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SHORT REPORT

# Mechanism of action of the flippase Drs2p in modulating GTP hydrolysis of Arl1p

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## ABSTRACT

Small GTPase ADP-ribosylation factors (ARFs) are key regulators of membrane trafficking and their activities are determined by guanine-nucleotide-binding status. In *Saccharomyces cerevisiae*, Arl1p, an ARF-like protein, is responsible for multiple trafficking pathways at the Golgi. The GTP-hydrolysis activity of Arl1p is stimulated by its GTPase-activating protein Gcs1p, and binding with its effector Imh1p protects Arl1p from premature inactivation. However, the mechanism involved in the timing of Arl1p inactivation is unclear. Here, we demonstrate that another Arl1p effector, the lipid flippase Drs2p, is required for Gcs1p-stimulated inactivation of Arl1p. Drs2p is known to be activated by Arl1p and is involved in vesicle formation through its ability to create membrane asymmetry. We found that the flippase activity of Drs2p is required for proper membrane targeting of Gcs1p *in vivo*. Through modification of the membrane environment, Drs2p promotes the affinity of Gcs1p for the Golgi, where it binds to active Arl1p. Together, Imh1p and Drs2p modulate the activity of Gcs1p by timing its interaction with Arl1p, hence providing feedback regulation of Arl1p activity.

**KEY WORDS: ARF, GTPase, Flippase, Golgin, Trans-Golgi network**

## INTRODUCTION

ADP-ribosylation factors (ARFs) and ARF-like proteins (ARLs) are highly conserved small GTPases that function as major regulators of vesicular trafficking and cytoskeletal reorganization (Donaldson and Jackson, 2011). The functions of ARF proteins require cycling between inactive GDP-bound and active GTP-bound forms, which is regulated by guanine-nucleotide-exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Casanova, 2007; Spang et al., 2010). Upon activation, cytosolic GDP-bound ARFs are converted into the membrane-bound ARF-GTP form and trigger membrane recruitment or activation of various effectors. GTP hydrolysis is promoted by an ARF GAP to inactivate the ARF, followed by its release from the membrane to the cytosol. Therefore, the cycle between activation and inactivation enable ARFs to serve as molecular switches that transduce upstream signals to downstream effectors (Gillingham and Munro, 2007; Cherfils and Zeghouf, 2013). Although the mechanisms underlying the regulation of the activity of ARF GEFs and GAPs are largely unknown, the sensitivity of ARF GAPs to membrane curvature have been demonstrated *in vitro* (Bigay et al., 2005; Nie et al., 2006). However, the regulation of its activity *in vivo* is unclear.

The *Saccharomyces cerevisiae* protein Arl1p and its mammalian homolog ARL1, localize to the trans-Golgi network (TGN) and participate in multiple membrane trafficking pathways (Jackson, 2003; Panic et al., 2003; Liu et al., 2006). In yeast, Arl1p is activated by the GEF Syt1p and inactivated by its GAP, Gcs1p (Liu et al., 2005; Chen et al., 2010). Activated Arl1p localizes to the TGN and then recruits the golgin protein Imh1p to the Golgi and activates the flippase Drs2p, thereby promoting vesicle transport from the Golgi (Tsai et al., 2013). Previously, we found that Imh1p prolongs Arl1p activity through positive-feedback regulation through a steric effect that modifies Gcs1p binding to Arl1p (Chen et al., 2012). How and when Gcs1p is allowed to access and stimulate Arl1p-GTP hydrolysis remained unknown. Here, we provide direct evidence of how the phosphatidylserine flippase Drs2p affects Gcs1p activity leading to Arl1p inactivation. By altering the lipid environment, Drs2p facilitates Gcs1p targeting to the TGN and hence its interaction with Arl1p. We present a model where there is sophisticated feedback regulation of Arl1p activity involving its effector and regulatory proteins.

## RESULTS AND DISCUSSION

### Drs2p flippase activity is required for Gcs1p-dependent GTP hydrolysis by Arl1p

We previously reported that Imh1p modulates the rate of Gcs1p-mediated GTP hydrolysis of Arl1p; thus, most Arl1p dissociates from the TGN in *imh1Δ* cells owing to a reduction in GTP binding (Chen et al., 2012; Fig. 1A). The Drs2p activity is also indispensable for maintaining Imh1p at the Golgi (Tsai et al., 2013). However, the Imh1p loss on the Golgi in *drs2Δ* does not lead to Arl1p inactivation as in *imh1Δ* (Tsai et al., 2013; Fig. 1A). These data suggest a complicated feedback regulation between Arl1p and its effectors.

To address how Drs2p and Imh1p regulate Arl1p activity, we examined the GTP-GDP cycle of Arl1p by analysing its subcellular localization in *drs2imh1Δ* cells. As shown in Fig. 1A, Arl1p–mRFP still colocalized with the TGN marker GFP–Sft2p in *arl1drs2imh1Δ* cells, indicating that a similar amount of Arl1p remained in the GTP-bound state to that in the presence of Drs2p and Imh1p. We quantified the amount of Arl1p-GTP in these cells by using a pull-down assay for active Arl1p, utilizing the C-terminal region of Imh1p (GST–Imh1-C177), which binds specifically to GTP-bound Arl1p (Chen et al., 2010). Consistent with the localization results (Fig. 1A), significantly more GTP-bound Arl1p was present in *drs2imh1Δ* cells than in *imh1Δ* cells (Fig. 1B), indicating that Drs2p is required for Arl1p inactivation in *imh1Δ* cells.

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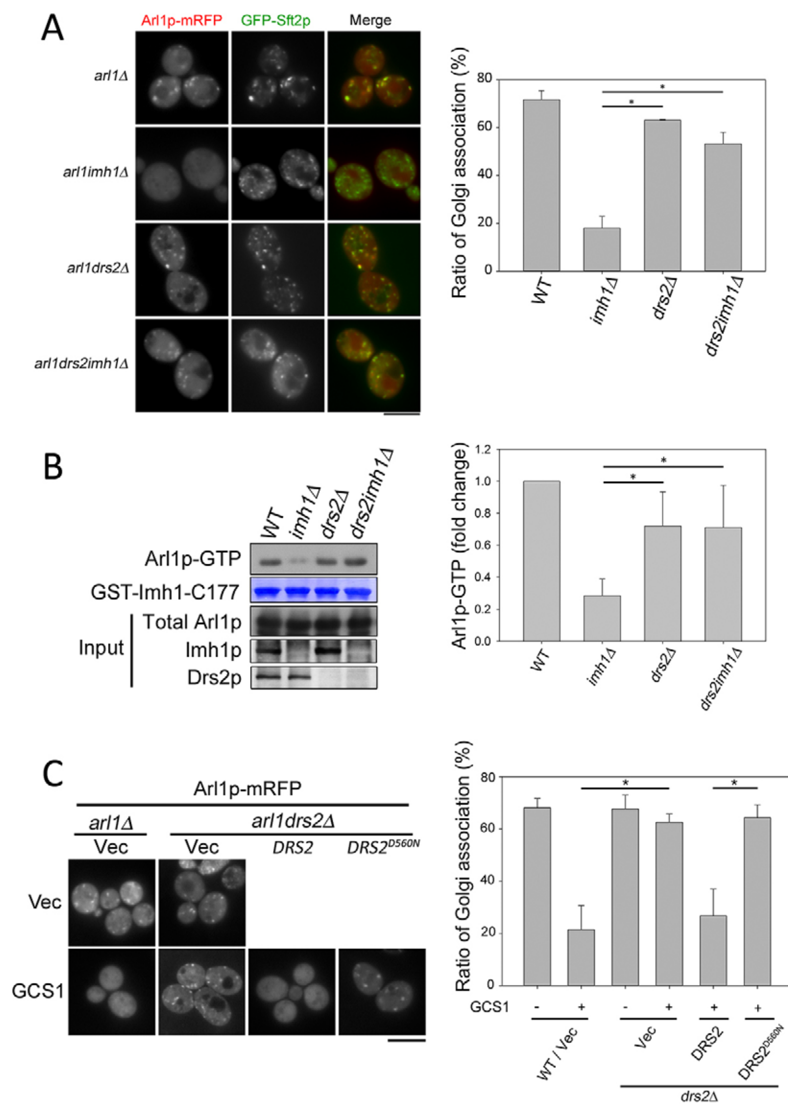


**Fig. 1. Drs2p is involved in the inactivation of Arl1p.** (A) *DRS2* depletion in *imh1*Δ cells alters the Arl1p Golgi localization. Arl1p–mRFP and GFP–Sft2p were co-expressed in different mutant yeasts as indicated. Cells in mid-log phase were observed by fluorescence microscopy. Punctuate signals of Arl1p–mRFP were quantified, summed and divided by the whole-cell signals (right panel; $n=200$). WT, wild type. (B) Arl1p-GTP pulldown assay with recombinant GST–Imh1-C177 and quantification of Arl1p-GTP pull-down normalized to signals from wild-type. (C) Gcs1p-mediated Arl1p inactivation depends on Drs2p flippase activity. Drs2p or Drs2p$^{D560N}$ was co-expressed with Gcs1p and Arl1p-mRFP in *arl1drs2*Δ. Arl1p-mRFP signals were observed by fluorescence microscopy. Punctuate signals of Arl1p–mRFP were quantified ($n=200$). Scale bars: 5 µm. The data are reported as the mean±s.d. of three experiments. *$P<0.05$

We have previously shown that Imh1p enhances Arl1p activation through attenuating the GAP activity of Gcs1p (Chen et al., 2012); therefore, we speculated that Drs2p participates in Gcs1p-stimulated Arl1p-GTP hydrolysis. To test this hypothesis, we examined the effect of Gcs1p overexpression in the presence or absence of Drs2p. Gcs1p is an Arl1p GAP, and Gcs1p overexpression promoted GTP hydrolysis of Arl1p, resulting in a diffuse cytosolic distribution of Arl1p–mRFP (Fig. 1C). Strikingly, when we ectopically expressed Gcs1p in *arl1drs2*Δ, Arl1p–mRFP retained its Golgi localization, indicating that Drs2p is required for Gcs1p-mediated inactivation of Arl1p (Fig. 1C). Furthermore, only wild-type Drs2p, not the flippase-inactive mutant Drs2p$^{D560N}$, which could not restore the cold sensitivity of *drs2*Δ (supplementary material Fig. S1), could rescue this phenotype (Fig. 1C). When Gcs1p was expressed in *arl1gea2*Δ cells, which have diminished Drs2p activity (Natarajan et al., 2009; Tsai et al., 2013), Arl1–mRFP localization phenocopied that seen in *arl1drs2*Δ (supplementary material Fig. S2). These data indicate that the Drs2p flippase activity is required for the Gcs1p-mediated inactivation of Arl1p.

### Drs2p is required for Gcs1p membrane targeting *in vivo*

How does Drs2p affect Gcs1p activity? Drs2p is a P-type ATPase localized at the TGN where it flips phosphatidylethanolamine and phosphatidylserine into the cytosolic membrane leaflet to generate membrane asymmetry and contribute to vesicle formation (Chen et al., 1999; Hua et al., 2002), whereas Gcs1p is a peripheral membrane protein with an amphipathic lipid-packing sensor (ALPS) motif, which is responsible for membrane binding and curvature sensing *in vitro* (Bigay et al., 2005). Therefore, we tested whether Drs2p could affect the subcellular localization of Gcs1p.

Gcs1p resides in both Golgi and endosomal compartments (Robinson et al., 2006), thus it partly colocalized with mRFP–Sft2p and the endosomal marker mCherry–Tlg1p (Fig. 2A,B). Strikingly, Gcs1p-GFP dramatically lost its colocalization with mRFP–Sft2p and mCherry–Tlg1p in *drs2*Δ cells (Fig. 2A,B). To quantify this effect, the distribution of endogenous Gcs1p was analyzed using sucrose gradient centrifugation. In wild-type cells, Gcs1p distributes between the cytosolic and light membrane fractions; however, Gcs1p was mainly cytosolic in the absence of Drs2p (Fig. 2C), suggesting that Drs2p might provide the proper membrane environment for Gcs1p targeting.

Gcs1p and its mammalian homolog ArfGAP1 possess ALPS motifs and bind avidly to highly curved membranes *in vitro* (Bigay et al., 2005). Similarly, we observed more Gcs1p bound to smaller liposomes using a flotation assay (supplementary material Fig. S3A,B). This result is consistent with the finding that Gcs1p

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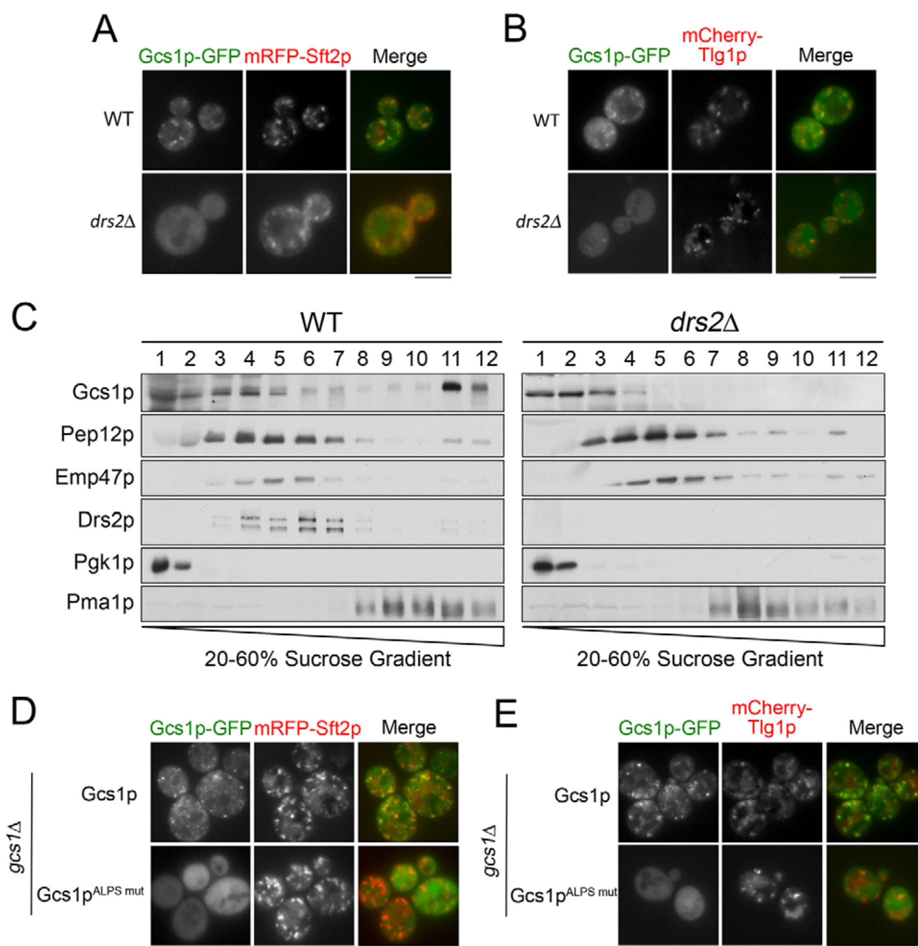


**Fig. 2. Drs2p is required for Gcs1p membrane targeting.** (A) Gcs1p–GFP localization in wild-type (WT) and *drs2Δ* cells also expressing mRFP–Sft2p. (B) Gcs1p–GFP localization in wild-type and *drs2Δ* cells also expressing mCherry–Tlg1p. (C) Sucrose gradient centrifugation analysis for cell lysates from wild-type and *drs2Δ* cells. Proteins from different fractions were analyzed using antibodies against Gcs1p, Drs2p (TGN marker), Pgk1p (cytosol marker), Pep12p (endosome marker) and Pma1p (plasma membrane marker). (D) Gcs1p–GFP or Gcs1p$^{\text{ALPS-mut}}$–GFP in *gcs1Δ* relative to mRFP-Sft2p. (E) Gcs1p–GFP or Gcs1p$^{\text{ALPS-mu}}$–GFP in *gcs1Δ* cells also expressing mCherry–Tlg1p. Scale bars: 5 µm.

preferentially binds liposomes <50 nm in diameter (Bigay et al., 2005). We also found that Gcs1 preferred to bind liposomes with a higher phosphatidylserine concentration (supplementary material Fig. S3D).

Three key residues in the ArfGAP1 ALPS motif, L207, W211 and F214, sense defects in lipid packing induced by membrane curvature and are essential for its membrane binding (Bigay et al., 2005). L246, W250 and F253 are the equivalent residues in Gcs1p, and their mutation to alanine residues (Gcs1$^{\text{ALPS-mut}}$) abolished the liposome binding of Gcs1p (supplementary material Fig. S3E). Moreover, Gcs1p$^{\text{ALPS-mut}}$–GFP lost its TGN- and endosomal-targeting ability *in vivo* (Fig. 2D,E). These data suggest that both membrane curvature and the phosphatidylserine asymmetry generated by Drs2p directly affect Gcs1p binding to the TGN and endosome, and that the Gcs1p ALPS motif is crucial.

### Membrane curvature determines Gcs1p binding and thus affects its GAP activity toward Arl1p

The membrane-curvature-sensitive GAP activity of Gcs1p towards Arf1p has been demonstrated *in vitro* (Bigay et al., 2005). However, it is unclear whether Gcs1p mediates Arl1p-GTP hydrolysis in response to membrane curvature. Using purified Gcs1 and myristoylated Arl1 to perform GAP assays with differently sized liposomes, we demonstrated that the GAP activity of Gcs1 towards Arl1 is also sensitive to membrane curvature (Fig. 3A). However, the membrane-binding-defective mutant Gcs1$^{\text{ALPS-mut}}$ lost its GAP activity even with highly curved liposomes (Fig. 3B). Notably, the association of myristoylated Arl1$^{\text{Q72L}}$ with liposomes is independent of membrane curvature (supplementary material Fig. S3C). Consistent with the curvature-dependent Gcs1p GAP activity towards Arl1p, Gcs1p$^{\text{ALPS-mut}}$ overexpression did not disrupt the Golgi localization of Arl1p–mRFP (Fig. 3C). To relate this observation to the Arl1p function on the Golgi, we examined the localization of Gas1p or Imh1p in cells overexpressing wild-type Gcs1p or Gcs1p$^{\text{ALPS-mut}}$. We also observed a Gas1p transport defect and Imh1p dispersion in Gcs1p-overexpressing yeast, but not in Gcs1p$^{\text{ALPS-mut}}$-expressing cells (supplementary material Fig. S4).

These biochemical and cell biological results indicate that the primary role of Drs2p in this pathway is to create a membrane environment that recruits Gcs1 to the Golgi. To determine whether membrane curvature has an additional role in regulating GAP activity, Gcs1p where the ALPS domain was swapped for a PH or FYVE domain (Gcs1p$^{\text{ALPS::PH}}$ and Gcs1p$^{\text{ALPS::FYVE}}$, respectively) were expressed in *drs2arl1Δ* cells to target these chimera to different compartments independently of Drs2p (Xu et al., 2013). We found that expression of the endosome-targeted Gcs1p$^{\text{ALPS::FYVE}}$, which binds to phosphatidylinositol 3-phosphate (PI3P), like Gcs1p$^{\text{ALPS-mut}}$, did not affect the Golgi localization of Arl1p–mRFP in *drs2arl1Δ* cells. However, Golgi-localized Gcs1p$^{\text{ALPS::PH}}$, which binds to phosphatidylinositol 4-phosphate (PI4P), induced Arl1p–mRFP dissociation from the Golgi (Fig. 3D). Our findings suggest that the GAP activity of Gcs1p on Arl1p-GTP hydrolysis is determined by the membrane curvature, which promotes their interaction.

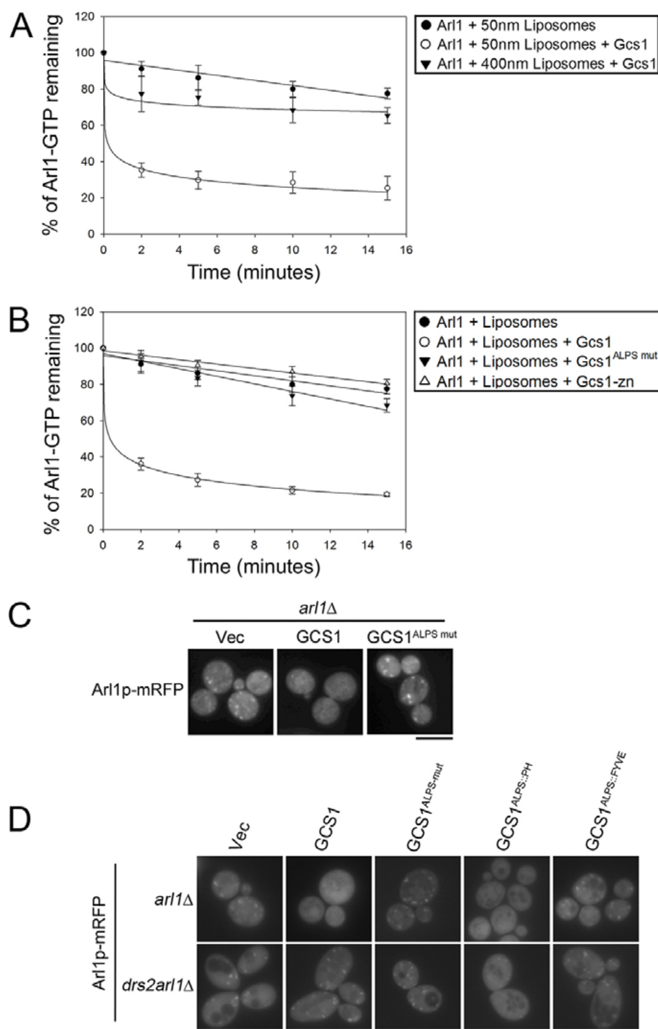


**Fig. 3. Gcs1p targeting to lipid membranes is required for Arl1p-GTP hydrolysis both *in vitro* and *in vivo*.** (A) Gcs1p GAP activity is sensitive to the membrane curvature. Recombinant myristoylated (myr-)Arl1 loaded with [$\gamma^{32}$P]GTP was incubated with liposomes of 50- or 400-nm diameter. After a 45-minute incubation, purified His-tagged Gcs1 was added to the reactions. Samples were collected at the indicated times. (B) Gcs1$^{ALPS\text{-}mut}$ does not exhibit GAP activity toward Arl1-GTP. Recombinant myr-Arl1 loaded with [$\gamma^{32}$P]GTP was incubated with 50-nm liposomes. After a 45-minute incubation, recombinant His–Gcs1, His–Gcs1-zn, a zinc-finger mutation which cannot bind to Arl1p (Liu et al., 2005), or His–Gcs1$^{ALPS\text{-}mut}$ was added. (C) Expression of Gcs1p, but not Gcs1p$^{ALPS\text{-}mut}$, in *gcs1Δ* cells leads to dissociation of Arl1p from the Golgi. Gcs1p or Gcs1p$^{ALPS\text{-}mut}$ was expressed in yeast with Arl1p–mRFP. Mid-log phase cells with Arl1p–mRFP signals were observed using fluorescence microscopy. (D) The indicated Gcs1 expression constructs were co-expressed with ARL1–mRFP in *arl1Δ* and *arl1drs2Δ* cells. Mid-log phase cells with Arl1p-mRFP signals were observed by fluorescence microscopy. Scale bars: 5 µm. Data are reported as the mean±s.d. of the percentage of [$\gamma$-$^{32}$P]GTP hydrolysis in three experiments.

### The Drs2p-regulated membrane environment modulates the Gcs1p–Arl1p interaction *in vivo*

Because we found that membrane curvature modulates the GAP activity of Gcs1p, we speculated that the underlying mechanism involved facilitation of the interaction between Gcs1p and Arl1p. In yeast two-hybrid assays, we found that both Gcs1p and Gcs1p$^{ALPS\text{-}mut}$ interacted with the constitutively active Arl1p-dN17$^{Q72L}$, but not the inactive Arl1p-dN17$^{T31N}$ (Fig. 4A), consistent with the finding that Gcs1p uses its zinc-finger motif to bind to active Arl1p (Liu et al., 2005). However, immunoprecipitation indicated that the interaction between Gcs1p$^{ALPS\text{-}mut}$ and Arl1p *in vivo* was diminished, suggesting that Gcs1p targeting to Golgi is essential for its interaction with Arl1p (Fig. 4B). Similarly, Gcs1p and Arl1p interaction is also largely disrupted in *drs2Δ* and *gea2Δ* cells (Fig. 4C). These data demonstrate that Drs2p-mediated membrane conditions regulate Gcs1p binding to the lipid membranes and in turn affect the interaction with Arl1p.

### Feedback regulation for Arl1p activation

Our study provides a missing piece of puzzle for the regulation of Arl1p activity on the Golgi. As Fig. 4D illustrates, (1) activated Arl1p-GTP binds to the Golgi and recruits or activates effectors to promote vesicle formation. (2) Specifically, Arl1p directly interacts and activates Drs2p flippase activity by forming an Arl1p–Gea2p–Drs2p complex to create a proper membrane environment for vesicle maturation and Imh1p targeting. Membrane-bound Imh1p interacts with active Arl1p and impedes GTP hydrolysis through steric protection from Gcs1p. (3) When the vesicle is mature and membrane curvature is established, substantial amounts of Gcs1p bind to this curved membrane micro-domain. Gcs1p might access Arl1p through a stochastic process or by curvature-induced Imh1p-disassociation to stimulate Arl1p inactivation. Our model illustrates that the Drs2p-mediated membrane environment controls Gcs1p membrane association. Lack of *DRS2* in yeast cells might slow the GAP activity of Gcs1p towards Arl1p owing to the loss of Gcs1p membrane binding.

Recently, it was reported that Drs2p is required for Gcs1p localization to the TGN and early endosomes, thus affecting Snc1p recycling (Xu et al., 2013). Although similar in principle, here, we provide further information regarding how Drs2p affects Gcs1p membrane binding, but not its GAP activity, and the subsequent effect on GTP hydrolysis of Arl1p through improvement of the association. Importantly, our data indicate that a sophisticated feedback regulation mediated by effector activity and binding, through Drs2p and Imh1p, determines the GTP-GDP switch of Arl1p on the Golgi. This kind of feedback regulation is also likely in other ARFs and small GTPases.

## MATERIALS AND METHODS

### Plasmids, strains and yeast culture

Standard protocols were used to generate yeast strains and culture yeast (Longtine et al., 1998). The yeast strains were transformed using the lithium acetate method (Ito et al., 1983). The plasmids and yeast strains used in the studies are listed in the supplementary material Tables S1, S2.

### Microscopy

All images of living cells were obtained after growth in synthetic medium to mid-log phase. Fluorescence microscopy was performed with a Zeiss Axioskop microscope and Cool Snap FX camera. Quantification of the ratio of Golgi association for Arl1p and Sft2p was calculated by measuring the fluorescence intensity values (arbitrary units) using Axio Vision Rel. 4.2 software by (1) measuring the punctate structure signals (PSS) >0.2 µm in diameter; (2) defining the PSS of Arl1p and Sft2p; and (3) calculating the ratio of Golgi association as a percentage of the fluorescence of Arl1p and Sft2p. Measurements were performed on images acquired with the same microscope settings.

### Liposomes preparation

Liposomes used in flotation and GAP activity assays were produced by extrusion (Bigay et al., 2005; Liu et al., 2005). The composition of Golgi-mix liposomes was (molar percentages) dioleoylphosphatidylcholine (DOPC, 65%), dioleoylphosphatidylethanolamine (DOPE, 20%),

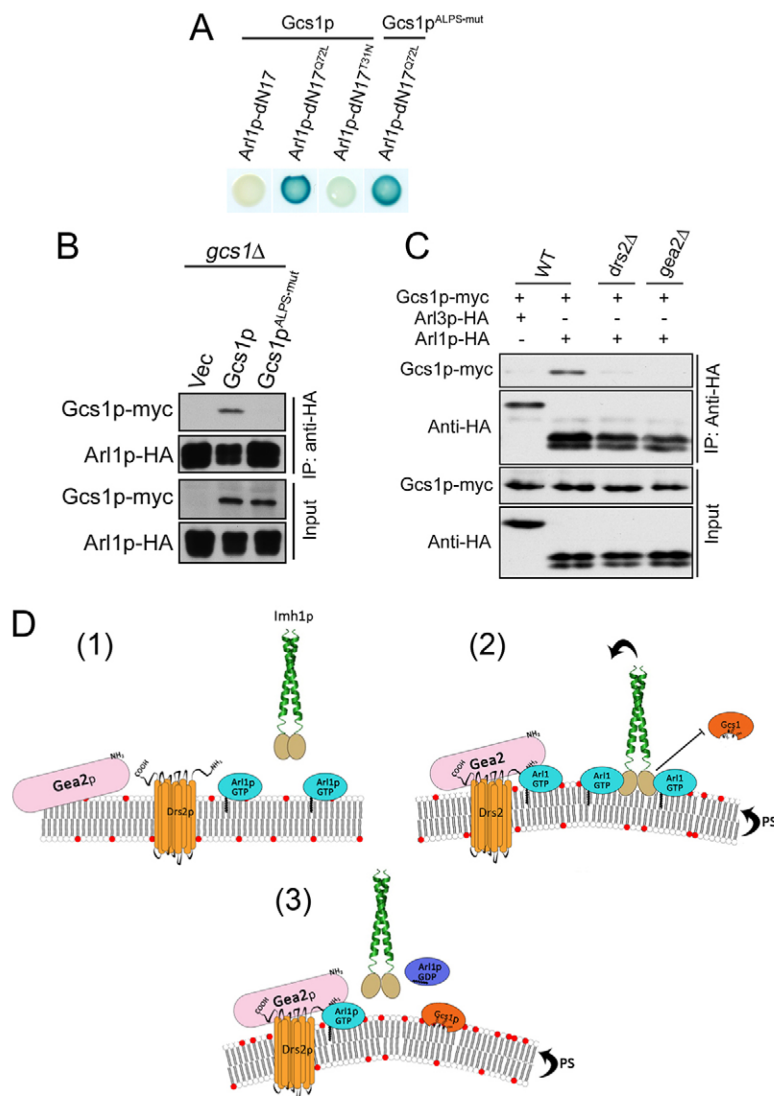


**Fig. 4. Drs2p-mediated membrane curvature and the ALPS motif of Gcs1p contribute to the association of Gcs1p with Arl1p.** (A) Gcs1p$^{ALPS\text{-}mut}$ interacts with Arl1p-dN17$^{Q72L}$ in a yeast two-hybrid assay. Various ARL1-dN17 constructs (wild-type, Q72L or T31N) and *GCS1* or *GCS1$^{ALPS\text{-}mut}$* were co-transformed into the reporter strain YEM1α and analyzed for β-galactosidase activity. (B) Gcs1$^{ALPS\text{-}mut}$–Arl1p interaction is disrupted *in vivo*. Gcs1p or Gcs1p$^{ALPS\text{-}mut}$ was co-expressed with Arl1p–HA, and co-immunoprecipitation was analyzed. (C) Gcs1p–Arl1p interaction in *drs2*Δ cells. Gcs1p and Arl proteins were expressed in cells as indicated. HA-tagged Arl1p or Arl3p was immunoprecipitated and assayed for Gcs1p binding. (D) Model for Arl1p inactivation. Activated Arl1p recruits Imh1p to the Golgi, which protects Ar1p from premature inactivation; Arl1p stimulates Drs2p flippase activity, thus providing a proper lipid environment for Gcs1p binding, leading to Arl1p-GTP hydrolysis. See text for further details.

dioleoylphosphatidylserine (DOPS, 10%) and phosphatidylinositol 4-phosphate (PI4P, 5%).

### GAP activity assay

The Gcs1 GAP activity assay was performed as described previously (Bigay et al., 2005; Liu et al., 2005). In the GAP activity assay with liposomes, liposomes with different diameters were pre-incubated with myristoylated-Arl1-bound [γ-$^{32}$P]GTP for 45 minutes at 30℃. Data are reported as the mean±s.d. of three experiments.

### Lipid flotation assay

The lipid flotation assay was performed as described previously (Bigay et al., 2005). Liposome–protein samples were added to the bottom of a centrifugation tube and layered sequentially with 25% and 0% sucrose. The samples were centrifuged at 240,000 ***g*** for 1.5 hours using a swinging bucket rotor. Fractions were collected, resuspended in SDS sample buffer and analyzed by western blotting.

### Biochemical experiments

Immunoprecipitation, active Arl1p pull-down, yeast two-hybrid assays, and sucrose gradient were performed as described previously (Huang et al., 2002; Liu et al., 2005; Chen et al., 2012).

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### Competing interests

The authors declare no competing interests.

### Author contributions

Z.-J.C., J.-W.H., Y.-W.L. and F.-J.S.L. designed the study and interpreted the results. Z.-J.C. and J.-W.H. performed the experiments and analyzed the data. J.-W.H., Y.-W.L. and F.-J.S.L. wrote and edited the manuscript.

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### Supplementary material

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.143057/-/DC1

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