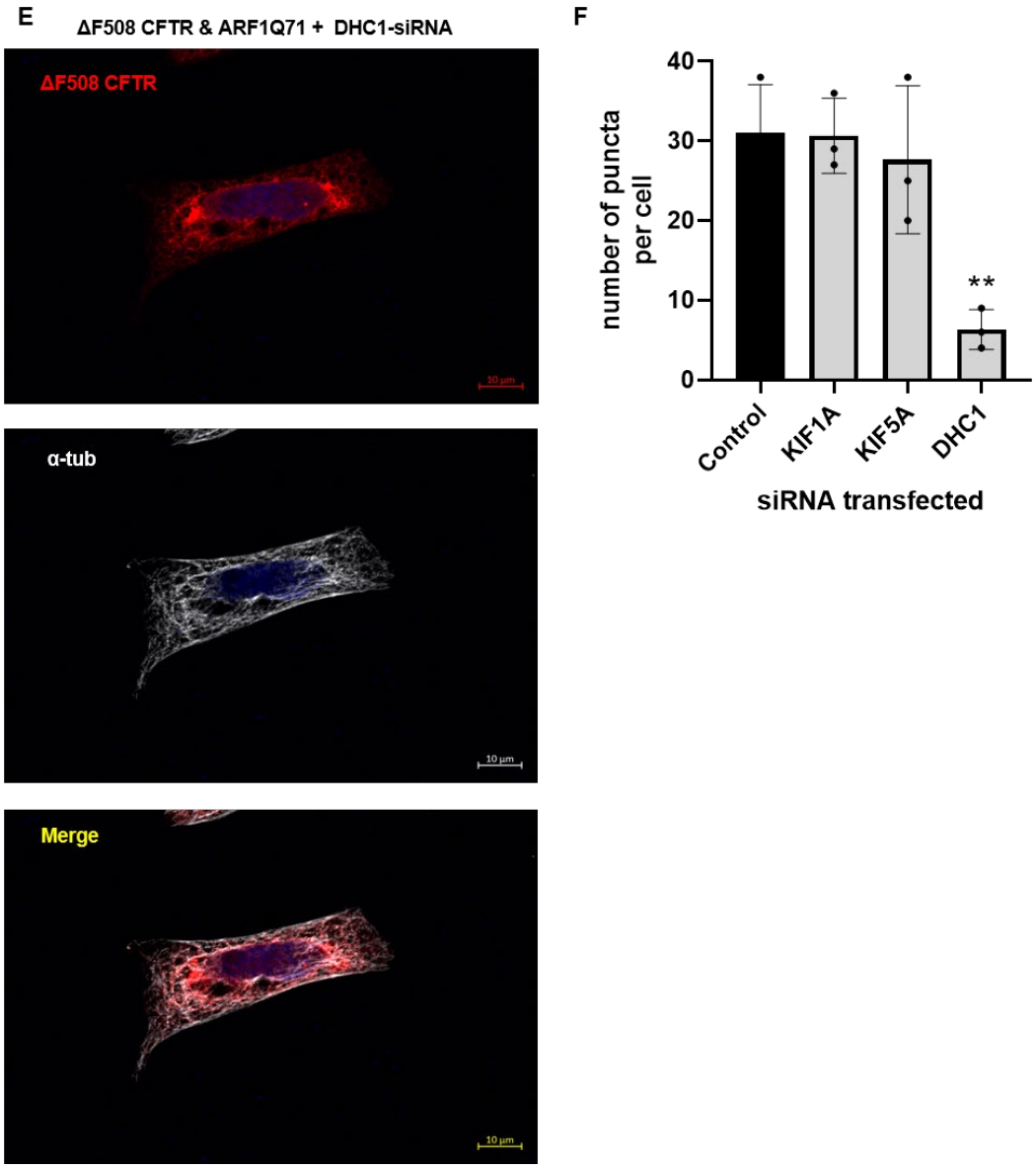


Figure 9. The different effects of KIF1A, KIF5A, and DHC1 knockdown on the formation and distribution of ΔF508 CFTR puncta.

Immunofluorescence analysis of ΔF508 CFTR in cells. HeLa cells were transfected with control siRNA (A, B) or target siRNAs (C:KIF1A, D:KIF5A, E:DHC1, 100nM each, 48hr) together with

plasmids encoding ΔF508 CFTR (A~E, 24hr), ARF1Q71L-HA was co-transfected in some cells (B~E). ΔF508 CFTR was stained with anti-ACL006 antibody (red, Alexa Fluor 568) and endogenous α-tubulin with anti-α-tubulin antibody (far-red, Alexa Fluor 647) The representative puncta were marked with a yellow. The number of puncta per cell in ARF1Q71L induced ER stress condition for each siRNA transfected group is counted (F).

3.4. Specific kinesins and dyneins are involved in the UPS of SARS-CoV-2 Spike.

To confirm whether specific motor proteins that were verified to be involved in the UPS pathway of ΔF508-CFTR were also involved in the UPS pathway of other cargos, we conducted additional experiments. There are several transmembrane proteins known to be unconventionally transported: CFTR, SARS-CoV-2 Spike, and pendrin[6]. Notably, previous reports indicated that SARS-CoV-2 utilizes lysosomes for egress instead of the conventional secretion pathway[3]. The Spike of SARS-CoV-2 is composed of two subunits, S1 which is for receptor binding and S2, which is for membrane fusion[25]. The Spike is cleaved into S1 and S2 fragment by furin which is enriched in the Golgi apparatus[26]. Therefore, when moving through UPS route bypassing the Golgi, we can observe the uncleaved Spike form instead of the cleaved Spike form.

To assess the effects of specific motor proteins on the UPS of Spike, we performed a surface biotinylation assay with silencing of previous set of kinesins (Figure 10, 11) and subunits of dynein (Figure 12). Interestingly, in line with previous data, the silencing of KIF1A, KIF5A, KIF14, KIF15, DHC1 and DLIC1 suppressed unconventional secretion of uncleaved Spike but not conventional secretion of cleaved Spike.

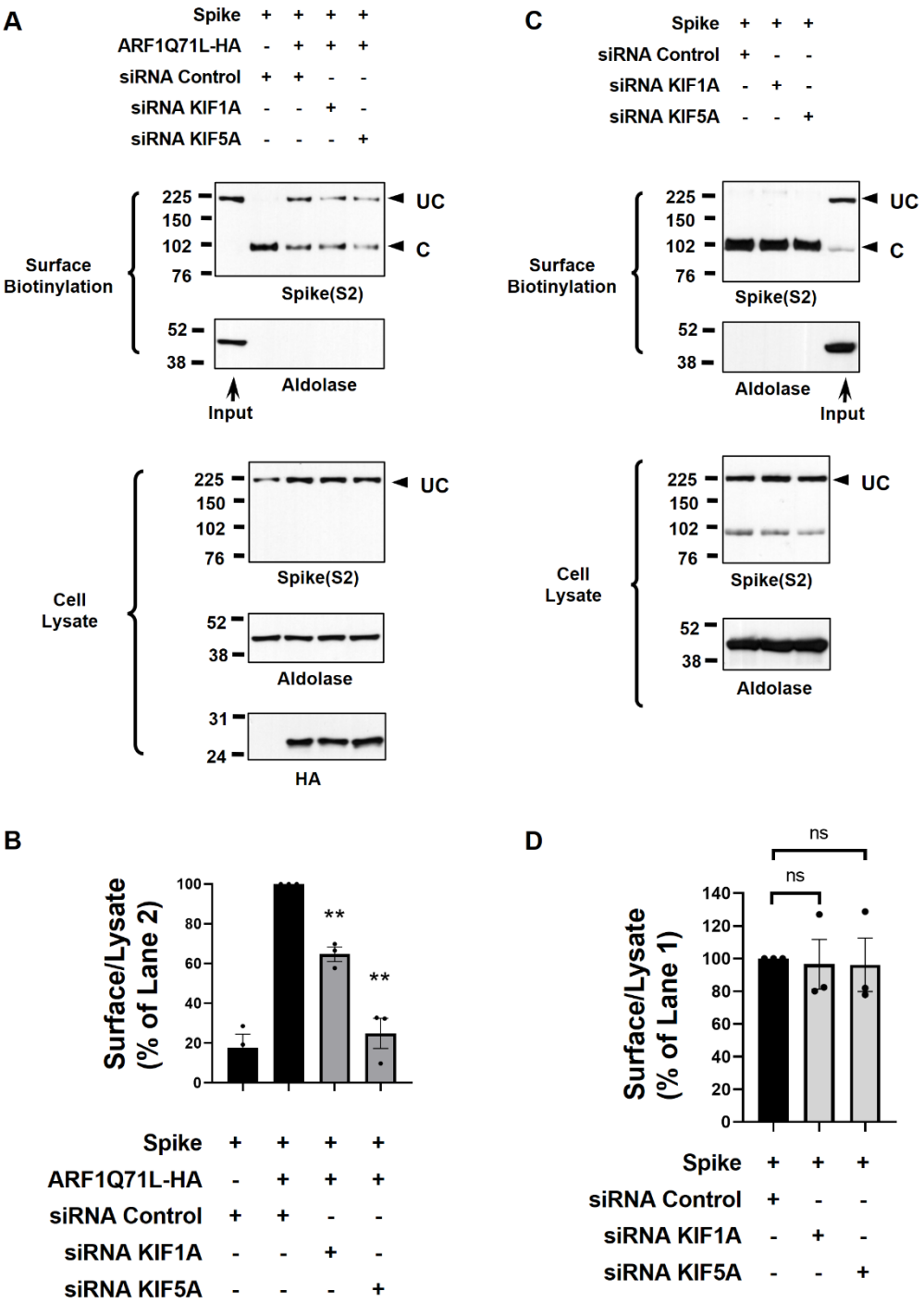


Figure 10. KIF1A and KIF5A are involved in unconventional trafficking of Spike but not in conventional trafficking of Spike.

Effects of silencing KIF1A and KIF5A genes on the unconventional trafficking of Spike (A) and conventional trafficking of Spike (C). HEK293 cells were used to perform a cell surface biotinylation assay after being transfected with either control (scrambled) siRNA or specific target siRNAs (50nM each, 48h), along with plasmids encoding Spike (24h). Some cells were also co-transfected with ARFQ71L-HA to induce ER stress. Surface proteins and cell lysates were quantified respectively (B, D). Bar graph data are expressed as mean ± SEM. *P<0.05, **P<0.01. Statistical analysis was conducted using one-way ANOVA, followed by Tukey's multiple comparison test. UC represents Golgi-bypassed uncleaved form of Spike; C represents Golgi-dependent cleaved form of Spike.

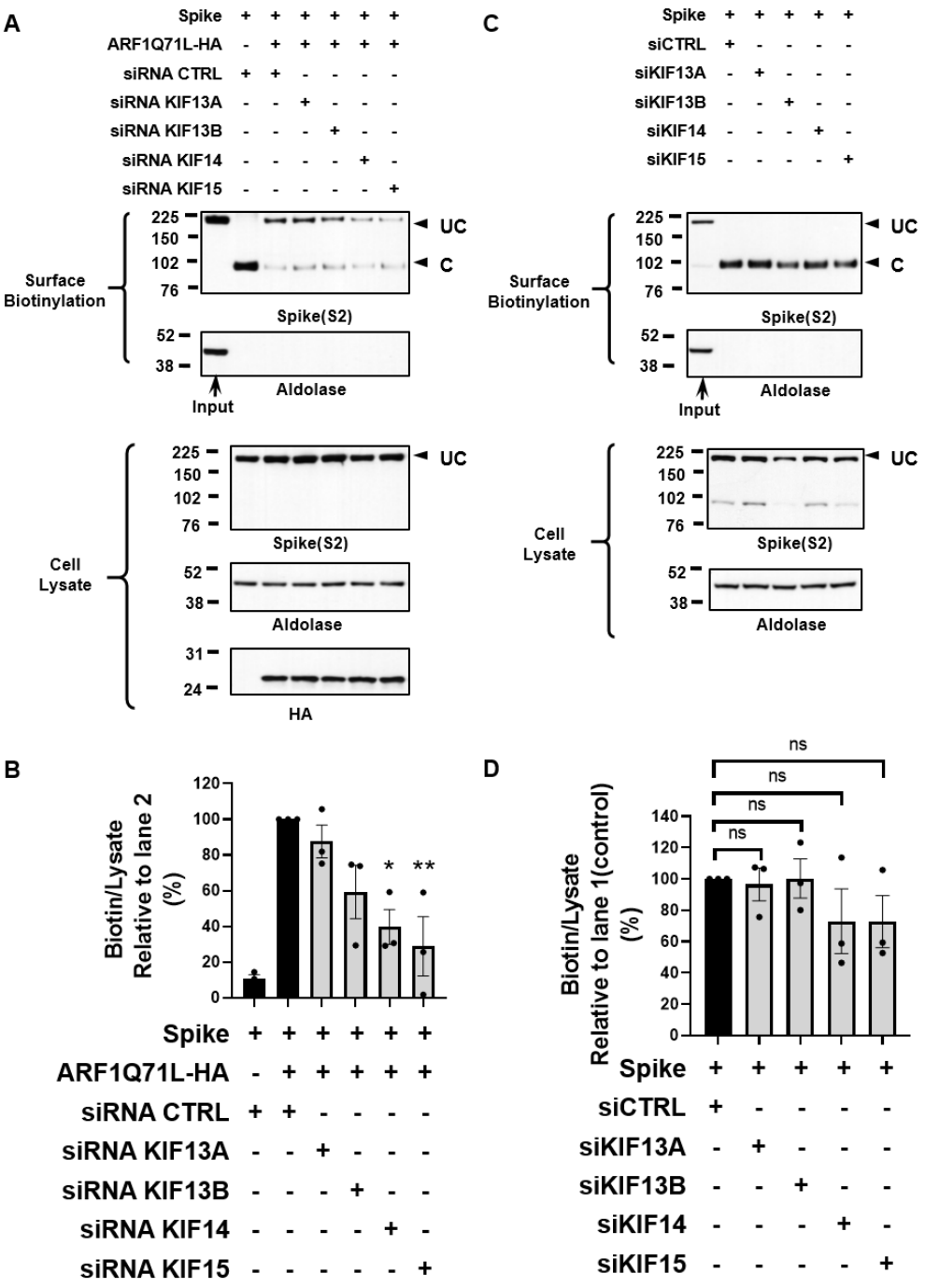


Figure 11. KIF14 and KIF15 are involved in unconventional trafficking of the Spike but not in conventional trafficking of Spike.

Effects of silencing KIF13A, KIF13B, KIF14 and KIF15 genes on the unconventional (A) and conventional (B) trafficking of Spike. EK293 cells were used to perform a cell surface biotinylation assay after being transfected with either control (scrambled) siRNA or specific target siRNAs (50nM each, 48h), along with plasmids encoding Spike (24h). Some cells were also co-transfected with ARFQ71L-HA to induce ER stress. Surface proteins and cell lysates were quantified respectively (B, D). Bar graph data are expressed as mean ± SEM. *P<0.05, **P<0.01. Statistical analysis was conducted using one-way ANOVA, followed by Tukey's multiple comparison test. UC represents Golgi-bypassed uncleaved form of Spike; C represents Golgi-dependent cleaved form of Spike.

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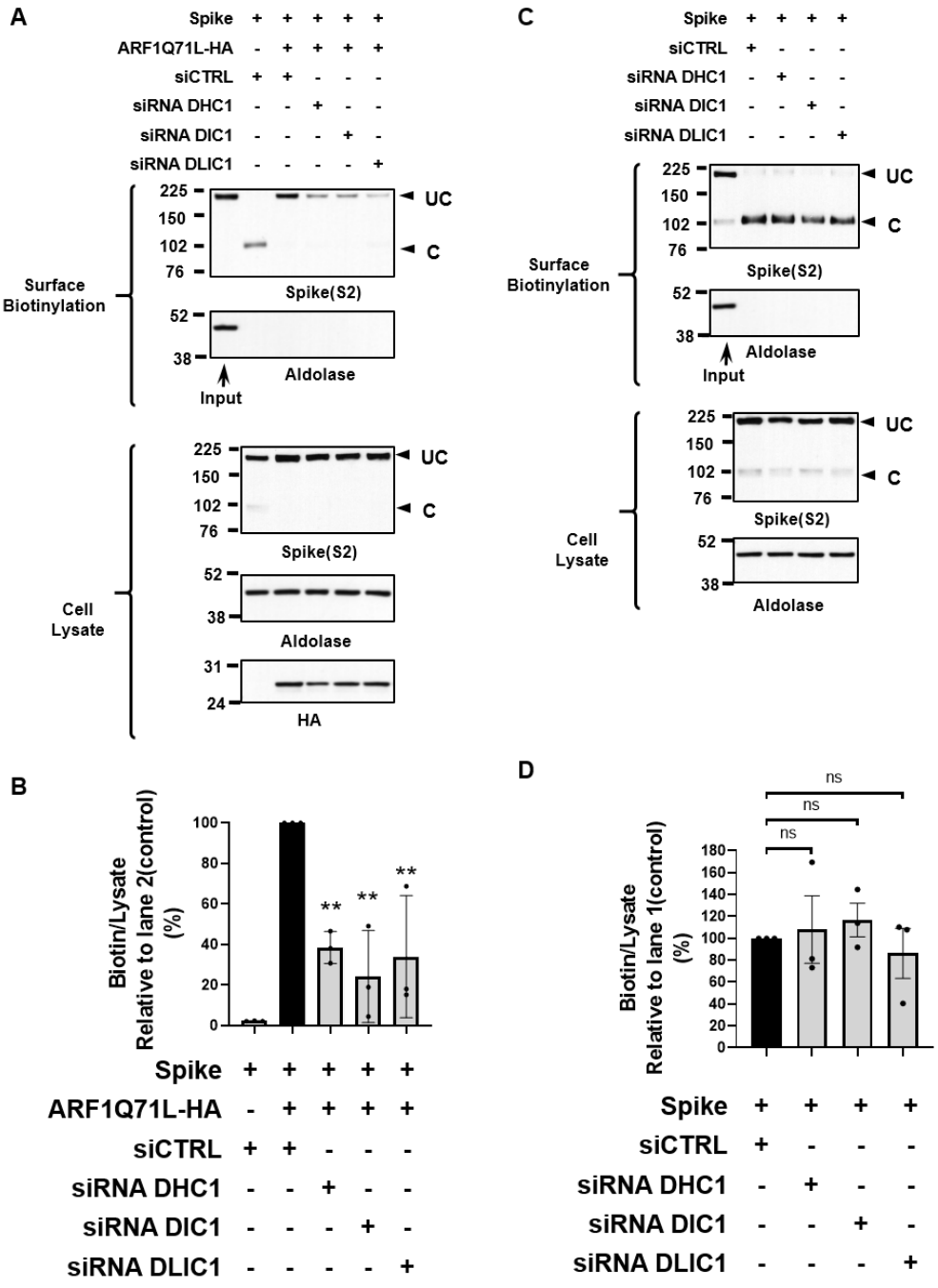

Figure 12. Dynein is involved in unconventional trafficking of Spike but not in conventional trafficking of Spike.

Effects of silencing each dynein subunit; DHC1, DIC1, and DLIC1 gene on the unconventional trafficking of Spike (A) and conventional trafficking of Spike (C). HEK293 cells were used to perform a cell surface biotinylation assay after being transfected with either control (scrambled) siRNA or specific target siRNAs (100nM each, 48h), along with plasmids encoding Spike (24h). Some cells were also co-transfected with ARFQ71L-HA to induce ER stress. Surface proteins and cell lysates were quantified respectively (B, D). Bar graph data are expressed as mean ± SEM. *P<0.05, **P<0.01. Statistical analysis was conducted using one-way ANOVA, followed by Tukey's multiple comparison test. UC represents Golgi-bypassed uncleaved form of Spike; C represents Golgi-dependent cleaved form of Spike.

3.5. Specific kinesin and dynein are involved in UPS by another ER stress inducer.

Next, we wanted to know if the motor proteins that we confirmed to be involved in the UPS pathway are also important for the UPS pathway when it is triggered by a different ER stress inducer, in addition to ARF1Q71L overexpression. Thapsigargin is a drug that inhibits the sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) pump, which functions to transport calcium ions from the cytosol into the lumen of the endoplasmic reticulum (ER) [27]. By inhibiting SERCA, thapsigargin depletes the ER of calcium, which can induce ER stress. According to the results of surface biotinylation experiments, among the previously identified motor proteins, all but KIF1A were found to significantly reduce the unconventional secretion of ΔF508-CFTR in a statistically meaningful manner (Figure 13A). Interestingly, a different study showed that KIF1A gene expression increased when cells were stressed with thapsigargin[28]. This suggests that thapsigargin treatment could offset the silencing effect of KIF1A siRNA. Although a p-value < 0.05 was not obtained during KIF1A knockdown (Figure 13B), a trend was observed that the UPS of ΔF508-CFTR by thapsigargin is inhibited even with KIF1A knockdown. Therefore, by increasing the siRNA concentration to sufficiently counteract the upregulation effect of KIF1A by thapsigargin or increasing the number of experimental repetitions, it may be possible to demonstrate the effectiveness of the ARF1Q71lL-induced UPS-specific motor proteins discovered in the previous study, even in the presence of thapsigargin.

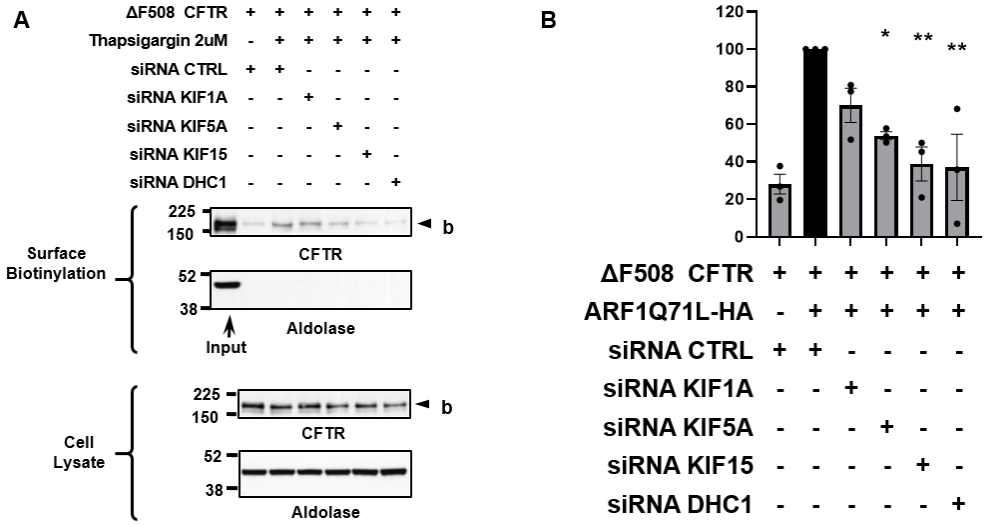

Figure 13. The KIF5A, KIF15 and DHC1 are involved in the thapsigargin-induced unconventional trafficking of ΔF508-CFTR.

Effects of silencing each DHC1, KIF15, KIF1A, and KIF5A gene on the unconventional trafficking of ΔF508-CFTR induced by thapsigargin (A). HEK293 cells were used to perform a cell surface biotinylation assay after being transfected with either control (scrambled) siRNA or specific target siRNAs (100nM each, 48h), along with plasmids encoding ΔF508 (24h). Thapsigargin (2uM, 12h) was treated in some cells to induce ER stress. Surface proteins and cell lysates were quantified respectively (B). Bar graph data are expressed as mean ± SEM. *P<0.05, **P<0.01. Statistical analysis was conducted using one-way ANOVA, followed by Tukey's multiple comparison test.

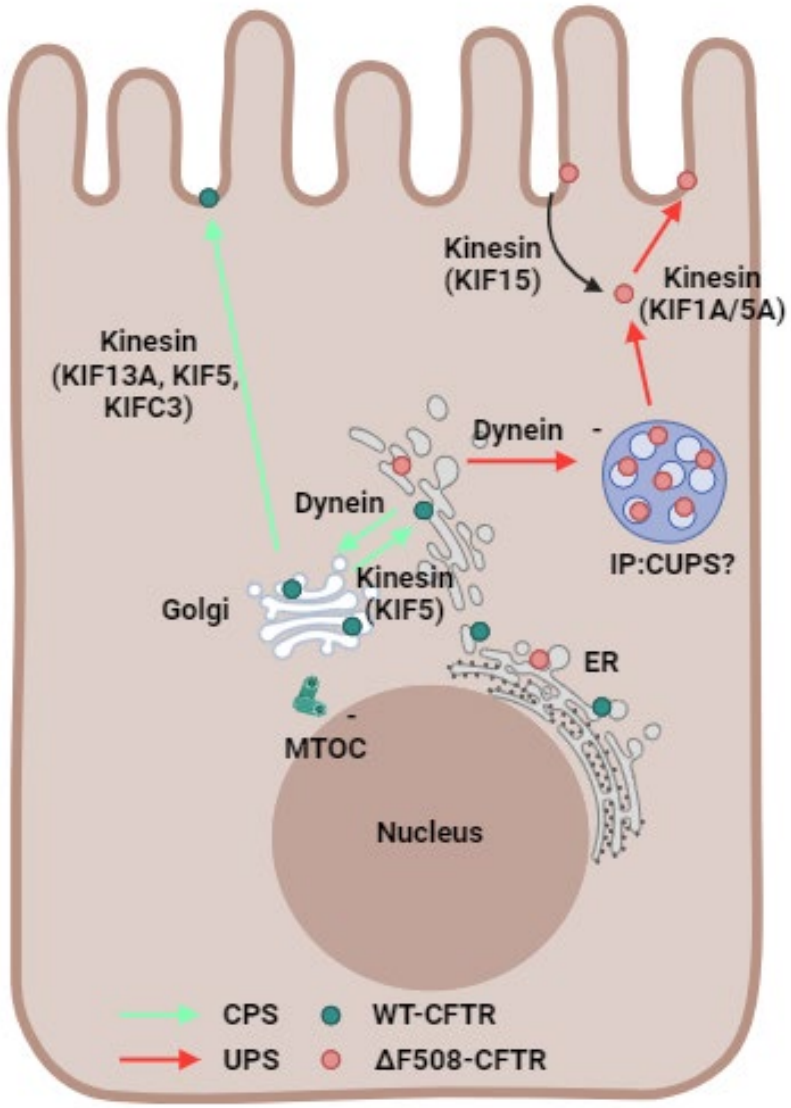

Figure 14. Graphical depiction of the intermediate point where dynein transfers UPS cargo to kinesin.

ER stress induced by ARF1Q71L expression induces unconventional transport of UPS cargo from the ER to cell membrane via intermediate points (IP). Dynein transports UPS cargo from ER to intermediate points and kinesin transports UPS cargo from intermediate point to cell membrane. This figure is created with BioRender.com.

4. DISCUSSION

Protein trafficking within the cell is essential for maintaining cellular function and homeostasis. Microtubules, cytoskeletal elements acting as intracellular highways, play a crucial role in this process by facilitating the long-distance transport of various cargos. Specialized motor proteins, such as kinesins and dyneins, navigate along these microtubule tracks, transporting vesicles and organelles containing essential cargo to their designated destinations. While significant progress has been made in identifying motor proteins responsible for specific cargo transport in the Golgi-mediated conventional secretion pathway, the transport of unconventionally secreted proteins, which bypasses the Golgi apparatus, remains poorly understood. In this study, we employed a two-pronged approach to identify the motor proteins involved in the UPS pathway. First, a text-mining approach was implemented to analyze a large corpus of scientific literature related to UPS and microtubule motor proteins. Second, a CRISPR-Cas9 screening was performed to identify essential genes required for UPS. This technique allowed for the systematic inspection of genes and subsequent analysis of their effect on the UPS pathway. As a result, we found that not only some specific kinesins (KIF1A, KIF5A, and KIF15), but also cytoplasmic dynein, and even specific adaptor proteins such as FYCO1 and SKIP, have an important role in the unconventional trafficking of CFTR. The results of gene silencing indicated that these motor proteins are involved in unconventional secretion of △F508-CFTR, but not in conventional secretion of wild type CFTR. Moreover, these motor proteins also participate in the unconventional transport of the Spike.

KIF1A is reported for its role in transporting dense-core vesicles and a subset of synaptic proteins[29], and KIF5A is known for its relevance of intracellular transport like TI-VAMP vesicles[30], collagen[31], and GABA-A receptor[32]. As a motor protein engaged in the transportation of microtubules towards the plus end, KIF1A and KIF5A appear to facilitate the movement of vesicles containing CFTR and Spike to the outer region of the cell. Our findings support this notion, as knocking down FYCO1 and SKIP, adaptor proteins associated with kinesin and involved in the vesicular trafficking of late endosomes and lysosomes, resulted in a decrease in the unconventional secretion of ΔF508-CFTR and Spike. Conversely, KIF13A and KIF13B, identified as participants in recycling endosomes and tubular endosomes, did not affect the unconventional secretion of ΔF508-CFTR and Spike in knockdown experiments using siRNA. It is noteworthy that prior reports

suggested SARS-CoV-2 utilizes lysosomes for egress rather than the conventional secretion pathway[3]. The specific vesicle type responsible for transporting ΔF508-CFTR and Spike during the UPS pathway remains uncertain. It is conceivable that a shared vesicle may convey both ΔF508-CFTR and Spike from the endoplasmic reticulum to the cell surface, utilizing previously identified motor and adaptor proteins in the UPS pathway. The detailed molecular mechanisms through which these kinesin motors facilitate the transport of UPS cargos from the endoplasmic reticulum to the cell surface, bypassing the Golgi apparatus, will be the focus of future investigations.

KIF15 is identified as a regulator of the endocytic trafficking of integrin, one of the major cell-surface adhesion receptors by mediating plasma membrane localization of the alternative clathrin adaptor Dab2[33]. Integrins, heterodimeric glycoproteins, are transmembrane receptors that mediate the attachment of cells to the extracellular matrix (ECM). The suppression of KIF15 expression by RNAi strongly inhibited the intracellular accumulation of α2 integrin, whereas expression of ectopic KIF15 induced α2 integrin accumulation. The internalization of integrin is often dependent on CLASPs (Clathrin-associated sorting proteins) and a CLASP Dab2 has been shown to be essential for the internalization of specific integrin[34].

Interestingly, it is already reported that Dab2 has a key role in endocytosis and post-endocytic trafficking of WT-CFTR protein.[35] Furthermore, downregulation of Dab2 increases the surface half-life and chloride channel activity of ΔF508-CFTR by inhibiting ΔF508-CFTR delivery to the late endosome for degradation.[36] As Dab2 and KIF15 work together to regulate the internalization of integrins, it is possible that they cooperate to internalize of CFTR in the same way, which is the opposite hypothesis of our experimental results. There are two ways to explain this contradiction. First, unlike the internalization of integrin, KIF15 is not involved in the internalization process of ΔF508-CFTR and only involved in outward transport of the cargo from ER to plasma membrane. Second, the portion of outward transport of ΔF508-CFTR is bigger than inward transport (internalization) of ΔF508-CFTR so that internalization effect could be masked. The surface biotinylation assay of ΔF508-CFTR with adding dynasore[37], which blocks endocytosis, is needed for further study to examine these hypotheses.

Dynein plays a crucial role in numerous cellular processes, including basic cargo transport[38] to intracellular organelle transport[39], axonal transport[40] and cell divisions. In addition, Cytoplasmic dynein is necessary for specific functions related to the positioning of the microtubule-organizing center (MTOC), such as the separation of centrosomes during the single-cell stage in Caenorhabditis

elegans embryo[41]. Dynein is a motor protein that moves organelles and other cargo inward towards the cell center. It does this by attaching to microtubules and walking along in the minus-end (towards the cell center) direction[24]. The findings, demonstrating that the silencing of cytoplasmic dynein hinders the unconventional transport of ΔF508-CFTR and Spike, suggest that the process of cargo movement from the ER to the cell surface is not solely characterized by centripetal (outward) motion. Instead, it involves dynein, which facilitates movement toward the minus end. In essence, when the cargo traverses the UPS pathway, there is a potential for it to follow specific segments along the minus end axis. Alternatively, one could infer that the coordinated interplay between kinesin and dynein plays a pivotal role in facilitating cargo movement.

We would like to discuss the reasons why KIF1A, KIF5A, and DHC1, excluding KIF15, are not included in the list of genes known to be associated with UPS, based on the current results of the CRISPR-Cas9 screening. Essentially, according to the principles of the CRISPR-Cas9 screening experiment, the crucial gene set related to UPS consists of the entire customized gRNA gene set, excluding the set inserted into cells where the HA-tagged protein is observed on the cell surface. Therefore, we are considering instances where more HA-tagged proteins have been transported to the cell surface. First, due to off-target effects, the target gene may not be knocked out, and HA-tagged ΔF508 CFTR can be transported to the cell surface. Second, although Cas9 stable cells were used in this experiment, there is a possibility that Cas9 function may decrease as the cell population increases. Third, despite efforts to find references to create the best gRNA for the target genes, the design of such gRNA is basically reliant on in silico methods, and in actual cell experiments, various factors may contribute to a reduction in gRNA functionality.

Finally, we would like to discuss the relationship between various motors and puncta in the UPS pathway. In Figure 6, it was confirmed that KIF1A co-localizes with CFTR puncta. In Figure 9, the number of CFTR puncta generated upon KIF1A or KIF5A knockdown was found to be comparable to the group treated with control siRNA. Therefore, UPS-specific kinesins are likely to play a crucial role in the transport pathway from puncta formation to the cell membrane. Conversely, in Figure 9, DHC1 knockdown resulted in a significant reduction in CFTR puncta formation, suggesting that dynein plays a crucial role in transporting CFTR from the perinuclear ER region to puncta. Here we postulate that there are “intermediate points" where the vesicles containing UPS cargo pauses on route from the ER to the plasma membrane. Then, the role of dynein could be to move the vesicle from ERES to “intermediate points” along microtubules by using a minus-end-directed mechanism.

And the role of kinesin could be to move the vesicle from “intermediate points” to the plasma membrane by using plus-end-directed mechanism. The identity of “intermediate points” is unknown, but we guess the puncta of ΔF508-CFTR are the point where unconventionally transported ΔF508-CFTR stays for a while. A graphical summary of this hypothesis is illustrated in Figure 14.

5. CONCLUSION

The exact mechanism of unconventional protein secretion (UPS) remains unclear. This study aimed to investigate the involvement of motor proteins in the UPS of CFTR and Spike proteins. In conclusion, the present findings revealed that

1. Multiple kinesins, KIF1A, KIF5A, KIF15 have an important role in UPS, but not in the conventional protein secretion, of CFTR and Spike.
2. Cytoplasmic dynein also plays a critical role in UPS, but not in conventional protein secretion of CFTR and spike.
3. Puncta are the intermediate points in the UPS pathway, where dynein transfers UPS cargo to kinesin

I hope that this study will help to further elucidate the UPS pathway and UPS.

References

1 Malhotra, V. Unconventional protein secretion: an evolving mechanism. *EMBO J* **32**, 1660-1664 (2013). https://doi.org:10.1038/emboj.2013.104

2 Noh, S. H. *et al.* Specific autophagy and ESCRT components participate in the unconventional secretion of CFTR. *Autophagy* **14**, 1761-1778 (2018). https://doi.org:10.1080/15548627.2018.1489479

3 Ghosh, S. *et al.* β-Coronaviruses Use Lysosomes for Egress Instead of the Biosynthetic Secretory Pathway. *Cell* **183**, 1520-1535.e1514 (2020). https://doi.org:10.1016/j.cell.2020.10.039

4 O'Sullivan, B. P. & Freedman, S. D. Cystic fibrosis. *Lancet* **373**, 1891-1904 (2009). https://doi.org:10.1016/S0140-6736(09)60327-5

5 Park, H., Shin, D. H., Sim, J. R., Aum, S. & Lee, M. G. IRE1α kinase-mediated unconventional protein secretion rescues misfolded CFTR and pendrin. *Sci Adv* **6**, eaax9914 (2020). https://doi.org:10.1126/sciadv.aax9914

6 Park, H. *et al.* TMED3 Complex Mediates ER Stress-Associated Secretion of CFTR, Pendrin, and SARS-CoV-2 Spike. *Adv Sci (Weinh)* **9**, e2105320 (2022). https://doi.org:10.1002/advs.202105320

7 El Khouri, E., Le Pavec, G., Toledano, M. B. & Delaunay-Moisan, A. RNF185 is a novel E3 ligase of endoplasmic reticulum-associated degradation (ERAD) that targets cystic fibrosis transmembrane conductance regulator (CFTR). *J Biol Chem* **288**, 31177-31191 (2013). https://doi.org:10.1074/jbc.M113.470500

8 Gee, H. Y., Noh, S. H., Tang, B. L., Kim, K. H. & Lee, M. G. Rescue of DeltaF508-CFTR trafficking via a GRASP-dependent unconventional secretion pathway. *Cell* **146**, 746-760 (2011). https://doi.org:10.1016/j.cell.2011.07.021

9 Abraham, Z., Hawley, E., Hayosh, D., Webster-Wood, V. A. & Akkus, O. Kinesin and Dynein Mechanics: Measurement Methods and Research Applications. *J Biomech Eng* **140**, 0208051-02080511 (2018). https://doi.org:10.1115/1.4037886

10 Hirokawa, N., Noda, Y., Tanaka, Y. & Niwa, S. Kinesin superfamily motor proteins and intracellular transport. *Nat Rev Mol Cell Biol* **10**, 682-696 (2009). https://doi.org:10.1038/nrm2774

11 Roberts, A. J. Emerging mechanisms of dynein transport in the cytoplasm versus the cilium. *Biochem Soc Trans* **46**, 967-982 (2018). https://doi.org:10.1042/BST20170568

12 Zhu, C. *et al.* Functional analysis of human microtubule-based motor proteins, the kinesins and dyneins, in mitosis/cytokinesis using RNA interference. *Mol Biol Cell* **16**, 3187-3199 (2005).

https://doi.org:10.1091/mbc.e05-02-0167

13 Guardia, C. M., Farias, G. G., Jia, R., Pu, J. & Bonifacino, J. S. BORC Functions Upstream of Kinesins 1 and 3 to Coordinate Regional Movement of Lysosomes along Different Microtubule Tracks. *Cell Rep* **17**, 1950-1961 (2016). https://doi.org:10.1016/j.celrep.2016.10.062

14 Joung, J. *et al.* Genome-scale CRISPR-Cas9 knockout and transcriptional activation screening. *Nat Protoc* **12**, 828-863 (2017). https://doi.org:10.1038/nprot.2017.016

15 Delevoye, C. *et al.* AP-1 and KIF13A coordinate endosomal sorting and positioning during melanosome biogenesis. *J Cell Biol* **187**, 247-264 (2009). https://doi.org:10.1083/jcb.200907122

16 Etoh, K. & Fukuda, M. Rab10 regulates tubular endosome formation through KIF13A and KIF13B motors. *J Cell Sci* **132** (2019). https://doi.org:10.1242/jcs.226977

17 Tong, Y. *et al.* Phosphorylation-independent dual-site binding of the FHA domain of KIF13 mediates phosphoinositide transport via centaurin alpha1. *Proc Natl Acad Sci U S A* **107**, 20346-20351 (2010). https://doi.org:10.1073/pnas.1009008107

18 Drechsler, H. & McAinsh, A. D. Kinesin-12 motors cooperate to suppress microtubule catastrophes and drive the formation of parallel microtubule bundles. *Proc Natl Acad Sci U S A* **113**, E1635-1644 (2016). https://doi.org:10.1073/pnas.1516370113

19 Roney, J. C., Cheng, X. T. & Sheng, Z. H. Neuronal endolysosomal transport and lysosomal functionality in maintaining axonostasis. *J Cell Biol* **221** (2022). https://doi.org:10.1083/jcb.202111077

20 Farias, G. G., Guardia, C. M., De Pace, R., Britt, D. J. & Bonifacino, J. S. BORC/kinesin-1 ensemble drives polarized transport of lysosomes into the axon. *Proc Natl Acad Sci U S A* **114**, E2955-E2964 (2017). https://doi.org:10.1073/pnas.1616363114

21 Khatter, D., Sindhwani, A. & Sharma, M. Arf-like GTPase Arl8: Moving from the periphery to the center of lysosomal biology. *Cell Logist* **5**, e1086501 (2015). https://doi.org:10.1080/21592799.2015.1086501

22 Matsuzaki, F., Shirane, M., Matsumoto, M. & Nakayama, K. I. Protrudin serves as an adaptor molecule that connects KIF5 and its cargoes in vesicular transport during process formation. *Mol Biol Cell* **22**, 4602-4620 (2011). https://doi.org:10.1091/mbc.E11-01-0068

23 Toropova, K. *et al.* Structure of the dynein-2 complex and its assembly with intraflagellar transport trains. *Nat Struct Mol Biol* **26**, 823-829 (2019). https://doi.org:10.1038/s41594-019-0286-y

24 Reck-Peterson, S. L., Redwine, W. B., Vale, R. D. & Carter, A. P. The cytoplasmic dynein transport machinery and its many cargoes. *Nat Rev Mol*

Cell Biol **19**, 382-398 (2018). https://doi.org:10.1038/s41580-018-0004-3

25 Watanabe, Y., Allen, J. D., Wrapp, D., McLellan, J. S. & Crispin, M. Site-specific glycan analysis of the SARS-CoV-2 spike. *Science* **369**, 330-333 (2020). https://doi.org:10.1126/science.abb9983

26 Ou, X. *et al.* Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV. *Nat Commun* **11**, 1620 (2020). https://doi.org:10.1038/s41467-020-15562-9

27 Egan, M. E. *et al.* Calcium-pump inhibitors induce functional surface expression of Delta F508-CFTR protein in cystic fibrosis epithelial cells. *Nat Med* **8(5)**, 485-492 (2002). https://doi.org:10.1038/nm0502-485.

28 Weldemariam, M. M., Woo, J. & Zhang, Q. Pancreatic INS-1 β-Cell Response to Thapsigargin and Rotenone: A Comparative Proteomics Analysis Uncovers Key Pathways of β-Cell Dysfunction. *Chem Res Toxicol* **35**, 1080-1094 (2022). https://doi.org:10.1021/acs.chemrestox.2c00058

29 Maday, S., Twelvetrees, A. E., Moughamian, A. J. & Holzbaur, E. L. Axonal transport: cargo-specific mechanisms of motility and regulation. *Neuron* **84**, 292-309 (2014). https://doi.org:10.1016/j.neuron.2014.10.019

30 Burgo, A. *et al.* A molecular network for the transport of the TI-VAMP/VAMP7 vesicles from cell center to periphery. *Dev Cell* **23**, 166-180 (2012). https://doi.org:10.1016/j.devcel.2012.04.019

31 Kamata, H. *et al.* KIF5A transports collagen vesicles of myofibroblasts during pleural fibrosis. *Sci Rep* **7**, 4556 (2017). https://doi.org:10.1038/s41598-017-04437-7

32 Nakajima, K. *et al.* Molecular motor KIF5A is essential for GABA(A) receptor transport, and KIF5A deletion causes epilepsy. *Neuron* **76**, 945-961 (2012). https://doi.org:10.1016/j.neuron.2012.10.012

33 Eskova, A. *et al.* An RNAi screen identifies KIF15 as a novel regulator of the endocytic trafficking of integrin. *J Cell Sci* **127**, 2433-2447 (2014). https://doi.org:10.1242/jcs.137281

34 Teckchandani, A. *et al.* Quantitative proteomics identifies a Dab2/integrin module regulating cell migration. *J Cell Biol* **186**, 99-111 (2009). https://doi.org:10.1083/jcb.200812160

35 Fu, L. *et al.* Dab2 is a key regulator of endocytosis and post-endocytic trafficking of the cystic fibrosis transmembrane conductance regulator. *Biochem J* **441**, 633-643 (2012). https://doi.org:10.1042/BJ20111566

36 Fu, L. *et al.* DeltaF508 CFTR surface stability is regulated by DAB2 and CHIP-mediated ubiquitination in post-endocytic compartments. *PLoS One* **10**, e0123131 (2015). https://doi.org:10.1371/journal.pone.0123131

37 Kirchhausen, T., Macia, E. & Pelish, H. E. Use of dynasore, the small molecule inhibitor of dynamin, in the regulation of endocytosis. *Methods Enzymol* **438**, 77-93 (2008). https://doi.org:10.1016/S0076-

6879(07)38006-3

38 Watanabe, T. M. & Higuchi, H. Stepwise movements in vesicle transport of HER2 by motor proteins in living cells. *Biophys J* **92**, 4109-4120 (2007). https://doi.org:10.1529/biophysj.106.094649

39 Wozniak, M. J. *et al.* Role of kinesin-1 and cytoplasmic dynein in endoplasmic reticulum movement in VERO cells. *J Cell Sci* **122**, 1979-1989 (2009). https://doi.org:10.1242/jcs.041962

40 Sleigh, J. N., Rossor, A. M., Fellows, A. D., Tosolini, A. P. & Schiavo, G. Axonal transport and neurological disease. *Nat Rev Neurol* **15**, 691-703 (2019). https://doi.org:10.1038/s41582-019-0257-2

41 Gönczy, P., Pichler, S., Kirkham, M. & Hyman, A. A. Cytoplasmic dynein is required for distinct aspects of MTOC positioning, including centrosome separation, in the one cell stage Caenorhabditis elegans embryo. *J Cell Biol* **147**, 135-150 (1999). https://doi.org:10.1083/jcb.147.1.135

Abstract in Korean

비전형적 수송 과정에서 운동 단백질의 역할

분비 단백질은 전통적으로 소포체(ER)를 통해 골지체(Golgi)로 운반되고, 그 후 세포막 또는 세포 밖으로 운반된다. 최근의 연구들에서 신호 펩타이드가 없는 많은 단백질이 소포체-골지체 수송 경로를 우회하는 비전형적 단백질 분비(UPS)를 통해 이동한다는 것을 발견되었다. 돌연변이로 인해 단백질 구조에 결함이 있는 낭포성 섬유증 막횡단 전도 조절자(CFTR) 단백과 중증 급성 호흡기 증후군 코로나바이러스 2(SARS-CoV-2)의 스파이크(Spike) 단백과 같은 여러 막관통단백들은 소포체 스트레스에 의해 유도된 비전형적 단백 분비를 통해 세포막으로 이동한다. 그러나 비전형적 단백 분비 과정의 구체적 경로는 충분히 밝혀지지 않았다. 이 연구에서 리보핵산 간섭(RNA interference) 실험을 통해 KIF1A, KIF5A 및 KIF15와 같은 키네신 뿐만 아니라 세포질 디네인도 돌연변이 낭포성 섬유증 막횡단 전도 조절자 단백의 비전형적 이동 과정에 중요한 역할을 한다는 것을 발견했다. 그러나 이러한 운동 단백들이 야생형 낭포성 섬유증 막횡단 전도 조절자 단백의 전통적 이동 경로에는 영향을 미치지 않음을 확인하였다. 더욱이 이러한 운동 단백질은 스파이크 단백의 비전형적 이동 경로에도 관여함을 확인하였다. 마지막으로, 면역세포화학(Immunocytochemistry) 염색에서 발견된 점 형태(puncta)의 구조체가 비전형적 이동 경로의 중간 지점이며, 이 지점에서 디네인과 키네신간에 화물(cargo) 단백이 교환된다고 제안한다.

핵심되는 말 : 낭포성 섬유증 막횡단 전도 조절자, 스파이크 단백, 비전형적 단백 분비, 키네신, 디네인