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# YIPF1, YIPF2, and YIPF6 are *medial-/trans*-Golgi and *trans*-Golgi network-localized Yip domain family proteins, which play a role in the Golgi reassembly and glycan synthesis

Jeerawat Soonthornsit[a,b], Noriko Sakai[c], Yurika Sasaki[d], Ryota Watanabe[d], Shiho Osako[d,e], Nobuhiro Nakamura[a,c,d,e,*]

[a] *Division of Engineering, Graduate School, Kyoto Sangyo University, Motoyama, Kamigamo, Kita, Kyoto 603-8555, Japan*
[b] *Department of Pre-clinic and Applied Animal Science, Faculty of Veterinary Science, Mahidol University, 999 Phutthamonthon Sai 4 Road Salaya, Phutthamonthon, Nakhon Pathom 73170 Thailand*
[c] *Graduate School of Natural Science and Technology and School of Pharmacy, Kanazawa University, Kakuma, Kanazawa 920-1192, Japan*
[d] *Department of Molecular Biosciences, Faculty of Life Sciences, Kyoto Sangyo University, Motoyama, Kamigamo, Kita, Kyoto 603-8555, Japan*
[e] *Division of Life Sciences, Graduate School, Kyoto Sangyo University, Motoyama, Kamigamo, Kita, Kyoto 603-8555, Japan*

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ABSTRACT

In this study, we attempted to explore the function of three uncharacterized mammalian homologs of yeast Yip domain family proteins—YIPF6, a homolog of Yip1p, and YIPF1 and YIPF2, which are homologs of Yif1p. Immunofluorescence staining revealed that YIPF1, YIPF2, and YIPF6 mainly localize in the *medial-/trans*-Golgi and also partially in the *trans*-Golgi network (TGN). On treatment with brefeldin A (BFA), the homologs co-migrated partly with *medial-/trans*-Golgi markers and also with a TGN marker in earlier time point, but finally redistributed within cytoplasmic punctate structures that were distinct from *medial-/trans*-Golgi and the TGN markers. YIPF6 formed a stable complex separately with YIPF1 and YIPF2, and knockdown of YIPF6 reduced YIPF1 and YIPF2 levels. These results suggest that YIPF6 forms complexes with YIPF1 and YIPF2 for their stable expression and localization within the Golgi apparatus. Knockdown experiments showed that YIPF1 and YIPF2, by contrast, are not necessary for the expression and localization of YIPF6. The structure of the Golgi apparatus and its disassembly after BFA treatment were not significantly affected by the knockdown of YIPF1, YIPF2, or YIPF6. However, reassembly of the Golgi apparatus after the removal of BFA was markedly delayed by the knockdown of YIPF1 and YIPF2, but not by that of YIPF6. These results strongly suggest that free YIPF6 after disassociating with YIPF1 and YIPF2 interferes with the reassembly of the Golgi apparatus. Knockdown of YIPF1 and YIPF2, but not that of YIPF6, also reduced intracellular glycans in HT-29 cells. Thus, we confirmed that YIPF1, YIPF2, and YIPF6 play a significant role in supporting normal glycan synthesis.

## 1. Introduction

Yip domain family (YIPF) proteins are mammalian homologs of yeast Yip1p and Yif1p, which interact with Ypt family small GTPases, including Ypt1p and Ypt31p [1]. Yip1p and Yif1p form a complex with each other and are found in the ER, Golgi apparatus, and COPII vesicles. They were hypothesized to play a role in vesicle budding from the ER and/or fusion with the Golgi apparatus under regulation by Ypt proteins [1–4]. This was supported by the findings that Yip1p interacts with ER to Golgi SNAREs (Bos1p and Sec22p) [4] and *YIP1* genetically interacts with genes coding an Rab-GDI (*GDI1*), COPII components (*SEC12*, *SEC13*, and *SEC23*), a COPI component (*SEC21*) and a Golgi tethering protein (*USO1*) genes [3,5].

Both Yip1p and Yif1p are predicted to have five transmembrane segments; an N-terminal segment, which is exposed to the cytoplasm; and a short C-terminal segment, which is exposed to the lumen of the secretory pathway [6,7]. Yip1p and Yif1p show significant similarity at the transmembrane segments (Yip domain); two other non-essential proteins, Yip4p and Yip5p, also share the Yip domain [8]. Homologs of Yip1p and Yif1p are found in most eukaryotes, including fungi, protozoa, plants, and animals, indicating their essential role in cell functioning. However, the precise mode of their function remains

*Abbreviations:* YIPF, Yip domain family; ERGIC, ER-Golgi intermediate compartment; TGN, *trans*-Golgi network; BFA, brefeldin A; GnT-I, *N*-acetylglucosaminyltransferase-I; RFP, red fluorescent protein; GalT-I, β4-galactosyltransferase-I; PAS staining, Periodic acid-Schiff staining

* Corresponding author at: Department of Molecular Biosciences, Faculty of Life Sciences, Kyoto Sangyo University, Motoyama, Kamigamo, Kita, Kyoto 603-8555, Japan.
*E-mail address:* osaru3@cc.kyoto-su.ac.jp (N. Nakamura).

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obscure [9].

Nine YIPF members are found in human cells [6,10]. Phylogenetic analyses have previously shown that YIPF4, YIPF5/YIP1A, YIPF6, and YIPF7/YIP1B are Yip1p homologs, while YIPF1, YIPF2, YIPF3, YIF1A, and YIF1B are Yif1p homologs (Fig. S1) [6]. While characterizing the human homologs, we previously found that each human Yip1p homolog (YIPF5 and YIPF4) works with a specific Yif1p homolog partner (YIF1A and YIPF3, respectively) [9,10]. Cellular distribution of each pair is different—YIPF5 and YIF1A mainly localize within the ER-Golgi intermediate compartment (ERGIC), while YIPF4 and YIPF3 mainly localize within the *cis*-Golgi. It is possible that each pair of YIPF proteins functions independently in their residing compartment by associating with a different set of proteins, in addition to functioning together in these compartments. Knockdown of Yip1p homologs (YIPF4 or YIPF5) causes marked reduction in the levels of their partner Yif1p homologs (YIPF3 or YIF1A), suggesting that Yif1p homologs are unstable in the absence of Yip1p homologs. The knockdown of YIPF4, YIPF3, YIPF5, and YIF1A causes significant fragmentation of the Golgi apparatus, highlighting their role in maintaining the Golgi structure.

Here, we report that the Yip1p homolog YIPF6 forms complexes with the Yif1p homologs YIPF1 and YIPF2, and they localize in the *medial-/trans*-Golgi and TGN. Gene knockdown experiments showed that YIPF6, free of YIPF1 and YIPF2, interferes with the reorganization of the Golgi apparatus and glycan synthesis.

## 2. Materials and methods

### 2.1. Cell culture, drug treatment, immunofluorescence staining, cell extraction, and immunoprecipitation

These experiments were performed as described previously [10]. For examining the Golgi reassembly, HeLa cells were treated with a growth medium (Dulbecco's modified Eagles medium containing 10% fetal bovine serum) containing 0.25 μg/mL BFA for one hour to four hours. Then, the BFA containing medium was removed and the cells were washed with the growth medium for three times, and were incubated in the growth medium for one hour. HT-29 cells were originally purchased from ATCC and were kindly donated by Profs. A. Kurosaka and K. Sato (Kyoto Sangyo University) and cultured with the growth medium.

### 2.2. Plasmids and transfection

A plasmid encoding *N*-acetylglucosaminyltransferase-I (GnT-I) tagged with red fluorescent protein (GnT-I-RFP) was produced by conjugating a cDNA encoding the monomeric red fluorescent protein [11] to the 3′-end of the GnT-I cDNA encoding GnT-I [12], and was inserted into pcDNA3 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Another plasmid encoding β4-galactosyltransferase-I (GalT-I) tagged with RFP (GalT-I-RFP) was purchased from Evrogen (pTagRFP-Golgi; Moscow, Russia).

### 2.3. Antibodies

Rabbit polyclonal antibodies for YIPF1, YIPF2, and YIPF6 were produced and affinity-purified as described previously by using recombinant proteins [10]. Rabbit anti-human GM130 (GST-human GM130) was kindly donated by Drs. Sohda (Niigata University) and Misumi (Fukuoka University) [13]. The following antibodies were also purchased—mouse monoclonal anti-GM130 antibody (BD Biosciences, San Diego, CA, USA), CY3-conjugated anti-rabbit IgG, CY5-conjugated anti-rabbit IgG, CY5-conjugated anti-mouse IgG (Jackson ImmunoResearch Labs. Inc., West Grove, PA, USA), Alexa488-conjugated anti-mouse IgG (Molecular Probes, Thermo Fisher Scientific), and HRP-conjugated anti-rabbit IgG (Jackson ImmunoResearch).

### 2.4. Pull-down analysis

Pull-down analysis was performed as described previously [10]. Briefly, HeLa cells were incubated in a buffer containing digitonin and centrifuged to obtain a soluble extract. A part of the extract was kept as a sample of the extract, and the remaining extract was separated into two equal parts. Affinity-purified antibody for a YIPF protein was added to one part of the extract and affinity-purified IgG was added to another part. Protein A Sepharose CL-4B (GE Healthcare Bio- Science AB, Uppsala, Sweden) was used to pull down antibodies and bound materials.

### 2.5. SDS-PAGE, western blotting, and densitometry

SDS-PAGE and western blotting were performed as described previously [10]. Chemiluminescence images were taken using LAS1000 and LAS4000mini (Fuji Photo Film. Inc., Tokyo, Japan). Densitometry was performed using ImageJ (National Institute of Health, Bethesda, USA).

### 2.6. siRNA transfection

siRNA transfection was performed as described previously [10]. Briefly, HeLa cells or HT-29 cells were transfected with Stealth RNAi™ siRNAs (YIPF1: HSS122872, YIPF2: HSS149042, and YIPF6: equal amount mixture of HSS138898, HS138899, and HS138900, Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA USA) by using Lipofectoamine 2000 and incubated for three days with a daily change in the medium before analyses. *Silencer*® Select Negative Control No. 1 siRNA (Thermo Fisher Scientific Inc.) was used as control.

### 2.7. Immunofluorescent staining

This was performed as described previously [14].

### 2.8. Periodic acid-Schiff (PAS) staining

$2\times10^5$ HT-29 cells were seeded on to coverslips in petri dishes 3.5 cm in diameter and were transfected with siRNA as described above. The cells were then stained using the PAS Kit (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) according to the manufacturer's instructions, but with slight modifications. Briefly, the cells were fixed with a formalin–ethanol fixative solution (4% formaldehyde in 95% ethanol) and gently washed three times with tap water. The cells were then treated with the PAS solution for 5 min and washed five times with distilled water. Finally, the cells were treated with the Schiff reagent for 15 min and washed 10 times with tap water. The coverslips, containing the stained cells, were mounted in glycerol and observed under light microscope. Counter-staining with hematoxylin was omitted for the following image analysis. Micrographs were taken using a bright-field microscope with identical illumination and exposure settings for all samples. For each sample, 6–8 images were used for quantification with ImageJ. Two areas of cell clusters were selected from an image by the free-hand selection mode and two cell-free areas were selected from the same image. Mean intensity of an area was divided by pixel number of the area to estimate the area's cell density. The average density of two cell areas was subtracted by that of the two cell-free areas to obtain a specific staining density of an image. Finally, an average specific staining density of 6–8 images was calculated for each sample. For each experiment, the average specific densities of YIPF knocked-down cells were divided by that of negative control transfected cells to ascertain relative staining density of the cells. Statistical analysis was performed using StatMate V (ATMS Co., Ltd, Tokyo, Japan).