

# Action of Arl1 GTPase and golgin Imh1 in Ypt6-independent retrograde transport from endosomes to the *trans*-Golgi network

Yan-Ting Chen<sup>a,†</sup>, I-Hao Wang<sup>a,†</sup>, Yi-Hsun Wang<sup>a</sup>, Wan-Yun Chiu<sup>a</sup>, Jen-Hao Hu<sup>a</sup>, Wen-Hui Chen<sup>a</sup>, and Fang-Jen S. Lee<sup>a,b,c,\*</sup>

<sup>a</sup>Institute of Molecular Medicine, College of Medicine, National Taiwan University, Taipei 100, Taiwan; <sup>b</sup>Department of Medical Research, National Taiwan University Hospital, Taipei 100, Taiwan; <sup>c</sup>Center of Precision Medicine, College of Medicine, National Taiwan University, Taipei 10002, Taiwan

**ABSTRACT** The Arf and Rab/Ypt GTPases coordinately regulate membrane traffic and organelle structure by regulating vesicle formation and fusion. Ample evidence has indicated that proteins in these two families may function in parallel or complementarily; however, the manner in which Arf and Rab/Ypt proteins perform interchangeable functions remains unclear. In this study, we report that a Golgi-localized Arf, Arl1, could suppress Ypt6 dysfunction via its effector golgin, Imh1, but not via the lipid flippase Drs2. Ypt6 is critical for the retrograde transport of vesicles from endosomes to the *trans*-Golgi network (TGN), and its mutation leads to severe protein mislocalization and growth defects. We first overexpress the components of the Arl3-Syt1-Arl1-Imh1 cascade and show that only Arl1 and Imh1 can restore endosome-to-TGN trafficking in ypt6-deleted cells. Interestingly, increased abundance of Arl1 or Imh1 restores localization of the tethering factor Golgi associated retrograde-protein (GARP) complex to the TGN in the absence of Ypt6. We further show that the N-terminal domain of Imh1 is critical for restoring GARP localization and endosome-to-TGN transport in ypt6-deleted cells. Together, our results reveal the mechanism by which Arl1-Imh1 facilitates the recruitment of GARP to the TGN and compensates for the endosome-to-TGN trafficking defects in dysfunctional Ypt6 conditions.

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## INTRODUCTION

The ADP-ribosylation factors (Arfs) are a family of small GTPases that govern vesicular trafficking in eukaryotic cells (D'Souza-Schorey and

Chavrier, 2006; Gillingham and Munro, 2007). Arfs have been reported to function in vesicle formation, organelle integrity, and signal transduction (Boman and Kahn, 1995; Donaldson and Jackson, 2011; Jackson and Bouvet, 2014). These small GTPases perform their functions based on their conformation, cycling between a GTP-bound active state and a GDP-bound inactive state regulated by guanine nucleotide-exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively (Donaldson and Jackson, 2000; Pasqualato *et al.*, 2002).

The class of Arf-like proteins (Arls), which function similarly to Arfs, has been identified as having more than 20 members in mammals and only two in yeast (Lee *et al.*, 1997; Kahn *et al.*, 2006). Among them, Arf-like protein 1 (Arl1) is conserved from yeast to humans and is also the most studied Arl. In yeast, the activity of Arl1 is regulated by its GEF, Syt1, and GAP, Gcs1 (Liu *et al.*, 2005; Chen *et al.*, 2010). Arl1 is known to enforce the transport of the glycosylphosphatidylinositol (GPI)-anchored protein Gas1 to the plasma membrane to maintain cell wall integrity (Liu *et al.*, 2006). Arl1 has also been shown to be involved in selective autophagy under

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<sup>†</sup>These authors contributed equally to this work.

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\*Address correspondence to: Fang-Jen S. Lee ([fangjen@ntu.edu.tw](mailto:fangjen@ntu.edu.tw)).

Abbreviations used: Arf, ADP-ribosylation factor; Arl, Arf-like protein; GAP, GTPase-activating protein; GARP, Golgi-associated retrograde protein; GEF, guanine nucleotide exchange factor; TGN, *trans*-Golgi network.

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normal growth conditions; this involvement occurs together with Arl3, which is necessary to activate Arl1 and its downstream effectors (Setty *et al.*, 2003, 2004; Wang *et al.*, 2017). Moreover, we have demonstrated that active Arl1 forms a ternary complex with the flippase Drs2 and the Arf GEF Gea2, which mediates the recruitment of the GRIP domain-containing golgin Imh1 to late Golgi (Tsai *et al.*, 2013). Furthermore, Imh1 not only is a downstream effector of Arl1 but also acts as a feedback regulator to maintain and protect the activity of Arl1 from Gcs1-activated GTP hydrolysis (Chen *et al.*, 2012).

Golgins are Golgi-localized proteins that contain coiled-coil regions and are required for tethering events in membrane fusion and for maintenance of the Golgi structure (Munro and Nichols, 1999; Munro, 2011; Witkos and Lowe, 2015). Some golgins target the *trans*-Golgi membrane via the GRIP domain at their carboxyl terminus. Four GRIP domain-containing golgins have been identified in mammalian cells, Golgin-97, Golgin-245/p230, GCC185, and GCC88, whereas Imh1 is the only one identified in yeast (Munro and Nichols, 1999; Brown *et al.*, 2001; Gillingham and Munro, 2016). Golgin-97, Golgin-245, and Imh1 are recruited to the Golgi membrane by Arl1 and act as downstream effectors (Lu and Hong, 2003; Panic *et al.*, 2003a; Setty *et al.*, 2003; Wu *et al.*, 2004). Despite extensive study of mammalian GRIP domain-containing golgins, the exact and detailed function of Imh1 in vesicular trafficking remains unclear.

Regarding the functions of Imh1, its strong genetic interaction with Ypt6 provides valuable hints, as the deletion of *YPT6* along with either *ARL1* or *IMH1* shows synthetic lethality (Siniossoglou *et al.*, 2000; Setty *et al.*, 2003). Ypt6, the yeast homologue of mammalian Rab6, is associated with the Golgi complex and has been demonstrated to be required for retrograde trafficking from early endosomes to late Golgi (or the *trans*-Golgi network [TGN] in mammalian cells) (Tsukada *et al.*, 1999; Siniossoglou *et al.*, 2000; Bensen *et al.*, 2001). However, Ypt6 does not appear to localize exclusively to the TGN, because more than 50% of GFP-Ypt6 fluorescence overlaps with the *cis*-Golgi marker. Recent studies suggest that mammalian Rab6 is important not only for membrane trafficking but also for the organization and maintenance of the Golgi (Starr *et al.*, 2010; Storrie *et al.*, 2012). Ypt6 promotes endosome-derived vesicle fusion via the recruitment of the tethering factor Golgi associated retrograde-protein (GARP) complex, which comprises four subunits, Vps51/52/53/54 (Conibear and Stevens, 2000; Conibear *et al.*, 2003; Donaldson and Jackson, 2000; Siniossoglou and Pelham, 2001). The interaction between Ypt6 and the GARP complex occurs through its Vps52 subunit. Removal of Ypt6 or GARP blocks recycling of the late Golgi SNAREs Tlg1 and Tlg2 and the exocytic SNARE Snc1 from endosomes back to the Golgi (Siniossoglou and Pelham, 2001, 2002).

Arl1 and Ypt6 have been suggested to have overlapping functions and to be in redundant pathways (Bensen *et al.*, 2001; Panic *et al.*, 2003b; Tong *et al.*, 2004; Yang and Rosenwald, 2016). Although Arl1 has also been reported to interact with Vps53 (Siniossoglou and Pelham, 2001; Panic *et al.*, 2003b), disruption of *ARL1*, unlike that of *YPT6*, does not affect the localization of the GARP complex at the Golgi or the recycling of Snc1 from endosomes back to the Golgi (Panic *et al.*, 2003b; Liu *et al.*, 2006). Therefore, the role of Arl1 in Ypt6-independent endosome-to-Golgi transport pathways remains to be further elucidated. In this study, we demonstrated that Imh1 mediates recruitment of the GARP complex in the absence of Ypt6. Additionally, increased abundance of Arl1 or Imh1, but not of the N-terminally truncated Imh1, can rescue Ypt6-mediated endosome-to-TGN transport. Our findings provide

new insight into the participation of the Arl1-Imh1 complex in Ypt6-mediated retrograde transport.

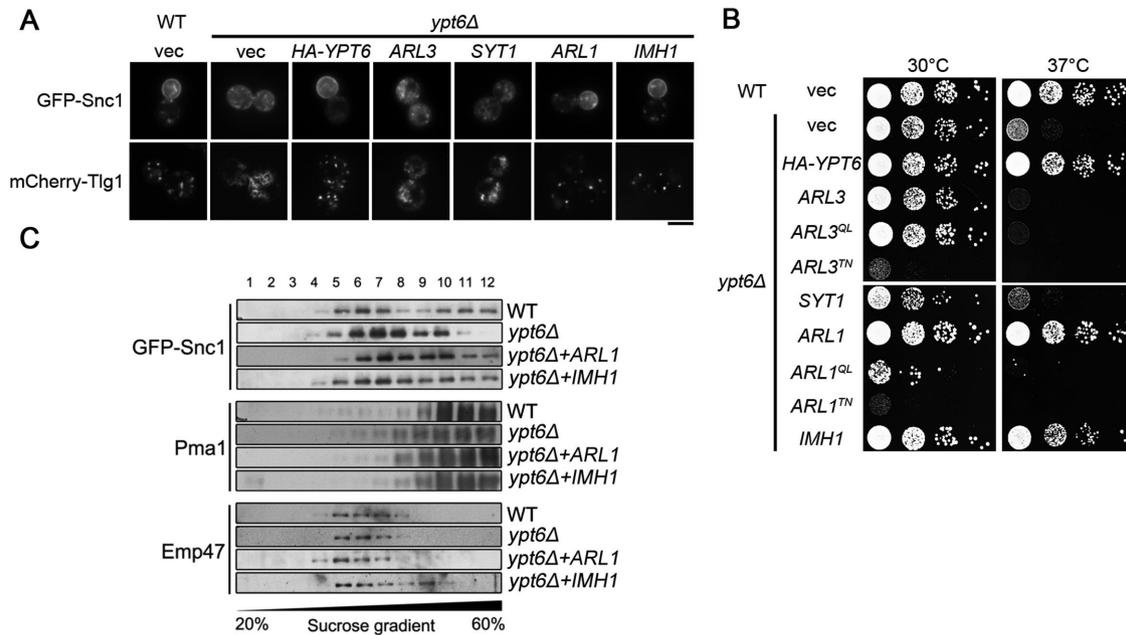
## RESULTS

### Overexpression of Arl1 or Imh1 restores endosome-to-TGN transport and suppresses temperature-sensitive growth defects in *ypt6Δ* cells

The Arl1 and Ypt6 pathways have been proposed to have overlapping functions in regulating vesicle-tethering events at the TGN membrane (Panic *et al.*, 2003b; Graham, 2004). To dissect the functional redundancy between Arl1 and Ypt6, we first determined the defects that resulted from *YPT6* deletion. We examined organelle protein markers in the secretory pathway, especially in the Golgi apparatus, in the absence of Ypt6. Consistent with a previous report (Siniossoglou and Pelham, 2001), we observed that the SNARE proteins Snc1 and Tlg1 mislocalized in *ypt6Δ* cells from the plasma membrane and endosome/Golgi, respectively, to an intracellular, obscure pattern (Supplemental Figure S1). However, Sec7 (late-Golgi marker), Gos1 (mid-Golgi marker), Sed5 (*cis*-Golgi marker), FYVE (endosome marker), Vps74 (PtdIns4P marker), and GOLPH3 (PtdIns4P marker) appeared to be unaffected in *ypt6Δ* cells. These results suggest a defect in targeting rather than a general disruption of the Golgi, as the distribution of several Golgi-localized proteins was unaffected in the *ypt6Δ* strain.

Although Ypt6 is known for its function on regulating the fusion of endosome-derived vesicles to the late Golgi in yeast (Conibear and Stevens, 2000; Bensen *et al.*, 2001; Siniossoglou and Pelham, 2001; Luo and Gallwitz, 2003; Suda *et al.*, 2013; Makaraci *et al.*, 2018), Rab6, the mammalian homologue of Ypt6, was shown to be involved in the secretory pathway from Golgi to the plasma membrane (Grigoriev *et al.*, 2007; Iwanami *et al.*, 2016). To clarify whether Snc1 mislocalization in *ypt6Δ* cells is a direct result of blocking the retrograde pathway from endosomes to the late Golgi or exocytic transport from the late Golgi to the plasma membrane, we examined the subcellular localization of endocytic internalization defective Snc1, Snc1-PM (Xu *et al.*, 2017), in wild-type, *ypt6Δ*, *arl1Δ*, or *imh1Δ* cells (Supplemental Figure S2A). We observed that GFP-Snc1-PM was properly transported to the plasma membrane in wild-type, *ypt6Δ*, *arl1Δ*, and *imh1Δ* cells, suggesting that Ypt6, Arl1, and Imh1 do not directly participate in the transport of Snc1 from the late Golgi to the plasma membrane (Supplemental Figure S2A). We also examined the colocalization of GFP-Snc1 and the late Golgi marker Sec7-mRFP in wild-type, *ypt6Δ*, *arl1Δ*, and *imh1Δ* cells. We observed that most of the Snc1 puncta in wild-type, *arl1Δ*, or *imh1Δ*, but not *ypt6Δ*, cells are colocalized with the late Golgi (Supplemental Figure S2B). These data indicate that Snc1 dissociation from the plasma membrane in *ypt6Δ* cells is due to Snc1-containing vesicles failing to fuse to the late Golgi and confirm that Ypt6 functions in the retrograde pathway from endosomes to the late Golgi.

We next examined whether Arl1 or Imh1 could restore the mislocalization of Snc1 and Tlg1 in the absence of Ypt6. While the deletion of *ARL1* or *IMH1* did not affect the localization of Snc1, Tlg1, and other markers in the secretory pathway (Supplemental Figure S3A), the overexpression of Arl1 or Imh1 restored the plasma membrane distribution of Snc1 and the late Golgi localization of Tlg1 and also suppressed the temperature sensitivity of *ypt6Δ* cells (Figure 1, A and B, and Supplemental Figure S4A). Interestingly, Arl3 and Syt1, upstream of the Arl1-Imh1 cascade, failed to rescue these phenotypes in *ypt6Δ* cells. These findings were further verified using sucrose-density gradient centrifugation by observing the



**FIGURE 1:** Overexpression of Arl1 or Imh1 restores endosome-to-TGN transport and suppresses temperature-sensitive growth defects in *ypt6Δ* cells. (A) Overexpression of Arl1 or Imh1, but not Syt1 or Arl3, restores Snc1 and Tlg1 transport defects in *ypt6Δ* cells. Fluorescently tagged Snc1 or Tlg1 was coexpressed with Arl3, Syt1, Arl1, or Imh1 in *ypt6Δ* cells. Live cells were observed in mid-log phase using fluorescence microscopy (scale bar, 5  $\mu$ m). (B) Overexpression of Arl1 or Imh1 suppresses the temperature-sensitive growth defects in *ypt6Δ* cells. *ypt6Δ* cells transformed with Ypt6, different forms (wild-type [WT], constitutively active [QL], and constitutively inactive [TN]) of Arl1 or Arl3, and Imh1 were serially diluted 10-fold as indicated, spotted on plates, and incubated at 30 or 37°C. (C) Arl1 and Imh1 restore the distribution of GFP-Snc1 in *ypt6Δ* cells. WT and *ypt6Δ* cells were transformed with Arl1 and Imh1. Lysates of spheroplasts from cells were fractionated by sucrose gradient (20–60%) centrifugation. Proteins in the fractions were precipitated, separated by SDS-PAGE, and analyzed by immunoblotting. Pma1 (plasma membrane marker), GFP-Snc1, and Emp47 (early Golgi marker) were identified with specific antibodies and detected using an ECL system. Gradient fractions were numbered from the top.

distribution of Snc1. Under the disruption of *YPT6*, Snc1 mislocalized from the higher-density plasma membrane fractions to the lower-density fractions, whereas overexpressing Arl1 or Imh1 restored its distribution (Figure 1C). Our results indicate that Arl1 and Imh1 are able to maintain the retrograde transport of Snc1 and Tlg1 in the absence of Ypt6.

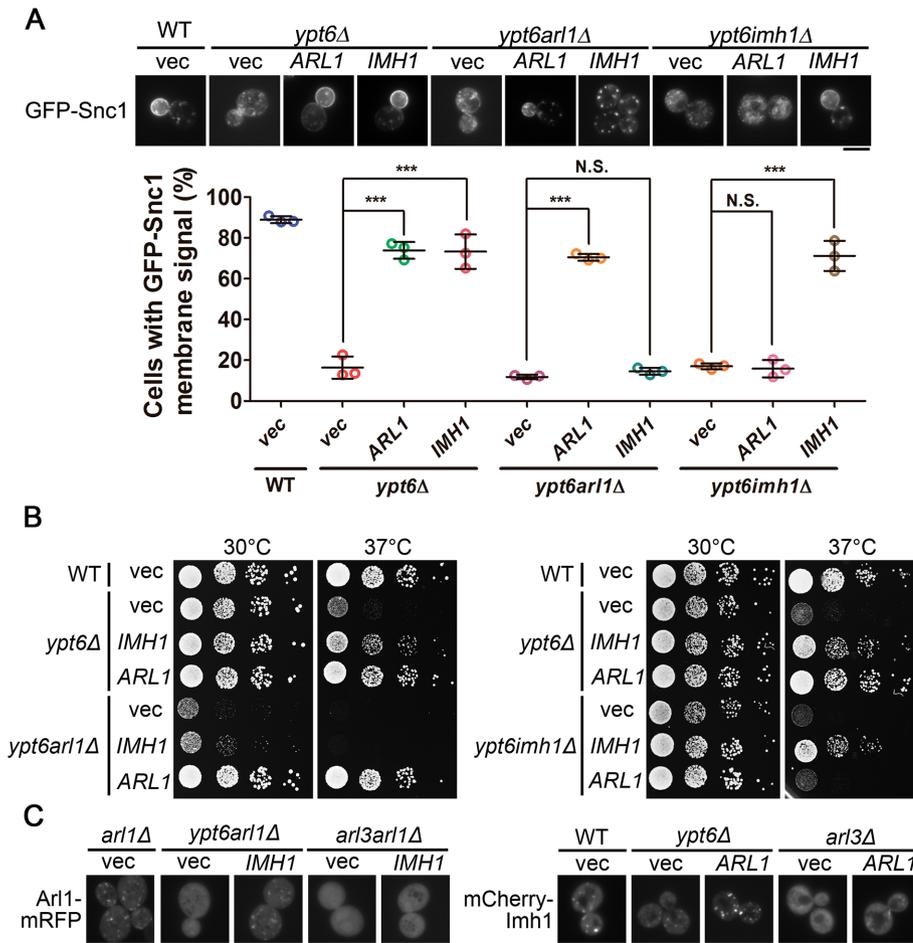
We further tested whether other proteins in the Arl3-Syt1-Arl1-Imh1 cascade could suppress temperature-sensitive growth defects in the *ypt6Δ* strain (Supplemental Figure S3B). We found that the malfunction of any gene in the Arl3-Syt1-Arl1-Imh1 cascade would not exhibit temperature-sensitive growth defects (Supplemental Figure S3B). Similarly to the results above, only Arl1 and Imh1, not Arl3 or Syt1, suppressed temperature-sensitive growth defects in *ypt6Δ* cells. Moreover, both the constitutively active Arl1<sup>Q72L</sup> (Arl1<sup>QL</sup>) and the constitutively inactive Arl1<sup>T32N</sup> (Arl1<sup>TN</sup>) failed to suppress defective growth at 37°C in *ypt6Δ* cells, suggesting that Arl1 required proper cycling between the GTP-bound active state and the GDP-bound inactive state to perform its functions (Figure 1B). These findings reveal the functional redundancy between Arl1-Imh1 and Ypt6, suggesting that Arl1 and Imh1 regulate trafficking and compensate for the absence of Ypt6.

### Arl1 and Imh1 function interdependently in suppressing defects of endosome-to-TGN transport and temperature-sensitive growth in *ypt6Δ* cells

To determine whether Arl1 and Imh1 function in concert to suppress defects in the absence of Ypt6, we first generated a conditional knockout strain by replacing the native promoter of *ARL1* with the

*GAL1* promoter (hereafter termed *ypt6arl1Δ*). This manipulation mimics *ARL1* and *YPT6* double deletion in glucose-containing culture medium and avoids the synthetic lethality between *ARL1* and *YPT6*. Compared with *ypt6Δ* cells, *ypt6arl1Δ* cells exhibit similar defects in the localization of Snc1 and Tlg1. We observed that overexpression of Arl1, but not of Imh1, restored the subcellular localization of Snc1 and Tlg1 in *ypt6arl1Δ* cells, suggesting that Imh1 depends on Arl1 for its suppression abilities (Figure 2A and Supplemental Figure S4B). Similarly, overexpression of Imh1, but not of Arl1, restored the trafficking defects of Snc1 and Tlg1 in *ypt6imh1Δ* cells (Figure 2A and Supplemental Figure S4B). Additionally, Arl1 and Imh1 exhibited interdependence in suppressing temperature-sensitive growth (Figure 2B). These results indicate that Arl1 and Imh1 function coordinately to suppress endosome-to-Golgi trafficking and high temperature-sensitive growth in the absence of Ypt6.

Previous studies have shown that Ypt6 is essential for the Golgi localization of Arl1 and Imh1 (Benjamin *et al.*, 2011). Because we demonstrated the strong interdependence of Arl1 and Imh1 in suppressing the dysfunction of Ypt6, we speculated that the overexpression of either Arl1 or Imh1 would be capable of restoring their localization in the absence of Ypt6. In *YPT6*-deletion cells, Arl1-mRFP and mCherry-Imh1 were diffuse throughout the cytosol. The dispersed patterns of Arl1-mRFP and mCherry-Imh1 were restored by overexpressing Imh1 and Arl1, respectively (Figure 2C). However, the overexpression of Arl1 or Imh1 did not restore their localization in the absence of Arl3 (Figure 2C), which excluded a potential dominant off-target effect of overexpression. These results again demonstrated the positive-feedback regulation between Arl1 and Imh1,



**FIGURE 2:** Arl1 and Imh1 act interdependently to suppress defects in endosome-to-TGN transport and temperature-sensitive growth in *ypt6Δ* cells. (A) Arl1 and Imh1 require each other to suppress the transport defects in *ypt6Δ* cells. Arl1 or Imh1 was coexpressed with GFP-Snc1 in the indicated cells. Live cells were observed in mid-log phase using fluorescence microscopy. Cells showing the GFP-Snc1 plasma membrane signal were quantified ( $n = 100$ ). The data are reported as the mean  $\pm$  SD of three independent experiments ( $***p < 0.001$ ; N.S. not significant; one-way ANOVA; scale bar, 5  $\mu$ m). (B) Both Arl1 and Imh1 are required to suppress temperature-sensitive growth defects in *ypt6arl1Δ* and *ypt6imh1Δ* cells. Yeast strains transformed with Arl1 or Imh1 were serially diluted 10-fold as indicated, spotted on plates, and incubated at 30 or 37°C. (C) Cytosolic distribution of Arl1 and Imh1 in *ypt6Δ* cells can be restored by overexpressing Imh1 or Arl1, respectively, in an Arl3-dependent manner. Arl1-mRFP or mCherry-Imh1 was coexpressed with Imh1 or Arl1, respectively, in the indicated cells. Live cells were observed in mid-log phase using fluorescence microscopy (scale bar, 5  $\mu$ m).

suggesting that they depend on each other for their Golgi localization in *ypt6Δ* cells.

### The Arl1- and Imh1-mediated suppression of Ypt6 dysfunction is independent of the Arf GEF Syt1

Our previous studies have demonstrated that the Arf GEF Syt1 is the only characterized GEF of Arl1. Syt1 is the GEF that is specifically responsible for activating Arl1 to promote the recruitment of the golgin Imh1 to the TGN (Chen *et al.*, 2010). To our surprise, Arl1 and Imh1 were still capable of exerting their suppression effects in the absence of Syt1 (Supplemental Figure S5). In contrast, the ability of Arl1 and Imh1 to suppress high-temperature growth defects in *ypt6arl3Δ* cells was attenuated. Moreover, we demonstrated that the overexpression of Syt1 was incapable of suppressing defects in endosome-to-Golgi trafficking of *ypt6Δ* cells (Figure 1A). Together,

these data imply that Arl1 is potentially regulated by another unknown GEF and thus can bypass Syt1-mediated activation to support trafficking in *ypt6Δ* cells.

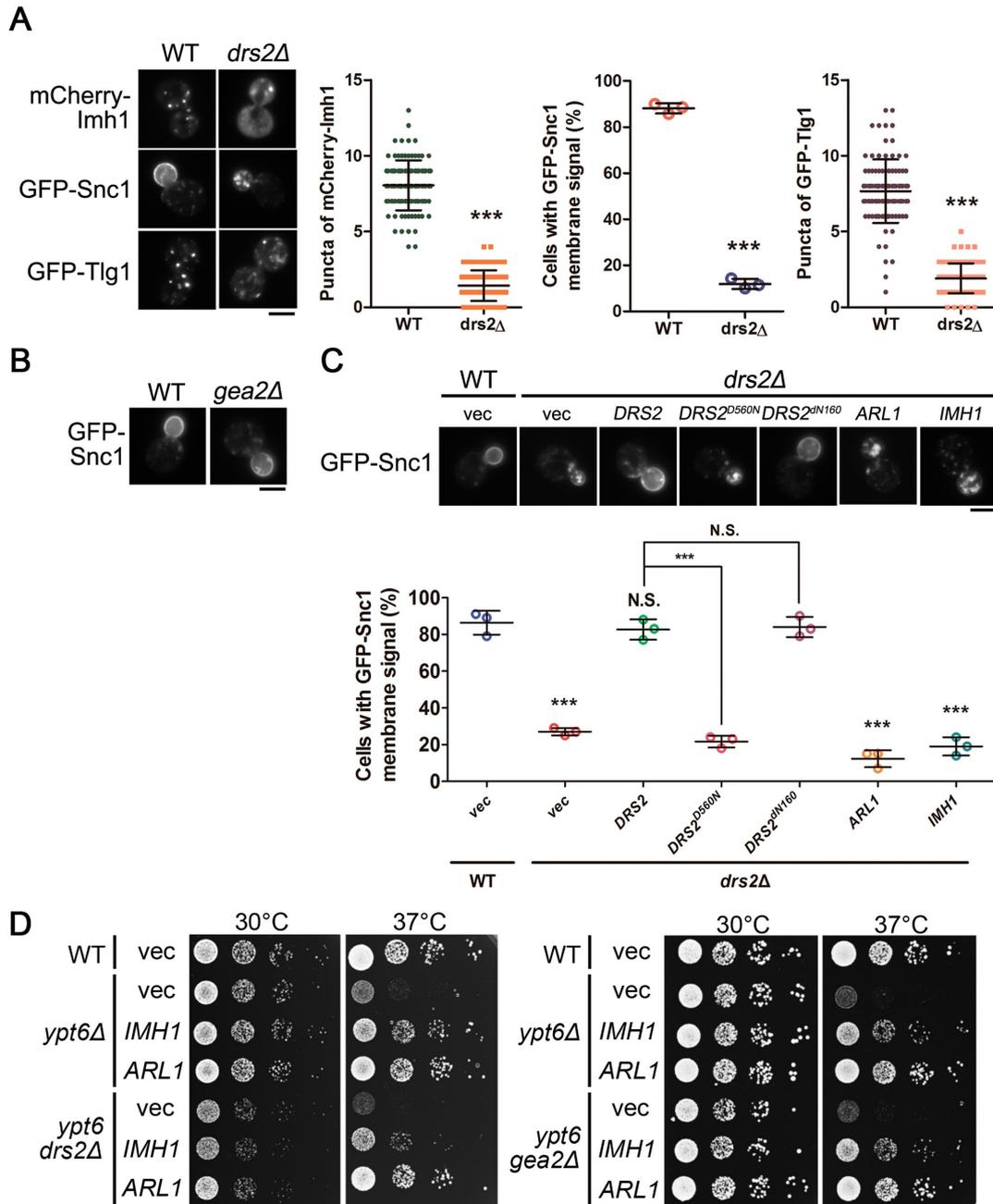
### The Arl1-Drs2-Gea2 ternary complex is not involved in the suppression of defects in endosome-to-TGN transport and temperature-sensitive growth in *ypt6Δ* cells

Our previous findings showed that Arl1 forms a complex with Drs2 and the Arf GEF Gea2 at the TGN that mediates the recruitment of the golgin Imh1 (Tsai *et al.*, 2013). To test whether this ternary complex is involved in Arl1- and Imh1-mediated suppression events under conditions of Ypt6 dysfunction, we examined the retrograde transport of Snc1 with *DRS2* or *GEA2* disruption. Consistent with the previous findings (Hua *et al.*, 2002; Furuta *et al.*, 2007), GFP-Snc1 and GFP-Tlg1 failed to maintain their proper localization in *drs2Δ* cells (Figure 3A). Interestingly, unlike Drs2, Gea2 did not seem to be involved in the transport of Snc1 (Figure 3B). Therefore, we speculate that instead of functioning as a Drs2-Gea2 complex, Drs2 might perform its own independent function in mediating the retrograde transport of Snc1. To test this hypothesis, we rescued *drs2Δ* cells with wild-type and different mutant forms of Drs2 and found that wild-type Drs2, but not the flippase activity-defective Drs2<sup>D560N</sup>, was able to restore the transport of Snc1 in *drs2Δ* cells. Intriguingly, Drs2<sup>dN160</sup>, which cannot interact with Arl1 (Tsai *et al.*, 2013), was able to rescue Snc1 retrograde transport in *drs2Δ* cells (Figure 3C). Moreover, overexpression of Arl1 or Imh1 failed to suppress Snc1 mislocalization in *drs2Δ* cells (Figure 3C). These results indicate that Drs2 functions in regulating Snc1 in an Arl1-independent manner. We further showed that overexpression of Arl1 or Imh1 restored Snc1 and Tlg1 localization in *ypt6gea2Δ* cells but not in *ypt6drs2Δ* cells (Supplemental Figure S6).

Moreover, overexpression of Arl1 or Imh1 was still capable of restoring temperature-sensitive growth defects in *ypt6drs2Δ* and *ypt6gea2Δ* cells (Figure 3D). Conclusively, Arl1 mediates retrograde transport via an Imh1-dependent, but Drs2-Gea2 complex-independent, pathway in *ypt6Δ* cells.

### The recruitment of the GARP complex to the TGN by Arl1 and Imh1 is critical for suppressing defects of endosome-to-TGN transport in *ypt6Δ* cells

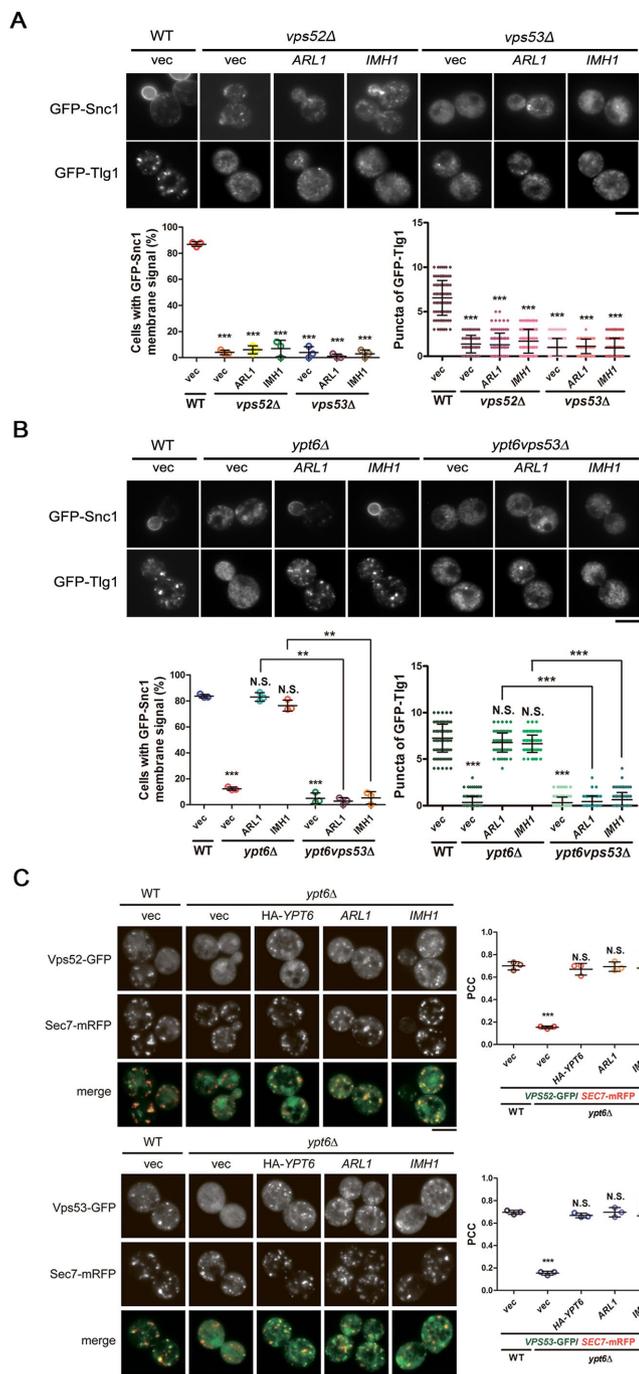
The GARP complex, the effector of Ypt6, is a multisubunit tethering complex (MTC) that is responsible for tethering vesicles in endosome-to-Golgi transport at late Golgi (Bonifacino and Hierro, 2011). Ypt6, but not Arl1 or Imh1, is known to regulate the recruitment of the GARP complex under normal conditions (Panic *et al.*, 2003b), and malfunction of the GARP complex also leads to mislocalization



**FIGURE 3:** The Arl1-Drs2-Gea2 ternary complex is not involved in the suppression of defects in endosome-to-TGN transport and temperature-sensitive growth in *ypt6Δ* cells. (A) Drs2 mediates the transport of Snc1 and Tlg1. Fluorescently tagged Imh1, Snc1, or Tlg1 was transformed into WT or *drs2Δ* cells (scale bar, 5  $\mu$ m). Cells showing the GFP-Snc1 plasma membrane signal were quantified ( $n = 100$ ) and the data are reported as the mean  $\pm$  SD of three independent experiments ( $***p < 0.001$ ; one-way ANOVA). Cells showing the mCherry-Imh1 or GFP-Tlg1 puncta were quantified ( $n = 100$ ), and the data are reported as the mean  $\pm$  SD of three independent experiments ( $***p < 0.001$ ; paired t test). (B) Gea2 is not required for the proper transport of Snc1. GFP-Snc1 was transformed into WT or *gea2Δ* cells and examined by fluorescence microscopy (scale bar, 5  $\mu$ m). (C) Arl1 is not involved in Drs2-mediated Snc1 transport. GFP-Snc1 was expressed in wild-type or coexpressed with Arl1, Imh1, or different forms of Drs2 in *drs2Δ* cells. Live cells were observed in mid-log phase by fluorescence microscopy. Cells showing the GFP-Snc1 plasma membrane signal were quantified ( $n = 100$ ). The data are reported as the mean  $\pm$  SD of three independent experiments ( $***p < 0.001$ ; N.S. not significant; one-way ANOVA; scale bar, 5  $\mu$ m). (D) The ternary complex of Arl1-Drs2-Gea2 is not involved in Arl1 and Imh1 suppressing temperature-sensitive growth defects in *ypt6Δ* cells. The indicated strains (wild-type, *ypt6Δ*, *ypt6Δdrs2Δ*, *ypt6Δgea2Δ*) transformed with Arl1 or Imh1 were serially diluted 10-fold as indicated, spotted on plates, and incubated at 30 or 37°C.

of Snc1 and Tlg1, which is similar to the situation in *ypt6Δ* cells (Conibear *et al.*, 2003; Supplemental Figures S1 and S7). Therefore, we speculated that the GARP complex might be involved in the Arl1-

and Imh1-mediated suppression of endosome-to-TGN transport defects in *ypt6Δ* cells. To test our hypothesis, we first examined whether overexpression of Arl1 or Imh1 restores endosome-to-Golgi



**FIGURE 4:** The recruitment of the GARP complex to the TGN by Arl1 and Imh1 is critical for suppressing defects in endosome-to-TGN transport in *ypt6Δ* cells. (A) Overexpression of Imh1 or Arl1 is unable to suppress GFP-Snc1 and GFP-Tlg1 mislocalization in GARP-complex subunit-deletion strains. Arl1 or Imh1 was coexpressed with GFP-Snc1 or GFP-Tlg1 in *vps52Δ* and *vps53Δ* cells. Live cells were observed in mid-log phase by fluorescence microscopy. Cells showing the GFP-Snc1 plasma membrane signal or the GFP-Tlg1 puncta were quantified ( $n = 100$ ). The data are reported as the mean  $\pm$  SD of three independent experiments ( $***p < 0.001$ ; one-way ANOVA; scale bar, 5  $\mu$ m). (B) The GARP complex is required for Arl1 and Imh1 to suppress Snc1 and Tlg1 transport defects in *ypt6Δ* cells. Fluorescently tagged Snc1 or Tlg1 was coexpressed with Arl1 or Imh1 in *ypt6Δ* or *ypt6Δvps53Δ* cells. Live cells were observed in mid-log phase using fluorescence microscopy. Cells showing the GFP-Snc1 plasma membrane signal or the GFP-Tlg1 puncta were quantified ( $n = 100$ ).

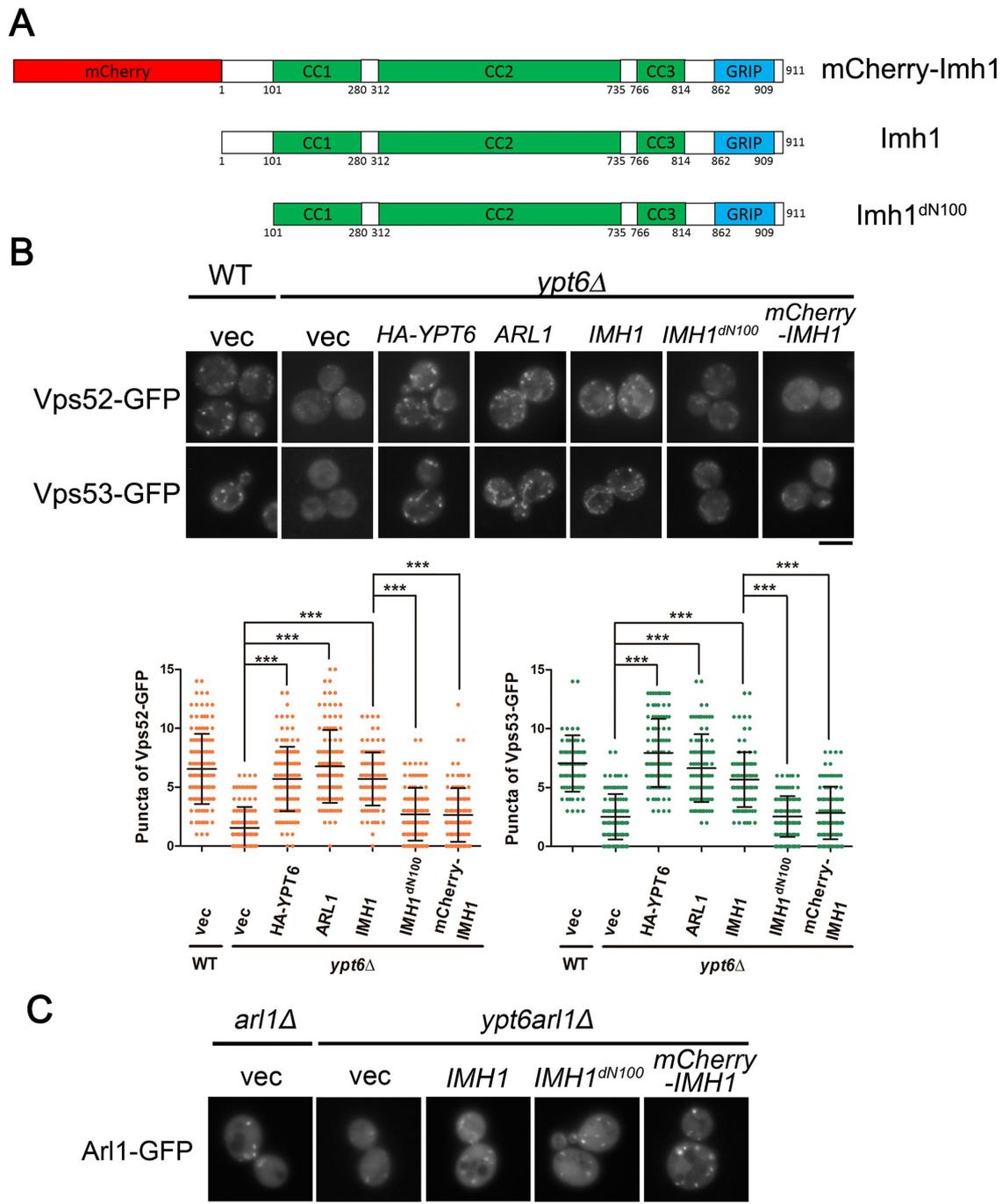
trafficking defects in the absence of the GARP complex. We observed that increased abundance of Arl1 or Imh1 failed to suppress the mislocalization of Snc1 and Tlg1 in *vps52Δ* and *vps53Δ* cells (Figure 4A), indicating that the GARP complex is strictly required for proper endosome-to-Golgi trafficking. Likewise, *ypt6Δvps53Δ* cells still displayed defective transport of Snc1 and Tlg1 upon overexpression of Arl1 or Imh1, again showing that the suppression ability of Arl1 and Imh1 required the presence of the GARP complex (Figure 4B).

We thus hypothesized that the overexpression of Arl1 and Imh1 in *ypt6Δ* cells might act to restore proper recruitment of the GARP complex, thus facilitating endosome-to-Golgi trafficking. Therefore, we examined the localization of GARP complex subunits in *ypt6Δ* cells overexpressing Arl1 or Imh1. We found that increased abundance of Arl1 or Imh1 indeed restored the dispersed pattern of GARP complex subunits into a late Golgi distribution in *ypt6Δ* cells (Figure 4C). Altogether, our finding indicates that increased abundance of Arl1 or Imh1 is able to recruit the GARP complex back to the TGN in the absence of Ypt6 and further restore the defects of endosome-to-TGN transport in the absence of Ypt6.

### The N-terminus of Imh1 is critical for recruiting the GARP complex to the TGN in the absence of Ypt6

Golgins play important roles in vesicle trafficking and are required for tethering specific cargoes before membrane fusion (Witkos and Lowe, 2015). For most of the golgins, the N-terminal motif provides the ability to recognize and capture specific cargoes (Wong et al., 2017). Accordingly, we speculated that Imh1 also utilizes its N-terminal motif to restore the dispersed GARP complex in *ypt6Δ* cells. Imh1 contains three coiled-coil domains and one GRIP domain, which have been reported to be involved in dimerization and Arl1 interaction, respectively (Wu et al., 2004). We therefore tested the function of the uncharacterized domain before the first coiled-coil domain at the N-terminal region, deleting the first 100 amino acids of Imh1 (Imh1<sup>dN100</sup>). On the other hand, the fluorescent protein mCherry was fused to the N-terminus of Imh1 (mCherry-Imh1) to examine whether the tag would mask or obstruct the cargo recognition of Imh1 (Figure 5A). In *ypt6Δ* cells, the GARP complex preserved its TGN localization with Arl1 or Imh1 overexpression. However, cells overexpressing Imh1<sup>dN100</sup> and mCherry-Imh1 could not recruit the GARP complex to the Golgi (Figure 5B), suggesting that the intact N-terminal domain of Imh1 must be exposed for Imh1 to perform its function.

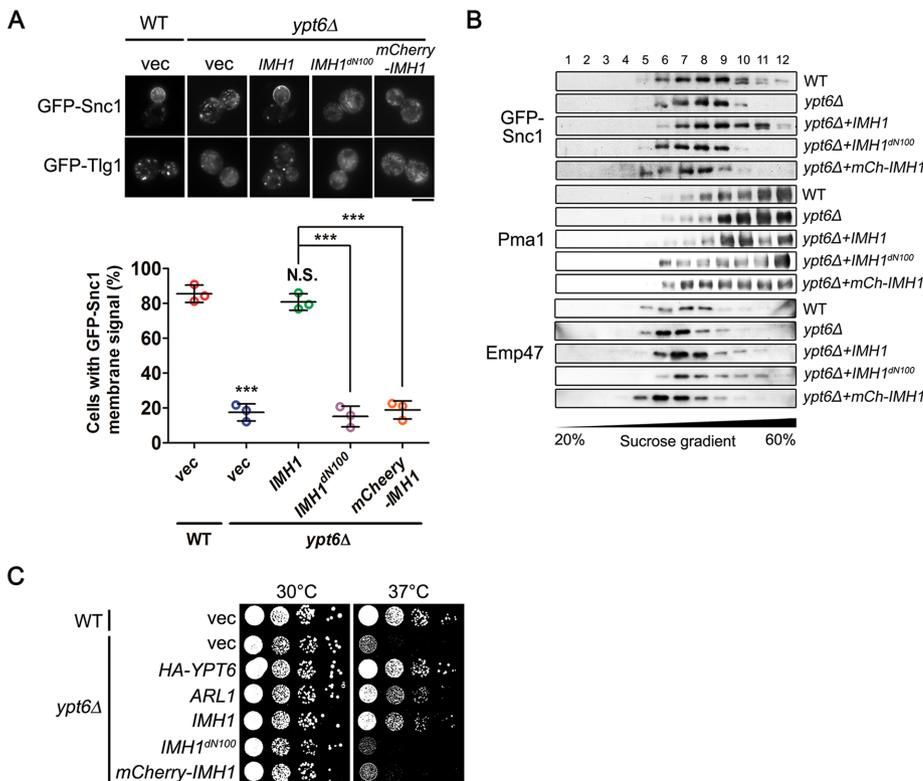
The data are reported as the mean  $\pm$  SD of three independent experiments ( $**p < 0.005$ ,  $***p < 0.001$ ; N.S. not significant; one-way ANOVA; scale bar, 5  $\mu$ m). (C) Overexpression of Arl1 or Imh1 restores the late Golgi puncta of the GARP complex in *ypt6Δ* cells. The Arl1-, Imh1-, or HA-Ypt6-overexpressed *ypt6Δ* cells were coexpressed with fluorescently tagged Vps52 or Vps53 and Sec7-mRFP (late Golgi marker). Live cells were observed in mid-log phase using fluorescence microscopy (scale bar, 5  $\mu$ m). At least 100 cells were included in the experiments from three independent biological repeats. The ratios of colocalization between Vps52/53-GFP and the late Golgi indicator (Sec7-mRFP) were represented as the Pearson correlation coefficient (PCC) using the ImageJ plug-in Just Another Colocalization Plugin with Costes Automatic Thresholding as described in *Materials and Methods*. The data are presented as the mean  $\pm$  SD, and the  $p$  values were determined applying one-way ANOVA ( $***p < 0.001$ ; N.S. not significant).



**FIGURE 5:** The N-terminal region of Imh1 is critical for recruiting the GARP complex to the TGN in the absence of Ypt6. (A) Schematic diagram of Imh1<sup>dN100</sup> and mCherry-Imh1 constructs. (B) The N-terminal region of Imh1 is required for restoring GARP complex localization in *ypt6Δ* cells. GFP-tagged Vps52 or Vps53 was coexpressed with wild-type Imh1, Imh1<sup>dN100</sup>, or mCherry-Imh1. Cells containing Vps52-GFP and Vps53-GFP puncta were quantified ( $n = 150$ ). The data are reported as the mean  $\pm$  SD of three biological repeats (\*\* $p < 0.001$ ; N.S. not significant; one-way ANOVA; scale bar, 5  $\mu$ m). (C) The N-terminal region of Imh1 is not involved in the localization of Arl1-GFP. Different forms of Imh1 were coexpressed with Arl1-GFP in *ypt6arl1Δ* cells to examine the localization of Arl1. Live cells were observed in mid-log phase using fluorescence microscopy (scale bar, 5  $\mu$ m).

Early findings provide evidence that Arl1 associates with the GARP complex through Vps53 (Panic *et al.*, 2003b); therefore, it is possible that Arl1 makes major contributions to GARP complex recruitment in the absence of Ypt6. However, we have demonstrated that the GRIP domain of Imh1 binds to Arl1 to preserve its activity (Chen *et al.*, 2012). The N-terminal mutants of Imh1 in this case,

showing defective GARP complex recruitment ability, should hypothetically be functional in regulating Arl1 activity. To confirm our speculation, we examined Arl1 localization in *ypt6arl1Δ* cells overexpressing Imh1<sup>dN100</sup> or mCherry-Imh1. Interestingly, full-length Imh1, Imh1<sup>dN100</sup>, and mCherry-Imh1 all restored the proper localization of Arl1 (Figure 5C). These results suggested that without the additional



**FIGURE 6:** The N-terminal region of Imh1 is essential for restoring defects in endosome-to-Golgi transport and temperature-sensitive growth in *ypt6Δ* cells. (A) Imh1<sup>dN100</sup> and mCherry-Imh1 failed to suppress the Snc1 and Tlg1 transport defects in *ypt6Δ* cells. Fluorescently tagged Snc1 or Tlg1 was coexpressed with different forms of Imh1 in *ypt6Δ* cells. Live cells were observed in the mid-log phase, and the percentages of cells with GFP-Snc1 membrane signals were quantified by fluorescence microscopy ( $n = 100$ ). The data are reported as the mean  $\pm$  SD of three biological repeats (\*\* $p < 0.001$ ; N.S. not significant; one-way ANOVA; scale bar, 5  $\mu$ m). (B) The N-terminal region is critical for Imh1 to restore the proper GFP-Snc1 cellular distribution in *ypt6Δ* cells. WT and *ypt6Δ* cells transformed with different forms of Imh1 were lysed and subjected to centrifugation in a 20–60% sucrose gradient as described in *Materials and Methods*. Fractions were collected and analyzed by Western blotting using antibodies against Pma1 (plasma membrane marker) and GFP-Snc1 and Emp47 (early Golgi marker). (C) Imh1<sup>dN100</sup> and mCherry-Imh1 are unable to suppress temperature-sensitive growth defects in *ypt6Δ* cells. *ypt6Δ* cells transformed with different forms of Imh1 were serially diluted 10-fold as indicated, spotted on plates, and incubated at 30 or 37°C.

function of the N-terminus of Imh1, Arl1 is insufficient for recruiting the GARP complex in the absence of Ypt6.

### The N-terminus of Imh1 is essential for suppressing defects of Ypt6 dysfunction

We next asked whether the N-terminus of Imh1 is also involved in suppressing endosome-to-Golgi trafficking defects and temperature-sensitive growth in the absence of Ypt6. We first examined the localization of Snc1 and Tlg1 in *ypt6Δ* cells overexpressing full-length Imh1, Imh1<sup>dN100</sup>, or mCherry-Imh1. We observed that full-length Imh1, but not Imh1<sup>dN100</sup> or mCherry-Imh1, restored the trafficking defects of these cargoes in *ypt6Δ* cells (Figure 6A). Consistently, the findings were supported by sucrose-density gradient centrifugation; only full-length Imh1, not Imh1<sup>dN100</sup> or mCherry-Imh1, suppressed the improper distribution of GFP-Snc1 in *ypt6Δ* cells (Figure 6B). We also examined the importance of the N-terminus of Imh1 in suppressing temperature-sensitive growth defects in *ypt6Δ* cells. In contrast to overexpression of full-length Imh1, overexpression of Imh1<sup>dN100</sup> or mCherry-Imh1 failed to suppress temperature-sensitive

growth defects in *ypt6Δ* cells (Figure 6C). In conclusion, we demonstrated that the ability of Imh1 to suppress endosome-to-TGN transport and to suppress temperature-sensitive growth defects depends on the N-terminus of Imh1, as it plays a role in recruiting the GARP complex back to the TGN.

### The Arl1-independent association between Imh1 and the GARP complex does not require the N-terminus of Imh1 but is insufficient for GARP complex recruitment

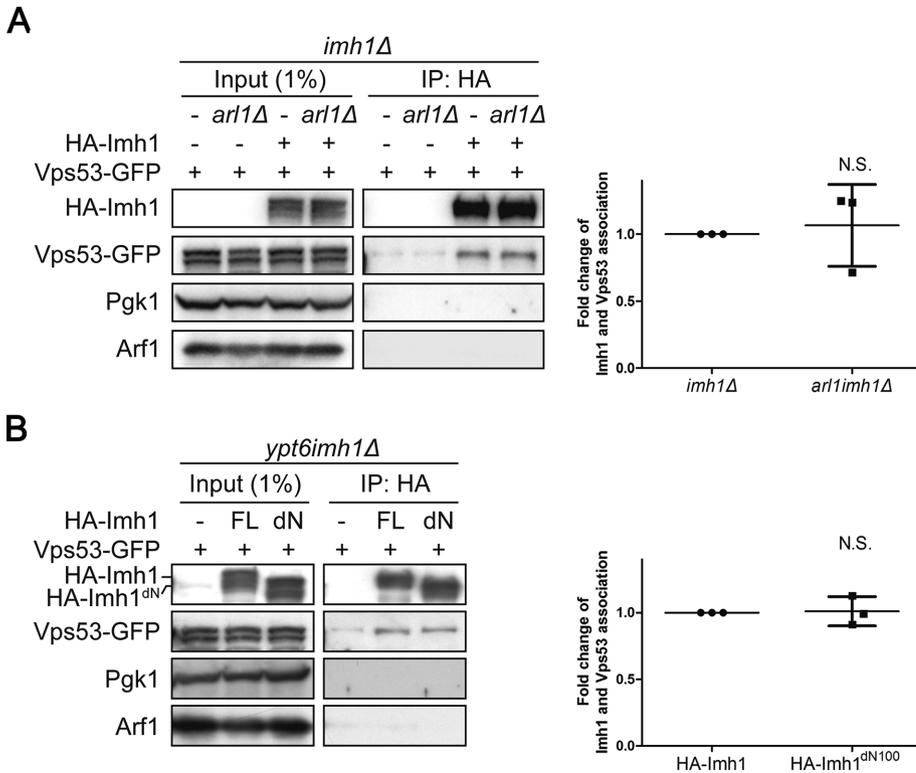
Given that the N-terminal mutants of Imh1 did not alter the localization of Arl1 (Figure 5C), we speculated that the recruitment of the GARP complex might rely mainly on the association with Imh1. To test this hypothesis, we carried out a coimmunoprecipitation assay and showed that Vps53-GFP coprecipitated with HA-Imh1 and that the depletion of Arl1 did not affect their association (Figure 7A). Strikingly, we found that in contrast to full-length Imh1, Imh1<sup>dN100</sup> showed no significant difference in Vps53 association (Figure 7B). These results suggest that the N-terminal 100 amino acids of Imh1 have additional functions in restoring trafficking defects in *ypt6Δ* cells other than associating with the GARP complex.

## DISCUSSION

The Arf and Ypt/Rab small GTPases are key regulators of membrane trafficking via their mediation of vesicle formation and membrane tethering versus vesicle fusion, respectively. Many reports have indicated that complementary function and cross-talk between these two families are important for membrane trafficking coordination in cells (Jones *et al.*, 1999; Deretic, 2013; McDonold and Fromme, 2014). In this study, we discovered that increased abundance of Arl1 and

its effector Imh1 interdependently recruits the GARP complex to the TGN in the absence of Ypt6, and this recruitment subsequently tethers endosome-derived vesicles and suppresses the growth defect. Our findings reveal the molecular mechanism of the functional redundancy between Arl1 and Ypt6 and provide further mechanistic insight into the cross-talk between the Arf and Ypt families.

Genetic interactions between components of the Arl1 and Ypt6 pathways have suggested overlapping functions (Benjamin *et al.*, 2011), while our data show that Arl1 and its effector Imh1 act interdependently to suppress defects of endosome-to-TGN transport and temperature-sensitive growth in *ypt6Δ* cells and that increased abundance of Arl1 must be activated by a GEF other than Syt1. It will be interesting to identify the unknown Arl1 GEF by screening genes involved in the Arl1-mediated suppression of Ypt6 dysfunction. In addition, our results show that both constitutively active Arl1<sup>Q72L</sup> and constitutively inactive Arl1<sup>T32N</sup> failed to suppress defective growth at 37°C in *ypt6Δ* cells, indicating that Arl1 required proper cycling between the GTP-bound active state and the GDP-bound inactive state to perform its functions.



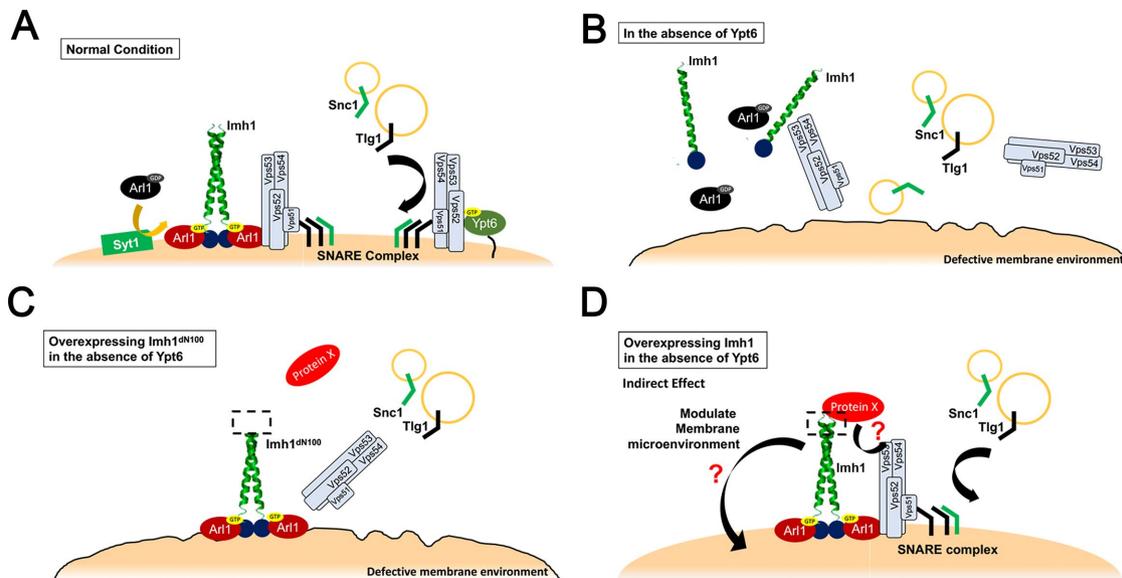
**FIGURE 7:** The Arl1-independent association between Imh1 and the GARP complex does not require the N-terminus of Imh1 but is insufficient for GARP complex recruitment. (A) The association of Imh1 and Vps53 is unaltered in the absence of Arl1. Yeast cells were lysed, centrifuged to collect clear lysate, and incubated with monoclonal anti-HA-agarose antibody. The bound proteins were analyzed by Western blotting. The intensities of bound HA-Imh1, bound Vps53-GFP, and input Vps53-GFP were measured by ImageJ Fiji software. The bound Vps53-GFP signals were normalized to the bound HA-Imh1 and input Vps53-GFP and then the readouts of *arl1imh1Δ* cells were compared with those of *imh1Δ* cells. The data are reported as the mean  $\pm$  SD of three independent experiments (N.S. not significant; paired t test). (B) The N-terminal truncated Imh1 does not disrupt the association between Imh1 and Vps53. Yeast cells were lysed, centrifuged to collect clear lysate, and incubated with monoclonal anti-HA-agarose antibody. The bound proteins were analyzed by Western blotting. The intensities of bound HA-Imh1, bound Vps53-GFP, and input Vps53-GFP were measured by ImageJ Fiji software. The bound Vps53-GFP signals were normalized to the bound HA-Imh1 and input Vps53-GFP and then the readouts of HA-Imh1<sup>dN100</sup>-expressing cells were compared with those of HA-Imh1-expressing cells. The data are reported as the mean  $\pm$  SD of three independent experiments (N.S. not significant; paired t test).

Membrane asymmetry, curvature, and dynamics are known to have major roles in vesicle transport (McMahon and Boucrot, 2015). Lipid translocases (flippases) have been implicated in vesicle formation through the generation of membrane curvature (Chen *et al.*, 1999). The Graham group previously reported that the Arf GEF Gea2 binds to and stimulates the activity of the flippase Drs2 (Chantalat *et al.*, 2004). Our previous study further demonstrated that activated Arl1 interacts with both Gea2 and Drs2, forming a ternary complex, and that each interaction is necessary for the stability of the complex and is critical for flippase activity at the TGN (Tsai *et al.*, 2013). This Arl1-Drs2-Gea2 complex is specifically required for recruiting the golgin Imh1 to the TGN. Consistent with a previous report that Drs2 is essential for supporting protein transport between endosomes and the TGN (Hua *et al.*, 2002), we show that GFP-Snc1 and GFP-Tlg1 failed to maintain their proper localization in *drs2Δ* cells (Figure 3A). However, we observed that unlike Drs2, Gea2 is not involved in the transport of Snc1 (Figure 3B), and that Drs2 functions in regulating Snc1 in an Arl1-independent manner.

Moreover, the findings from Tanaka's group have identified that Rcy1, an F-box protein that was reported to be involved in recycling plasma membrane proteins, would bind directly to the Drs2 C-terminal regulatory domain, implying that the role of Rcy1 is to act on Drs2 flippase activity, thus providing another putative Arl1-independent pathway in which Drs2 participates in endosome-to-late Golgi protein transport (Hanamatsu *et al.*, 2014). On the basis of our results and the report from Tanaka's group, we speculate that instead of functioning in an Arl1-Drs2-Gea2 complex at the TGN, Drs2 might regulate the retrograde transport of Snc1, which is not based on an Arl1-related mechanism. Therefore, we conclude that the ternary complex formed by Arl1-Drs2-Gea2 is not involved in Arl1- and Imh1-mediated suppression events in *ypt6Δ* cells.

Cross-talk between the Arl1 and Ypt6 pathways has been suggested by the fact that Arl1 and Ypt6 share the ability to bind GARP through Vps53 and Vps52, respectively (Panic *et al.*, 2003b). The GARP complex functions to tether and facilitate retrograde transport of cargoes, such as Snc1 or Tlg1, to the TGN (Conibear *et al.*, 2003). However, the deletion of *YPT6*, but not of *ARL1*, affects GARP complex localization at the TGN; thus, the major recruitment of the GARP complex to the TGN is via Ypt6 (Siniosoglou and Pelham, 2001). Our findings show that neither Imh1<sup>dN100</sup> nor mCherry-Imh1 could restore GARP localization and endosome-to-TGN transport in *ypt6*-deleted cells, although both Imh1<sup>dN100</sup> and mCherry-Imh1 can stabilize Arl1 on the Golgi. These results suggest that the interaction between Arl1 and the GARP complex is not sufficient to recruit GARP to the TGN, thus implying that Imh1 may interact with the GARP complex through its N-terminal region. Our results indeed show that Imh1 can associate with GARP *in vivo* in the absence of Arl1. However, the N-terminal region of Imh1 is not required for its association with the GARP complex, indicating that the association of the GARP complex and Imh1 is not sufficient to restore GARP complex recruitment. Therefore, we hypothesize that the N-terminus of Imh1 may recruit other factors that either interact with the GARP complex or sustain the integrity of the membrane environment that the GARP complex favors. This complex, in turn, performs its appropriate functions to facilitate retrograde transport with its vesicle-tethering ability.

We propose a model (Figure 8) in which, under normal conditions, endosome-to-TGN retrograde transport is mainly mediated by the Ypt6-dependent GARP complex. Dysfunction of *YPT6* may lead to a defective Golgi membrane environment, which impairs the TGN localization of Arl1, Imh1, and the GARP complex and leads to defects in endosome-to-TGN transport. Increased abundance of Arl1 is activated in a Syt1-independent manner and subsequently recruits the golgin Imh1 to help restore the GARP complex to the



**FIGURE 8:** Model of Arl1-Imh1 acting on the Ypt6-independent endosome-to-TGN retrograde transport. (A) Under normal conditions, endosome-to-TGN retrograde transport is mainly mediated by the Ypt6-dependent GARP complex. (B) Dysfunction of *YPT6* leads to a defective Golgi membrane environment, which impairs TGN localization of the GARP complex and endosome-to-TGN transport. (C) Overexpression of the N-terminal mutant Imh1 can stabilize active Arl1 at the TGN but cannot recruit the GARP complex to the TGN to promote proper targeting of endosome-derived vesicles. (D) We hypothesize that Imh1 might direct its function through indirect mechanisms, as the association of Imh1<sup>ΔN100</sup> with Vps53 is insufficient to restore GARP localization. Imh1 might recruit a protein X and either associate with the GARP complex through protein X or together with it contribute to restoring the defective membrane environment resulting from *YPT6* deletion.

TGN in the absence of Ypt6. Then, Imh1 might recruit a protein X and either associate with the GARP complex through protein X or together with it contribute to restoring the defective membrane environment resulting from *YPT6* deletion.

In conclusion, we reveal a novel action of Arl1-Imh1 in compensating for the defects of dysfunctional Ypt6 on endosome-to-TGN traffic by facilitating the recruitment of GARP to the TGN. Our findings identify an unexpected function of Imh1 in addition to fine-tuning Arl1 activation in the absence of Ypt6 and provide a link to Syt1-independent Arl1 activation. The N-terminal domain of Imh1 is critical for restoring GARP localization in *ypt6*-deleted cells. Previous findings indicate that GARP orchestrates retrograde transport from endosomes to the TGN by promoting vesicle tethering and the assembly of SNARE complexes in consecutive, independent steps (Perez-Victoria and Bonifacino, 2009), yet the molecular mechanism of the spatial and temporal control of GARP recruitment is still unknown. Imh1 is a multiphosphorylated protein and has been reported to potentially interact with many cellular proteins. Further analysis of the regulation of Imh1 will improve our understanding of the mechanism of Arl1 activity and Imh1 function at the late Golgi.

## MATERIALS AND METHODS

### Strains, plasmids, antibodies, and reagents

The yeast strains used in this study are listed in Supplemental Table S1. The plasmids used in this study are listed in Supplemental Table S2. The pRS416-GFP-SNC1 and pRS416-GFP-SNC1-PM plasmids were generously provided by Todd R. Graham, Vanderbilt University, Nashville, TN. The pRS313-VPS52-GFP and pRS313-VPS53-GFP plasmids were kindly provided by Li-Ting, Jang, National Taiwan University, Taipei, Taiwan.

The following antibodies were used for Western blotting: Emp47 (1:5000), HA (1:5000; Covance, Princeton, NJ), Pma1

(1:5000), GFP (1:5000; Molecular Probes), and Arl1 (1:3000). The anti-Emp47, Pma1, and Arl1 antibodies were generated in our laboratory. The procedures of generating rabbit anti-Emp47 and anti-Arl1 antibodies were referred to in our previous studies (Li *et al.*, 2007; Chiang *et al.*, 2019). *Oryctolagus cuniculus* (female New Zealand white rabbits) were immunized with purified recombinant Emp47<sup>N200</sup> protein (N-terminal 200 residues of Emp47) or Arl1 protein, respectively. Rabbit anti-Pma1 antibodies were prepared against a synthetic peptide corresponding to residues 905–918 of Pma1 (AAMQRVSTQHEKET). All the animal experiments were conducted obeying the institutional guidelines that were approved by the Institutional Animal Care and Use Committee (IACUC), National Taiwan University.

### Plating assay for high temperature sensitivity

Mid-log phase yeast cells were spotted with 10-fold serial dilution onto synthetic medium plates. The plates were incubated at 30°C or 37°C for 2 d before imaging.

### Microscopy and fluorescent images analysis

Images of live cells containing fluorescent fusion proteins were obtained after overnight culture in synthetic medium with 2% (wt/vol) glucose. The mid-log phase cells were examined, and images were captured using a Zeiss Axioskop microscope equipped with a Cool Snap FX-camera. For all microscopic examinations, the exposure times and image processing procedures were identical for each sample within an experiment. The ratio of cells with membrane signal was determined following the previous reports (Hankins *et al.*, 2015; Xu *et al.*, 2017). The intensities of plasma membrane GFP-Snc1 were detected by ImageJ Fiji software and compared with total fluorescence. At least 100 cells were included in each experiment with three biological repeats. The cells containing Vps52-GFP or

Vps53-GFP punctate signals were measured using ImageJ Fiji software ( $n = 150$ , three biological repeats). To quantify the colocalization between mCherry-Tlg1 and Sec7-GFP or GFP-Sed5, the Pearson correlation coefficient was assessed by the ImageJ plug-in Just Another Colocalization Plugin with Costes Automatic Thresholding (Bolte and Cordelières, 2006; Xu et al., 2017).

### Sucrose density–gradient centrifugation

Wild-type or *ypt6Δ* yeast cells exogenously expressing full-length Imh1 or Arl1 were cultured in 50 ml of minimal medium to mid-log phase ( $A_{600} \sim 1$ ). Approximately 30  $A_{600}$  units of yeast cells was resuspended in 1 ml of 20 mM  $\text{NaN}_3$  and NaF and then incubated with a Zymolyase-100T mixture in 1 ml of K-Pi buffer (190 mM  $\text{K}_2\text{HPO}_4$ , 310 mM  $\text{KH}_2\text{PO}_4$ , and 1.2 M sorbitol) containing  $\beta$ -mercaptoethanol to form spheroplasts at 30°C. Spheroplasts were lysed in 5 ml of cold lysis buffer (20 mM HEPES [pH 6.8], 1 mM EDTA [pH 8.0], 50 mM KOAc, 100 mM sorbitol, 1 mM dithiothreitol [DTT], and a protease inhibitor cocktail) with a nitrogen bomb. After  $600 \times g$  centrifugation for 10 min, equal amounts of clarified lysates were layered on top of a manually generated sucrose gradient (stacked with a 20–60% sucrose mixture with 500  $\mu\text{l}$  of each layer in a 5% interval) diluted in dilution buffer (40 mM HEPES [pH 6.8], 2 mM EDTA [pH 8.0], 100 mM KOAc), which was then subjected to centrifugation ( $179,640 \times g$  for 16 h at 4°C in a Beckman SW55-Ti rotor). Fractions were collected manually from the top, precipitated with 10% trichloroacetic acid, mixed with an SDS sample buffer, and analyzed by Western blot analysis.

### Protein interaction analysis

To analyze the interaction between Vps53 and Imh1, cells were exogenously transformed with GFP-tagged Vps53 and full-length Imh1 or N-terminal truncated Imh1. Cells were cultured in minimal medium containing 2% glucose lacking amino acids according to the selection of the plasmids. After overnight culture to mid-log phase, yeast cells (100  $A_{600}$  units) were harvested and washed with 1 ml of 20 mM  $\text{NaN}_3$  and NaF, lysed with glass beads in 1 ml of lysis buffer (50 mM Tris-Cl [pH 7.5], 50 mM NaCl, 10 mM  $\text{MgCl}_2$ , 1 mM EDTA [pH 8.5], 5% glycerol, 0.15% NP-40, and a protease inhibitor cocktail) at 4°C and subsequently centrifuged at  $3500 \times g$  for 10 min to collect clear cell lysate. The clarified cell lysates were then incubated with prewashed monoclonal anti-HA-agarose antibody (Sigma-Aldrich) for 2 h at 4°C and washed three times with lysis buffer. The bound proteins were then analyzed by Western blotting for the presence of GFP-tagged Vps53.

### Statistical analysis

All statistical data were analyzed by either paired *t* test or one-way analysis of variance (ANOVA) followed by Dunnett's post hoc multiple comparison tests using GraphPad Prism (GraphPad Software) with mean  $\pm$  SD. Significant statistical differences ( $***p < 0.001$ ) are indicated.

### ACKNOWLEDGMENTS

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