Control Systems of Membrane Transport at the Interface between the Endoplasmic Reticulum and the Golgi

Jorge Cancino,[1,2,6,*] Anita Capalbo,[1,2] Antonella Di Campli,[1] Monica Giannotta,[3,7] Riccardo Rizzo,[1,2] Juan E. Jung,[1,4] Rosaria Di Martino,[1] Maria Persico,[1,4] Petra Heinklein,[5] Michele Sallese,[3] and Alberto Luini[1,2,*]

[1]Istituto di Biochimica delle Proteine, Consiglio Nazionale delle Ricerche, Via Pietro Castellino 111, 80131 Napoli, Italy
[2]Telethon Institute of Genetics and Medicine, Via Pietro Castellino 111, 80131 Napoli, Italy
[3]Consorzio Mario Negri Sud, Via Nazionale 8/A, 66030 Santa Maria Imbaro (Chieti), Italy
[4]Istituto di Ricovero e Cura a Carattere Scientifico, Istituto di Ricerca Diagnostica e Nucleare (SDN), 80143 Napoli, Italy
[5]Institut für Biochemie Charité, Universitätsmedizin Berlin, CrossOver Charitéplatz 1/Sitz, Virchowweg 6, 10117 Berlin, Germany
[6]Departamento de Ciencias Biológicas, Facultad de Ciencias Biológicas, Universidad Andrés Bello, Quillota 980, Viña del Mar 2520000, Chile
[7]Present address: Unit of Vascular Biology, FIRC Institute of Molecular Oncology Foundation, 20139 Milan, Italy
*Correspondence: jorge.cancino@unab.cl (J.C.), a.luini@ibp.cnr.it (A.L.)
http://dx.doi.org/10.1016/j.devcel.2014.06.018

SUMMARY

A fundamental property of cellular processes is to maintain homeostasis despite varying internal and external conditions. Within the membrane transport apparatus, variations in membrane fluxes from the endoplasmic reticulum (ER) to the Golgi complex are balanced by opposite fluxes from the Golgi to the ER to maintain homeostasis between the two organelles. Here we describe a molecular device that balances transport fluxes by integrating transduction cascades with the transport machinery. Specifically, ER-to-Golgi transport activates the KDEL receptor at the Golgi, which triggers a cascade that involves Gs and adenylyl cyclase and phosphodiesterase isoforms and then PKA activation and results in the phosphorylation of transport machinery proteins. This induces retrograde traffic to the ER and balances transport fluxes between the ER and Golgi. Moreover, the KDEL receptor activates CREB1 and other transcription factors that upregulate transport-related genes. Thus, a Golgi-based control system maintains transport homeostasis through both signaling and transcriptional networks.

INTRODUCTION

A fundamental property of complex cellular functions is to maintain optimal activity in the face of exogenous and endogenous perturbations (Kitano, 2007; Stelling et al., 2004). It is therefore desirable to elucidate the mechanisms that underlie cellular homeostasis and robustness at the molecular and design level. Among the main cellular functions, protein folding in the endoplasmic reticulum (ER) and the cell cycle are well understood in this regard (Csikász-Nagy et al., 2006; Walter and Ron, 2011), whereas others, including membrane traffic, remain relatively unexplored.

Membrane traffic is a fundamental process by which secretory proteins are transported from their site of synthesis, the ER, through a series of anatomically separated membranous compartments until they reach their cellular destinations in correctly processed forms. Transport involves large membrane and protein fluxes across the transport stations, which can be subject to physiological and pathological perturbations as well as to spontaneous drifts away from equilibrium (Hirschberg et al., 1998; Mironov et al., 2001; Pulvirenti et al., 2008; Trucco et al., 2004). Such perturbations can be a serious threat to the homeostasis of the transport compartments, particularly if they occur at the interface between the ER and the Golgi complex. The ER is the largest transport organelle (Griffiths et al., 1984), and its membrane output is massive compared to the size of the receiving station, the Golgi complex (Griffiths et al., 1984; Klumperman, 2000; Martínez-Menárguez et al., 1999; Thor et al., 2009; Wieland et al., 1987). Thus, even minor changes in export from the ER would cause profound alterations in Golgi morphology and composition, and ultimately in the whole transport apparatus, if they were not compensated for by corresponding changes in retrograde transport from the Golgi to the ER (Klumperman, 2000; Martínez-Menárguez et al., 1999; Thor et al., 2009; Wieland et al., 1987) (see legend to Figures 7F and 7G; Figures S7A and S7B available online). The fact that the Golgi maintains or recovers its normal composition and morphology even when subjected to major challenges (Mironov et al., 2001; Trucco et al., 2004) provides evidence that efficient homeostatic mechanisms do exist. The nature of such mechanisms, however, remains poorly understood. Previous reports have indicated that protein kinase C (PKC) (De Matteis et al., 1993) and PKA modulate the secretory pathway in various ways (Bejarano et al., 2006; Martin et al., 2000; Mavillard et al., 2010; Muñiz et al., 1996, 1997) and that numerous kinases can affect the morphology and function of the Golgi (Chia et al., 2012), but whether these effects are involved in the maintenance of homeostasis is unknown.

We have previously proposed that the homeostasis of the transport organelles depends not only on the stoichiometry and self-assembly of the traffic machinery components (Gong et al., 2008; Heinrich and Rapoport, 2005; Sengupta and

Linstedt, 2011) but also, or mainly, on dedicated signaling circuits that monitor and govern transport rates across organelles (Giannotta et al., 2012; Pulvirenti et al., 2008). Specifically, we have reported the existence of a regulatory device based on the signaling properties of the KDEL receptor (KDELR), a seven-transmembrane-domain protein that resides in the intermediate compartment (IC) and the *cis*-Golgi (Giannotta et al., 2012; Pulvirenti et al., 2008). The KDELR is a member of a protein family (the PQ-loop family) (Saudek, 2012) that is distantly related to the G-protein-coupled receptor (GPCR) superfamily (Yee et al., 2013; Zhai et al., 2001) and resembles GPCRs in topology and fold of the transmembrane helices (Giannotta et al., 2012). It has two main transport functions: (1) it binds to and recycles chaperones that escape the ER and reach the Golgi during traffic back to the ER, and (2) it is required for intra-Golgi traffic both in yeast (Semenza et al., 1990) and in mammals (Lewis and Pelham, 1990). The latter role, at least in mammals, is mediated by a signaling reaction (Pulvirenti et al., 2008). When the KDELR binds to the KDEL C-terminal tail of chaperones, it is activated and stimulates a Golgi pool of the heterotrimeric G proteins, Gq (Giannotta et al., 2012), which stimulates a Golgi pool of Src-family kinases, which then in turn activate anterograde traffic through the Golgi (Giannotta et al., 2012; Pulvirenti et al., 2008). Thus, this KDELR-Gq-Src cascade coordinates intra-Golgi with ER-to-Golgi traffic. However, it does not activate retrograde transport. We have therefore searched for mechanisms that would regulate retrograde transport to the ER and maintain Golgi homeostasis.

Here we report that anterograde traffic fluxes from the ER to the Golgi activate another KDELR-dependent Golgi-localized signaling pathway that involves stimulatory heterotrimeric G protein (Gs) as well as specific adenylyl cyclases and phosphodiesterases and culminates in the activation of a Golgi pool of PKA. PKA phosphorylates several proteins, some of which are involved in traffic, and activates retrograde transport from the Golgi to the ER. This is likely to contribute significantly to the traffic balance between these two organelles. Additionally, PKA regulates the Gq-Src pathway and activates cAMP response element binding protein 1 (CREB1) and other transcription factors, which upregulate several hundreds of secretory machinery-related genes, presumably to prepare the transport apparatus to sustain a prolonged increase in traffic load. Thus, the KDELR-Gs-PKA axis is a Golgi-based cell-autonomous device, or control system, that couples with components of the transport apparatus and uses both signaling and transcriptional networks to maintain homeostasis at the ER-Golgi interface.

RESULTS

The goal of this study was to generate a complete outline of the control system that couples anterograde ER-to-Golgi transport with retrograde transport from the Golgi. Considering that (1) the KDELR coimmunoprecipitates with Gs (Giannotta et al., 2012); (2) Gs regulates PKA (Cooper, 2003); and (3) Gs and PKA localize at the Golgi (Maier et al., 1995; Nigg et al., 1985b), we examined whether the Gs-PKA pathway might be involved in the regulation of retrograde traffic.

ER-to-Golgi Traffic Activates a Canonical Gs-PKA Signaling Pathway at the Golgi Complex

Quiescent PKA comprises two catalytic (PKAcat) and two regulatory (PKAreg) subunits that are stably bound to the Golgi complex (Nigg et al., 1985a). When cAMP binds PKAreg subunits, the PKAcat subunits detach and become enzymatically active (Nigg et al., 1985a, 1985b). Thus, the dissociation of PKAcat from the Golgi is an index of PKA activation. To generate a traffic pulse, we used the synchronizable temperature-sensitive mutant of the vesicular stomatis virus G glycoprotein (tsO45VSVG; hereafter VSVG) in Cos7 cells (Mironov et al., 2001; Pulvirenti et al., 2008). The induction of a traffic pulse decreased the Golgi:total PKAcat ratio, which indicates that PKA was activated (Figure 1A). This PKA activation was confirmed by a Förster resonance energy transfer (FRET)-based method (Zaccolo and Pozzan, 2002) by the use of fluorescently tagged PKA subunits (Figure S1A). Further experiments were carried out using the Golgi PKA total:cat ratio, because this did not require transfection of PKA subunits, which might affect PKA activation dynamics.

PKA requires cAMP for activation and phosphorylates numerous proteins. We found that a traffic pulse induces a transient cAMP increase in the Golgi area (Figure 1B; Figures S1B and S1C) in line with the notion that cAMP can be spatially restricted by local mechanisms (Zaccolo and Pozzan, 2002) and enhances the PKA-specific phosphorylation of several proteins at the Golgi (Figure S1D). These collective data provide evidence that transport controls cAMP production and PKA activation at the Golgi.

Gs activates adenylyl cyclases, which produce cAMP, which is degraded by phosphodiesterases (Cooper, 2003). Silencing Gs small interfering RNAs (siRNAs) drastically reduced traffic-induced PKA activation (Figure 1C). To inhibit Gs, we also expressed a Gs "minigene," peptides designed to inhibit the activation of specific G proteins (Feldman et al., 2002; Gilchrist et al., 2002). The Gs minigene inhibited the traffic-induced PKA activation, whereas the Gq minigene had no effect (not shown). We also sought to acutely inhibit Gs using the Gs minigene fused with a membrane-permeant octaarginine "carrier" peptide (R8-Gs) (D'Ursi et al., 2006; Mazzoni et al., 2000). These polybasic peptides and related constructs are well characterized and nontoxic (Heitz et al., 2009; Verdurmen and Brock, 2011). R8-Gs inhibited traffic-dependent PKA activation (Figure 1D), whereas R8-Gq and R8-scrambled Gs were without effect (not shown). Gs was selectively activated at the Golgi by a traffic pulse (Figure S1E). Thus, different approaches based on Gs silencing, transfected and membrane-permeant Gs minigenes, and immunofluorescence and biochemical assays converge to indicate that Gs is required for traffic-induced Golgi PKA activation at the Golgi complex.

We then examined the role of the adenylyl cyclases (ACs) and phosphodiesterases (PDEs). There are nine AC isoforms, three of which are ubiquitous (AC4, AC7, and AC9) (Sunahara et al., 1996). We silenced the ubiquitous ACs and found that only the depletion of AC9 inhibited traffic-induced Golgi PKA activation (Figure 1E) and cAMP increase (not shown). We also determined that AC9 resides at the Golgi (Figure 1E). This is the first AC with a described role at the Golgi. There are 11 PDE isoforms (Francis et al., 2011), of which one, PDE7A1, localizes at the Golgi and regulates cAMP levels and PKA by both conventional and unconventional mechanisms (Han et al., 2006; Mavillard et al., 2010). The overexpression of PDE7A1 inhibited the
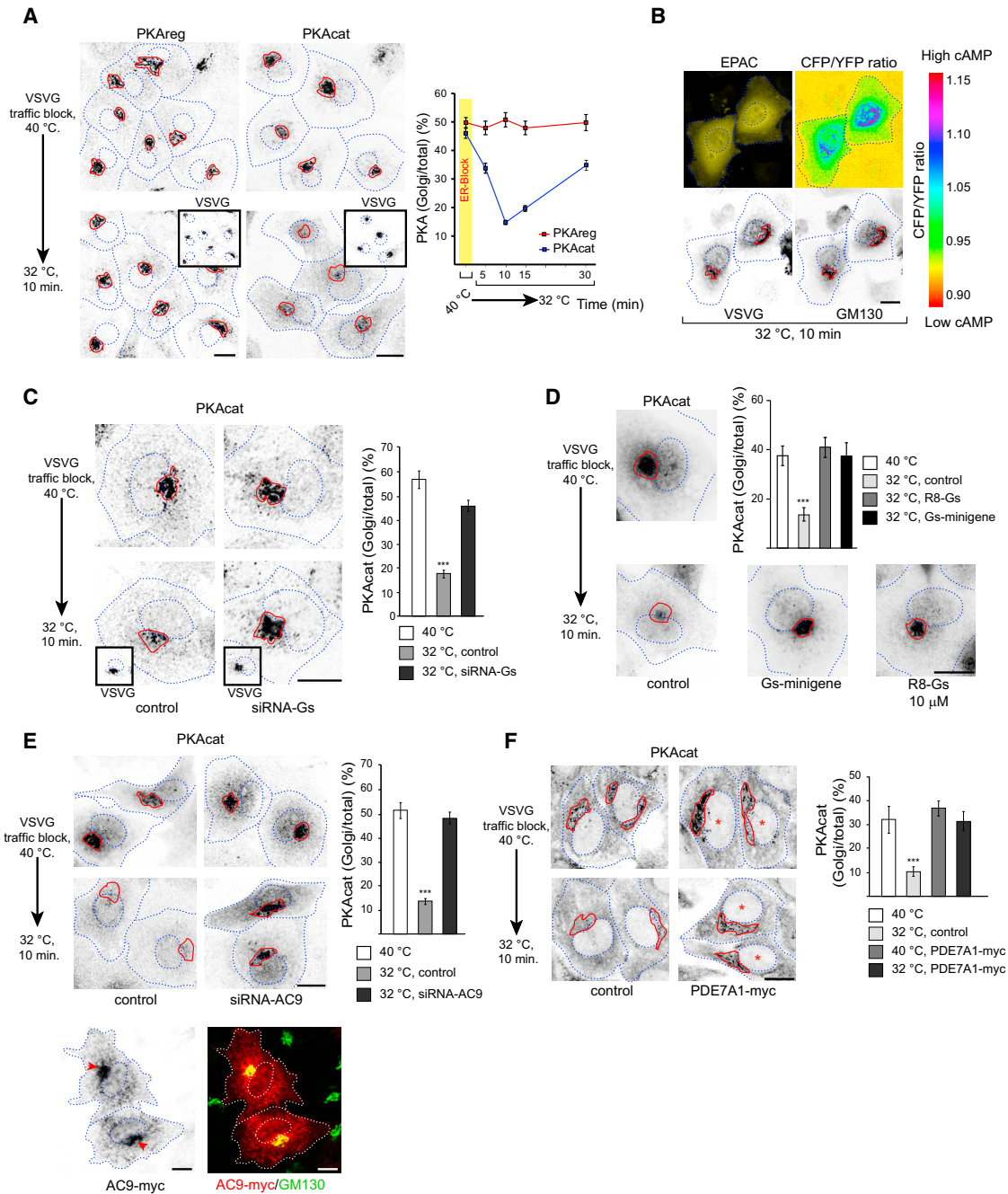
Figure 1. Synchronized Traffic Waves Activate PKA at the Golgi

(A) Cos7 cells were subjected to a VSVG traffic pulse and the fluorescence signal of the catalytic (PKAcat) and regulatory (PKAreg) PKA at the Golgi was quantified. The PKA immunofluorescence signal is shown in grayscale with inverted contrast (lower panels) to facilitate the appreciation of dim structures. The Golgi region in this figure and in all other figures is outlined by a solid red line.

(B) Cells expressing CFP-EPAC-(dDEP)-YFP and VSVG-mCherry. cAMP levels are shown by a pseudocolored image of the CFP:YFP ratio.

(C) Cos7 cells treated with siRNAs against Gs.

(D) Cos7 cells expressing the Gs minigene or treated with R8-Gs peptides.

(E) Control (mock) and AC9 siRNA-treated Cos7 cells. Cos7 cells expressing AC9-myc.

(F) HeLa cells expressing PDE7A1-myc.

All data represent means ± SEM (n = 10–30 cells). ***p < 0.001 (Student's t test). Scale bars represent 10 μm. See also Figure S1.

traffic-induced activation of Golgi PKA (Figure 1F), presumably reflecting enhanced degradation of the traffic-induced increase in cAMP levels at the Golgi. These data indicate that AC9 and PDE7A1 play a key role in the traffic-induced PKA activation at the Golgi (although they do not exclude the involvement other ACs and PDEs in this process).
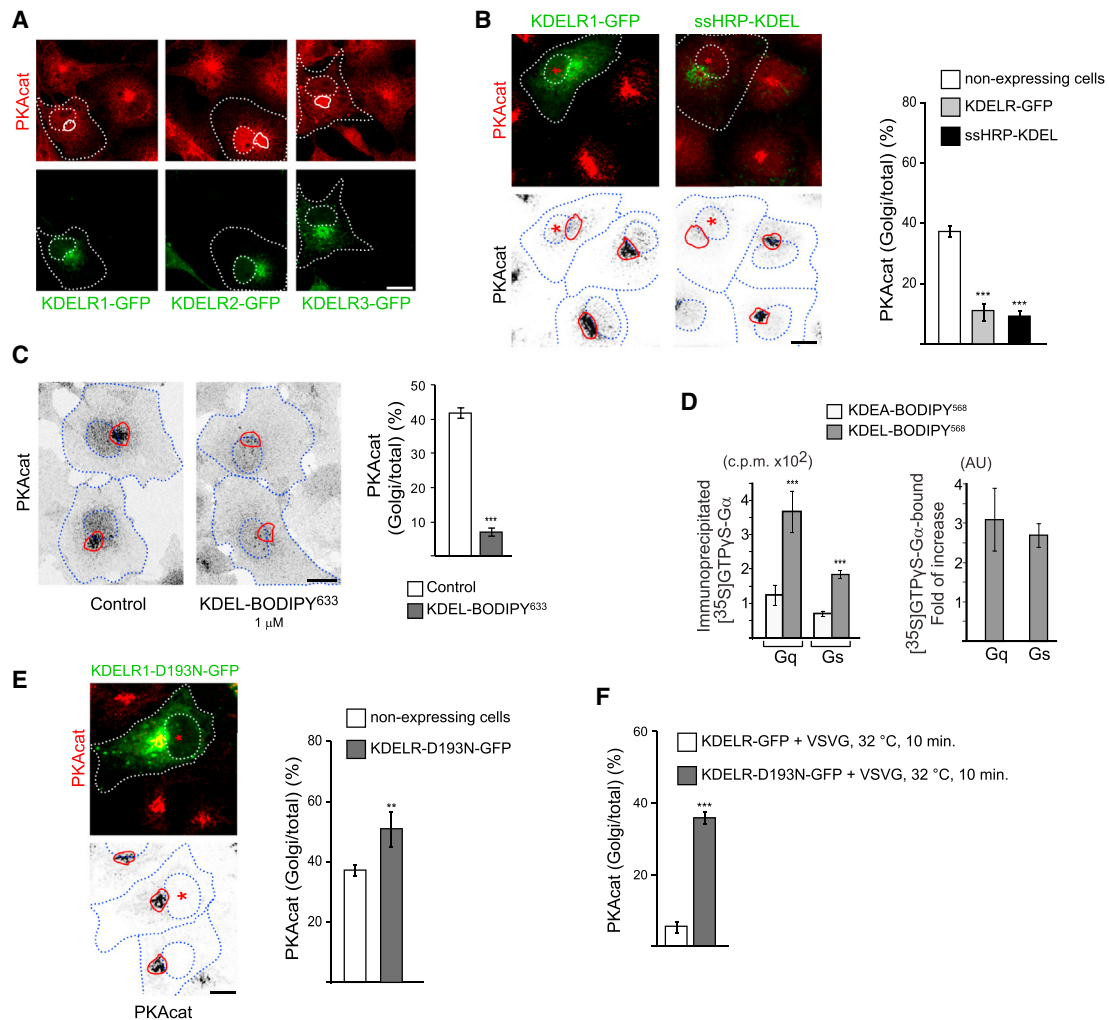
Figure 2. The KDELR Mediates the Traffic-Induced Activation of Gs and PKA at the Golgi

(A) Cos7 cells expressing KDELR1-GFP, KDELR2-GFP, or KDELR3-GFP.

(B) Cos7 cells expressing KDELR1-GFP or KDEL-containing cargo (ssHRP-KDEL).

(C) Cos7 cells incubated with KDEL-BODIPY^{633} peptide (1 μM) for 30 min.

(D) Golgi-enriched membranes from rat liver incubated with [^{35}S]GTPγS and KDEL-BODIPY^{568} peptide (1 μM) or KDEA-BODIPY^{568} peptide (1 μM). AU, arbitrary units.

(E) Cos7 cells microinjected to express KDELR1-D193N-GFP.

(F) Cos7 cells comicroinjected to express either KDELR-GFP or KDELR1-D193N-GFP with VSVG-mCherry.

All data represent means ± SEM (B, n = 40 cells; C, n = 20 cells). **p < 0.01; ***p < 0.001 (Student's t test). Scale bars represent 10 μm. See also Figure S2.

To control for possible temperature effects on PKA activation, we induced traffic pulses using a human growth hormone construct (hGH-GFP-FM) that is retained in the ER and synchronously released in a temperature-independent fashion (Gordon et al., 2010). hGH-GFP-FM traffic pulse activated both Gs and PKA at the Golgi (Figure S1F). Thus, the traffic-induced activation of the Gs-PKA pathway is independent of temperature shifts. Notably, these results indicate that the Golgi Gs-PKA signaling pathway can be activated by different cargoes. To further test this notion, we used another cargo, the synchronizable cargo procollagen I, to induce traffic pulses (Bonfanti et al., 1998), and found similar effects on the Gs-PKA pathway (not shown). These results indicate that the induction of this pathway by transport is a general phenomenon, although they cannot formally exclude that other cargo types might induce different behaviors.

The Traffic-Induced Activation of Golgi PKA Is Mediated by the KDELR

The KDELR "autoactivates" when overexpressed (Hsu et al., 1992), and can be activated by traffic pulses (Pulvirenti et al., 2008) or by KDEL-bearing ligands (Lewis and Pelham, 1992; Majoul et al., 2001; Pulvirenti et al., 2008; Townsley et al., 1993). The overexpression (Aoe et al., 1997; Hsu et al., 1992; Pulvirenti et al., 2008) of the KDELR1 isoform strongly activated Golgi PKA. KDELR2 was less potent, and KDELR3 was inactive (Figure 2A). Both artificial KDEL-bearing ligands (Giannotta et al., 2012; Pulvirenti et al., 2008) ssHRP^{KDEL} (Figure 2B) and
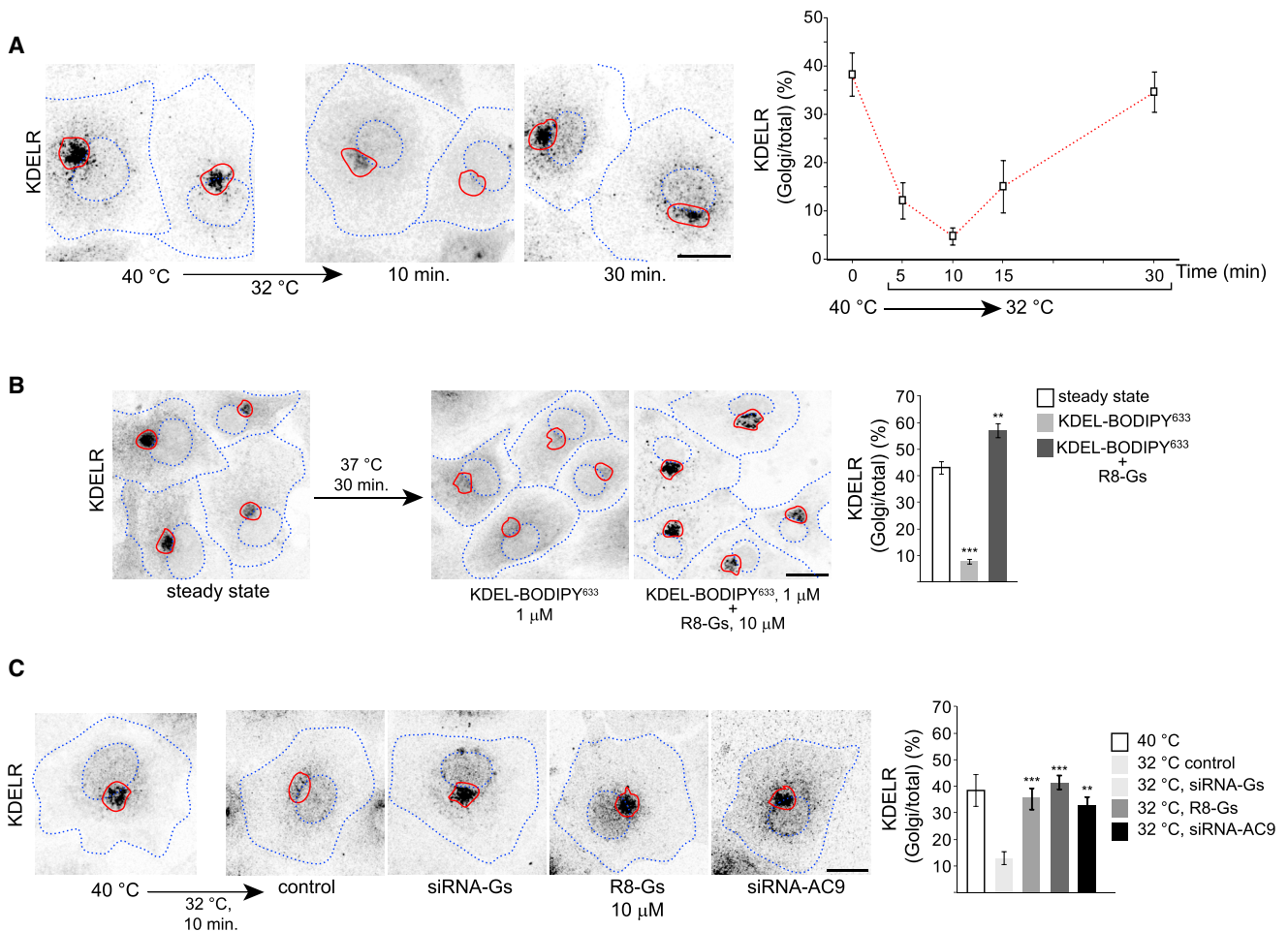
Figure 3. Traffic-Induced Activation of the Gs-PKA Pathway Stimulates the Retrograde Transport of the KDELR
(A) Cos7 cells subjected to a traffic pulse.
(B) Cos7 cells incubated with KDEL-BODIPY[633] and R8-Gs peptide.
(C) Cos7 cells depleted of Gs or AC9 or treated with the R8-Gs peptide.
All data represent means ± SEM (n = 20–30 cells). **p < 0.01; ***p < 0.001 (Student's t test). Scale bars represent 10 μm. See also Figures S3 and S4.

KDEL-BODIPY[633] (Figure 2C) activated Golgi PKA. KDEL-BODIPY[633] also activated Gs in vitro (Figure 2D). To inhibit the KDELR, we used the characterized dominant-negative KDELR-D193N-GFP (Pulvirenti et al., 2008; Townsley et al., 1993), which does not activate PKA (Figure 2E) and inhibits traffic-induced Golgi PKA activation (Figure 2F). These data indicate that the activation of the KDELR is necessary to mediate, and sufficient to mimic, the traffic-dependent activation of Golgi PKA.

The Gs-PKA Pathway Controls Golgi-to-ER Transport as well as Golgi Size and Morphology
The KDELR cycles between the Golgi and the ER and localizes mainly at the cis-Golgi at steady state (Griffiths et al., 1994). When we activated the Gs-PKA Golgi pathway using traffic pulses or KDEL-BODIPY[633], the KDELR moved retrogradely to the ER (Figure 3A), as expected (Lewis and Pelham, 1992) (Figure 3B), and this relocation of the KDELR was prevented by Gs or AC9 depletion (Figure 3C). The inhibition of the Gq-Src pathway had no such effect (Figure S2A). Thus, the traffic- and

ligand-induced ER relocation of the KDELR selectively requires the Gs-PKA pathway. On the other hand, upon release from the ER, the synchronizable cargo hGH-GFP-FM traverses the Golgi in a Gq-dependent way in 20–40 min (Giannotta et al., 2012). The inhibition of Gq by R8-Gq blocked the exit of the cargo from the Golgi, as expected (Giannotta et al., 2012), without affecting ER-to-Golgi transport (Figure 4A). The inhibition of Gs (by R8-Gs) had no such effect; rather, it accelerated the exit of cargo from the Golgi. This acceleration might be explained by the fact that the inhibition of Gs retains the KDELR in the Golgi, resulting in enhancement in Gq-Src signaling (Figures 4B and 4C). These data confirm that Gs controls anterograde, and Gq retrograde, transport from the cis-Golgi (Giannotta et al., 2012; Pulvirenti et al., 2008).

Next, we asked whether increasing or decreasing the activity of Golgi PKA might affect the location of the KDELR independent of the activation of the KDELR by traffic or ligands. We silenced AC9 or PDE7A1, which should result in decreased and increased cAMP levels, and hence in inhibiting or stimulating PKA,
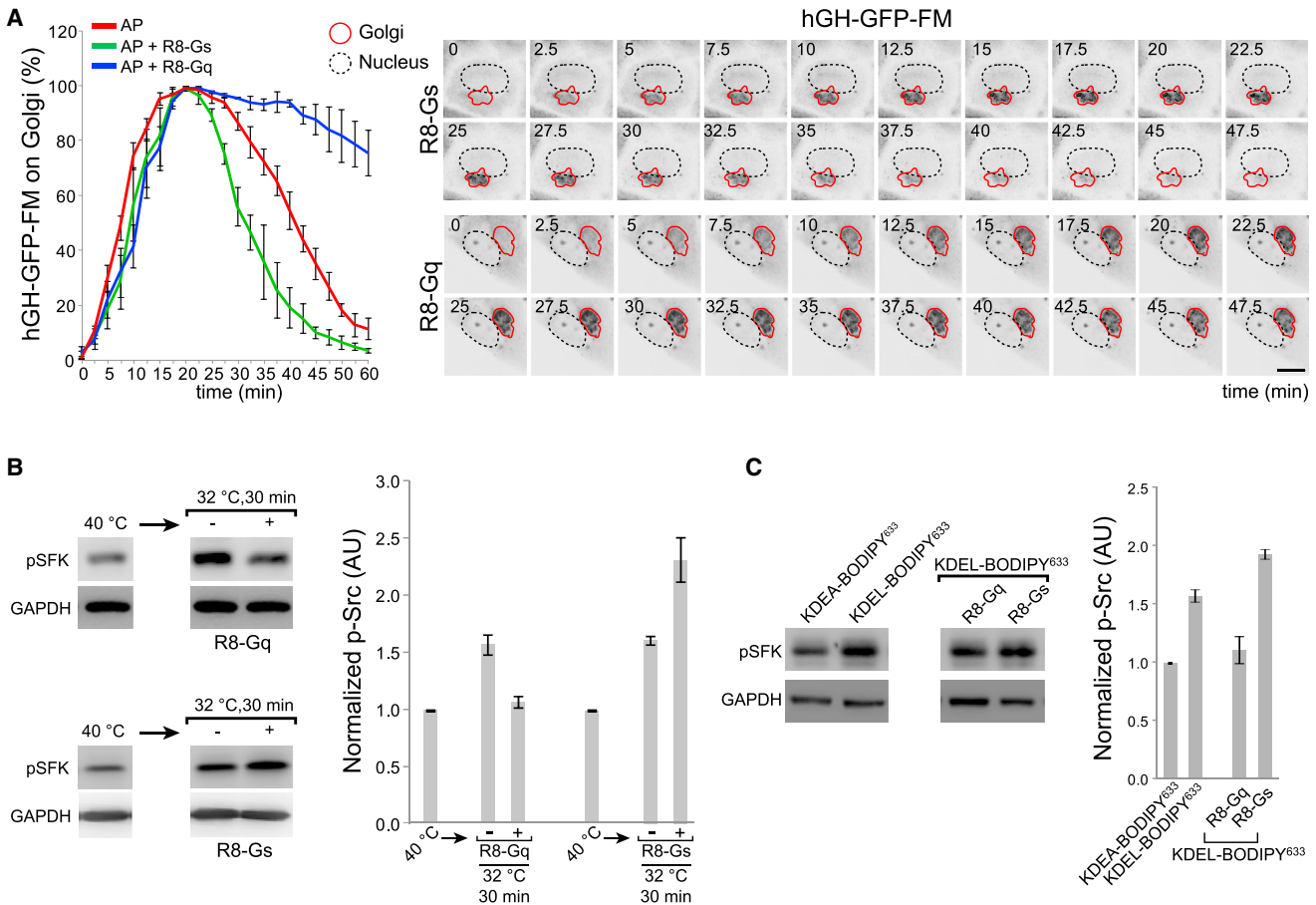
Figure 4. The Gs-PKA Pathway Indirectly Controls Anterograde Transport by Activating Gq/Src

(A) HeLa cells expressing hGH-GFP-FM subjected to a VSVG traffic pulse in the presence or absence of either R8-Gs (10 μM) or R8-Gq (100 μM).

(B) HeLa cells treated with R8-Gq (100 μM) or R8-Gs (10 μM) and immunoblotted for p-Src (pSFK).

(C) HeLa cells incubated with KDEL-BODIPY^{633} peptide (1 μM) for 30 min in the presence of either R8-Gq (100 μM) or R8-Gs (10 μM) and immunoblotted for p-Src (pSFK).

All data represent means ± SEM (A, n = 20–30 cells; B, n = 2 cells). The scale bar represents 10 μm.

respectively, at the Golgi. Silencing AC9 inhibited, and silencing PDE7A1 stimulated, the retrograde transport of the KDELR (Figures S3A and S3B). Further along this line, we used the KDELR-VSVG chimera (Cole et al., 1998), which, like the KDELR, cycles between the ER and the Golgi at 32°C and localizes at the Golgi but unfolds at 40°C and remains trapped on the ER (Cole et al., 1998). While in control cells, KDELR-VSVG moved to the ER at 40°C, as expected; in cells where the Gs-PKA pathway was inhibited, KDELR-VSVG remained at the Golgi complex (Figure S2C). These data indicate that the Gs-PKA pathway is required for the recycling of the KDELR and that this pathway exerts a tonic control on retrograde traffic between the Golgi and ER.

Finally, we examined the traffic of several other retrograde transport markers, such as ER-Golgi intermediate compartment (ERGIC)53, Syntaxin 5 (coat complex protein [COP]I dependent), and Shiga toxin (COPI independent). After KDELR activation, all of them moved from the Golgi to the ER and their relocation was dependent on the activity of the Gs-PKA pathway, as shown for the KDELR (Figure S4). Accordingly, the activation of the Gs-PKA

pathway through the KDELR represents a general mechanism of retrograde transport regulation. Additionally, this pathway modulates the anterograde Golgi-to-plasma membrane (PM) pathway by controlling the intensity of Gq signaling (Figure 4).

As noted, retrograde transport is expected to play a major role in Golgi and ER homeostasis (Griffiths et al., 1984; Klumperman, 2000; Martínez-Menárguez et al., 1999; Thor et al., 2009; Wieland et al., 1987). Thus, the inhibition of the Gs-PKA pathway (and hence of retrograde traffic from the Golgi to the ER) should result in a rapid increase in size and in morphology changes of the Golgi. To verify this, we first used a dual-fluorescence emission Golgi-lipid probe (BODIPY-FL-C$_5$-ceramide) whose fluorescence peak shifts as a function of probe concentration in lipid membranes (Pagano et al., 1991). When applied in the extracellular medium, BODIPY-FL-C$_5$-ceramide is incorporated (and metabolized) into cellular membrane lipids and reaches high concentrations in the Golgi (Pagano et al., 1991). Cells were incubated with BODIPY-FL-C$_5$-ceramide and then treated with the Gs inhibitor R8-Gs. R8-Gs caused a rapid decrease of the Golgi red:green emission ratio, which reached a plateau in 20–30 min
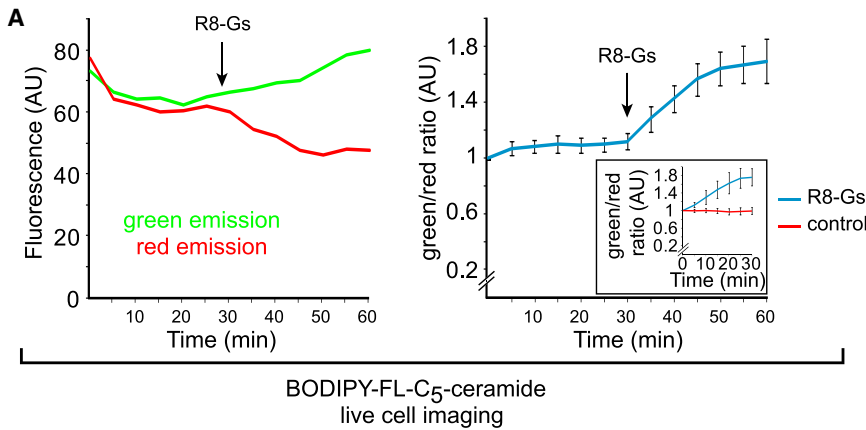
Figure 5. The KDELR-Dependent Gs-PKA Pathway Regulates Golgi Size and Morphology

(A) Cos7 cells incubated with BODIPY-FL-C$_5$-ceramide.

(B) After treatment as in (A), the cells were fixed and the green:red emission ratio was assessed in the Golgi area. Data in (A) and (B) represent means ± SEM (n = 20 cells). The scale bar represents 10 μm.

(C) HeLa cells incubated with R8-Gs for 15 min, fixed, and examined by electron microscopy. The scale bars represent 500 nm. The red arrows indicate intermediate compartments.
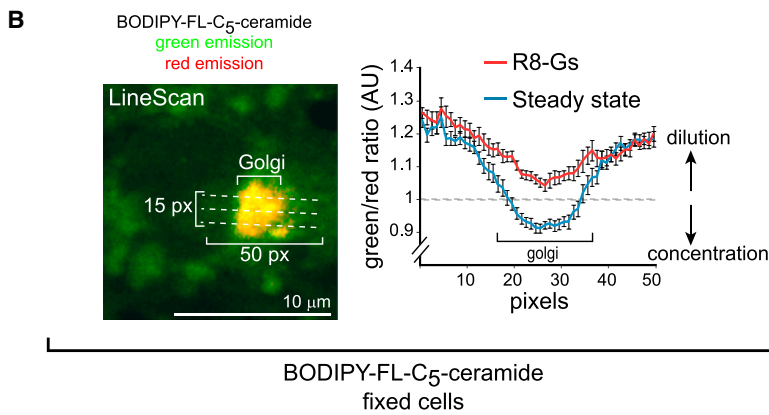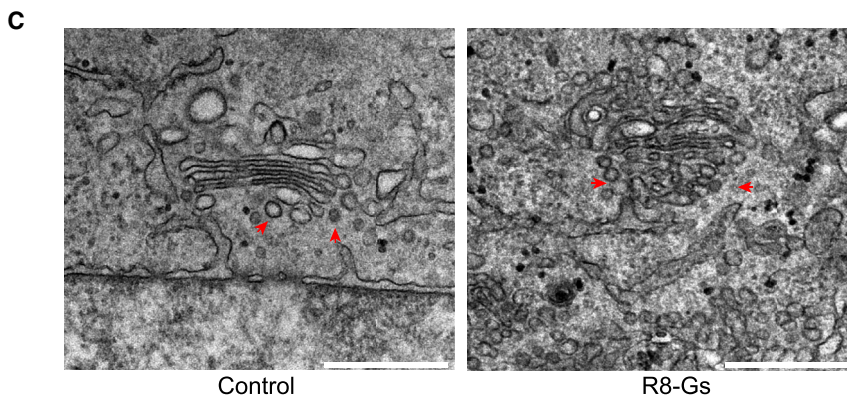
similar juxta-Golgi accumulation of tubular vesicular membranes has been reported to be induced by COPI inhibition and by the consequent impairment of retrograde transport (Pepperkok et al., 1993).

These collective results are consistent with a central role of the KDELR-Gs-PKA pathway in the maintenance of the composition, size, and morphology of the Golgi complex.

Molecular Mechanisms Underlying the Control of Retrograde Transport by the Gs-PKA Pathway

We next sought to identify at least some of the targets of PKA that are relevant for retrograde traffic (a complete molecular analysis of the regulation of transport by Golgi PKA is beyond the scope of this study). We induced a traffic pulse and examined protein phosphorylation using both antibody microarrays and PKA-specific phosphoproteomics both in HeLa cells and human fibroblasts. The combination of these phosphoproteomic approaches yielded a list of over 200 proteins, which included a large number of actin interactors and regulators and signaling proteins and a smaller number of chaperones, transcription factors, coat proteins, adaptors, and others (see Table S1). Among these, we selected a few components that have been implicated in transport to test the functional significance of these phosphorylations in retrograde traffic.

The group of actin-related phosphorylated proteins included the actin regulator LIMK and its substrate cofilin (Arber et al., 1998), in addition to actin itself (Table S1). We focused on these proteins because actin dynamics is known to have a role in a number of transport steps (Curwin et al., 2012; Salvarezza et al., 2009; Stamnes, 2002; Valderrama et al., 2001; von Blume et al., 2009) and because LIMK can be phosphorylated and activated by PKA (Nadella et al., 2009); LIMK then
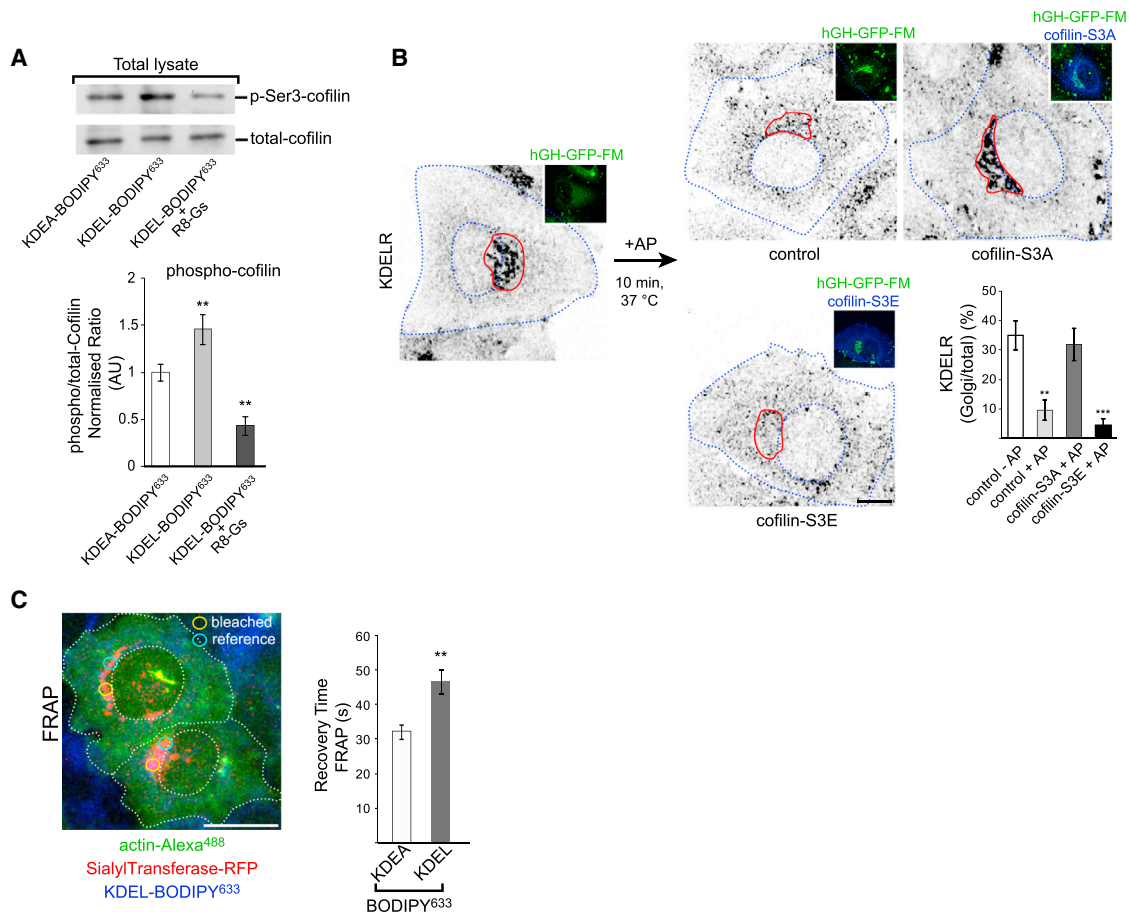
(Figure 5A). This is consistent with an increase in Golgi membranes resulting in probe dilution (Figure 5B). To test this notion directly, we examined the Golgi ultrastructure in cells where the retrograde transport was inhibited acutely for 20 min by addition of R8-Gs. Here, the stacks were slightly larger and more disorganized than in control cells, and they exhibited a large amount of tubular-vesicular membranes close to one of their poles, most likely representing an expanded IC-*cis*-Golgi network (Figure 5C, red arrows). The overall volume and surface of the IC-Golgi complex were therefore increased. These observations are consistent with the block of the recycling of the retrograde traffic markers that is seen under the same conditions (Figure S4). A

Figure 6. KDELR Activation Stimulates the Phosphorylation of Actin Cytoskeleton Proteins, Which Mediate the Activation of Retrograde Traffic

(A) HeLa cells incubated with KDEL-BODIPY^{633} (1 μM) or KDEA-BODIPY^{633} (1 μM) for 30 min at 37°C.

(B) HeLa cells expressing hGH-GFP-FM (green in inset) were transfected with cofilin-S3A (nonphosphorylatable; blue in inset) or cofilin-S3E (phosphomimetic; blue in inset). AP, adaptor protein.

(C) HeLa cells microinjected with cDNA coding for sialyltransferase-RFP cDNA in the nucleus and with purified actin-Alexa^{488} in the cytosol.

Data represent means ± SEM (n = 10–30 cells). **p < 0.01; ***p < 0.001 (Student's t test). The scale bars represent 10 μm. See also Figure S5.

phosphorylates cofilin at Ser3, resulting in cofilin inactivation and hence in inhibition of actin depolymerization (Arber et al., 1998). We first examined whether the activation of the KDELR causes the phosphorylation of cofilin at Ser3. The experiments in Figure 6A showed that this was indeed the case. Moreover, this phosphorylation of cofilin was abolished by inhibitors of the Golgi Gs-PKA pathway. The nonphosphorylatable, constitutively active cofilin-S3A (Salvarezza et al., 2009) impaired the traffic-pulse-induced relocation of the KDELR from the Golgi to the ER, whereas a phosphomimetic constitutively inactive form of cofilin (cofilin-S3E) (Meberg et al., 1998) had no such effect (Figure 6B). Additional experiments using the fluorescence recovery after photobleaching (FRAP) technique showed that the activation of the KDELR elicits a reduction of actin dynamics at the Golgi (Figure 6C), in line with the above effects on cofilin (Arber et al., 1998). These observations indicate that KDELR activation induces inhibition of cofilin by phosphorylation at the regulatory residue Ser3 (Yang et al., 1998), most likely through a PKA-LIMK-cofilin cascade (Nadella et al.,

2009), and that this reaction is required for Golgi-to-ER retrograde traffic.

Another group of phosphorylated actin-related players was the myosin light chain (MLC) and the kinase PAK, which phosphorylates and activates MLC (Sells et al., 1999). MLC has been previously shown to be required for retrograde Golgi-to-ER traffic (Durán et al., 2003). We hypothesized that Golgi PKA phosphorylates/activates PAK, which then activates MLC. We first examined the role of the Golgi PKA pathway and of PAK in the phosphorylation of MLC. MLC is phosphorylated in its activation site Ser19 during a traffic pulse, and this phosphorylation is prevented by inhibiting the Golgi PKA pathway as well as by PAK inhibitors (Figures S5A–S5C). We then tested whether PAK activation depends on KDELR and Gs-PKA activity. Activation of the KDELR induced the phosphorylation of PAK at the activation site Thr423, which was prevented by inhibitors of the Gs-PKA pathway. The experiments support a scheme in which Golgi PKA phosphorylates PAK, which phosphorylates and activates MLC, which finally contributes to the induction of retrograde
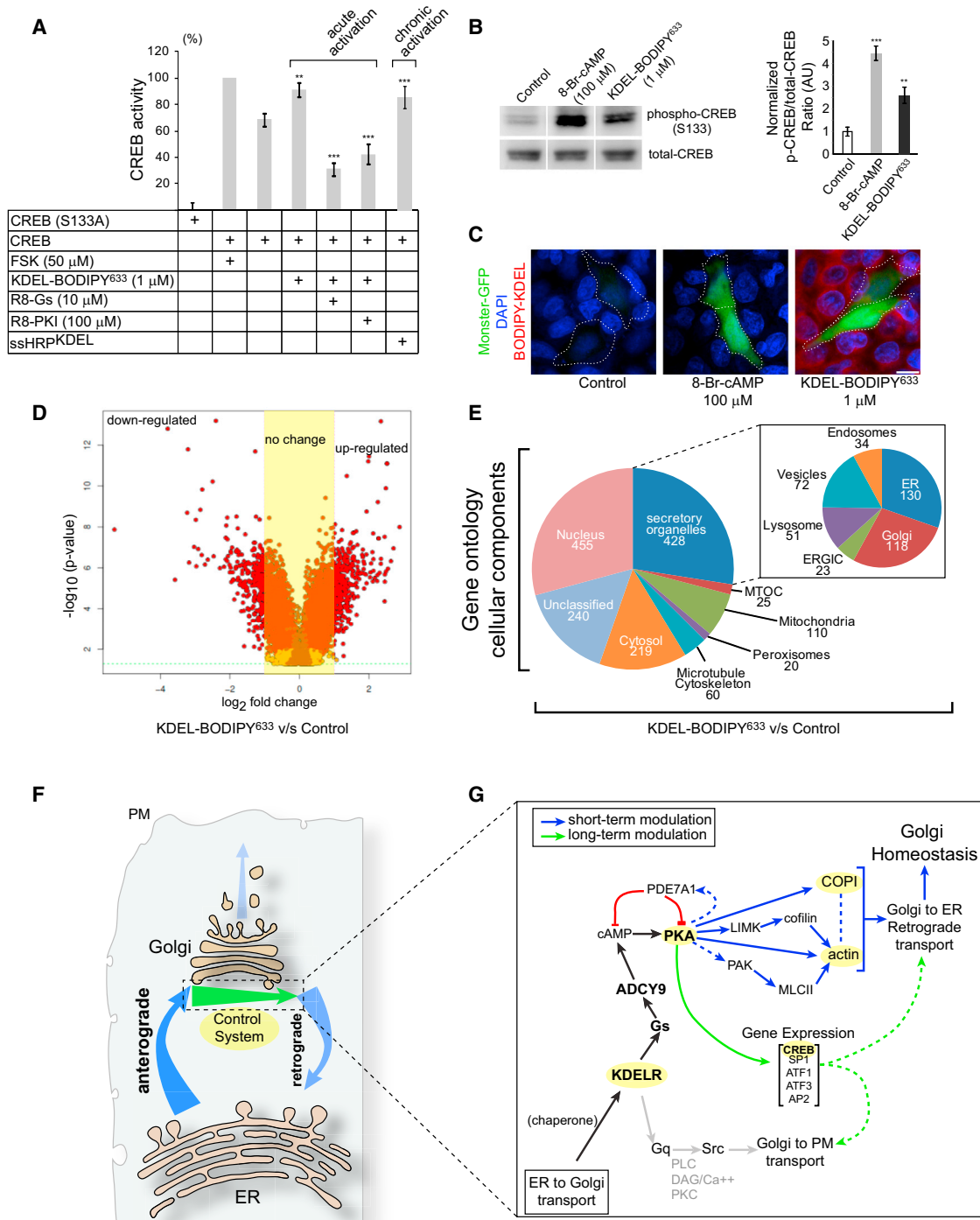
Figure 7. KDELR Activation Controls the Phosphorylation and Activation of CREB1 and Gene Expression

(A) Cos7 cells transfected with CREB1 or mutant inactive CREB1 (Ser133A) activation reporters.

(B) Cos7 cells incubated with KDEL-BODIPY[633] or 8-Br-cAMP for 30 min. Data in (A) and (B) represent means ± SEM (A, n = 20 cells; B, n = 3 cells). **p < 0.01; ***p < 0.001 (Student's t test).

(C) HeLa cells expressing monster-GFP under the control of CREB1. The scale bar represents 10 μm. DAPI, 4′,6-diamidino-2-phenylindole.

(D) HeLa cells incubated with KDEL-BODIPY[633] (1 μM) for 6 hr. mRNA levels were measured using an Affymetrix human gene array.

(E) Gene ontology analysis (cellular components). The number of genes of each compartment upregulated is shown.

(legend continued on next page)

traffic by the KDELR. The above data on actin interactors are in broad agreement with a recent kinome screening study that showed that the phosphorylation of actin cytoskeleton proteins exerts a variety of effects on Golgi structure and function (Chia et al., 2012).

Finally, a group of COPI subunits, α, δ, ε, and ζ, was also phosphorylated. The COPI complex cycles rapidly on and off the Golgi membranes, driving vesicle turnover and controlling intra-Golgi and Golgi-to-ER transport (Rothman and Wieland, 1996). We found that COPI subunits are phosphorylated in a KDELR- and PKA-dependent manner (Figure S5D). We used the mutant ldlF Chinese hamster ovary (CHO) cell line, in which the endogenous ε-COP subunit is replaced by ε-COP-YFP in the COPI complex (Presley et al., 2002). Upon activation of the KDELR, the FRAP recovery time of ε-COP decreased, whereas it increased after subsequent inhibition of Gs by the R8-Gs peptide (Figure S5E). These data indicate that the KDELR-Gs-PKA pathway can regulate (presumably by controlling the phosphorylation of the COPI subunits) the turnover of COPI at the Golgi membranes, which might be relevant to the activation of retrograde traffic.

In summary, several molecular targets appear to be phosphorylated via PKA and to activate retrograde traffic during an ER-to-Golgi traffic pulse. PKA has also been proposed to phosphorylate the KDELR itself and to accelerate its recycling (Cabrera et al., 2003). Thus, the KDELR-Gs-PKA pathway appears to control both general and cargo-specific mechanisms of retrograde traffic.

The KDELR-Gs-PKA Pathway Regulates the Expression of Traffic-Related Genes

A further protein that was phosphorylated during a traffic pulse was CREB1 (Table S1). Activated PKA is known to translocate into the nucleus (Meinkoth et al., 1993) and to phosphorylate/activate CREB1 on its residue Ser133 (Mayr and Montminy, 2001). CREB1 controls a large number of metabolism-, cell-cycle-, and membrane-transport-related genes (Romero et al., 2013; Zhang et al., 2005), and CREB-like factors have been shown to regulate the expression of transport machinery proteins (Fox et al., 2010; Reiling et al., 2013). We thus sought to determine whether the KDELR-Gs-PKA pathway regulates the activity of CREB1 and the expression of transport-related genes. For the first task, we used multiple approaches, including a FRET-based assay for CREB1 phosphorylation (Figure 7A), western blotting (Figure 7B), and a CREB-dependent transcription assay (Figure 7C). These experiments confirmed that CREB1 is phosphorylated at Ser133 and is activated by the KDELR-Gs-PKA pathway (Figures 7A–7C; Figures S6A and S6B). We then evaluated the gene expression profile of cells where the KDELR pathway is stimulated by KDELR ligands or by KDELR overexpression. The two treatments had nearly identical effects, as assessed by volcano plot analysis, and induced the upregulation of over 1,300 genes (Figure 7D; Figure S6D; Table S2). Gene set enrichment analysis showed that the biolog-

ical function categories represented by the upregulated genes include organelle organization/biogenesis, protein folding, metabolic processes (energy and lipid metabolism), the cell cycle, regulation of the actin cytoskeleton, protein kinase cascades, and others (Table S3). Using Gene Ontology, we categorized these genes according to their residence in cellular organelles. We found that a relatively large proportion of genes assigned to secretory-related organelles was upregulated, such as ER (143 out of 267), Golgi (118 out of 208), ERGIC (23 out of 23), lysosomal (58 out of 66), and endosomal (36 out of 66) genes (Figure 7E; Figure S6E). The membrane traffic genes included SNAREs, adaptor complexes, GTPases, chaperones, Golgi enzymes, kinases, and other molecules with key roles in secretion. Thus, the KDELR-Gs-PKA pathway controls the expression of a large number of genes, a significant fraction of which are relevant for membrane traffic, whereas another fraction is involved in different cellular functions. Promoter analysis of the upregulated genes (as assessed by TransFind; http://transfind.sys-bio.net) indicated that in addition to CREB1, other cAMP-regulated transcription factors such as SP1, AP2, ATF1, and ATF3 (García et al., 1999; Hai and Hartman, 2001; Rehfuss et al., 1991; Rohlff et al., 1997), as well as a few that are not cAMP dependent, mediate the effects of KDELR activation on transcription (Table S3).

In summary, the KDELR-dependent pathways control transcription factors that are mostly activated by cAMP/PKA and upregulate both traffic-related and -unrelated genes. From a functional standpoint, this might reflect an adaptive response that is aimed at expanding the capacity of the transport pathways when they are challenged by prolonged increases in traffic load.

DISCUSSION

This study describes a cell-autonomous regulatory circuit that senses membrane transport fluxes at the interface between the ER and the IC-Golgi complex and contributes to the compositional and morphological homeostasis of the two organelles. Based on current and previous results, this process includes the following steps: ER-derived transport carriers reach the Golgi and bring ER chaperones in contact with the KDELR, which stimulates a Golgi pool of Gs. Gs activates AC9 and the formation of cAMP, which is partially degraded and spatially restricted by PDE7A1, resulting in controlled activation of Golgi PKA. PKA then phosphorylates a large number of Golgi and cytosolic proteins, several of which are related to actin, rapidly activating retrograde transport.

In addition to Gs, the KDELR activates Gq, which activates transport through the Golgi. The mechanism by which the KDELR triggers two different downstream pathways is unclear. It is possible that different KDELR isoforms, or perhaps different KDELR homo- and heterodimers (as for many GPCRs, KDELR dimerization is associated with activation), control different G proteins (Rozenfeld and Devi, 2011; Vilardaga et al., 2010). The mechanistic details of the interaction between the KDELR and

---

(F and G) Transport fluxes that reach the Golgi carry chaperones that bind to, and activate, the KDELR. Gs leads to the activation of AC9 to increase cAMP levels. cAMP then activates PKA to activate retrograde transport via the phosphorylation of specific components of the actin machinery and COPI (short-term response). Prolonged activation of the KDELR and of PKA results in the phosphorylation and activation of CREB1 and in the upregulation of gene expression (long-term response). See also Figures S6 and S7.

G proteins will have to be elucidated before this question can be addressed.

The main role of the KDELR-Gs-PKA regulatory axis appears to be to balance the membrane and protein fluxes in and out of the Golgi. In its absence, retrograde transport from the Golgi to the ER is inhibited, and the IC-Golgi complex undergoes rapid and marked compositional and morphological alterations. It remains possible that a fraction of the retrograde traffic fluxes may not depend on the Golgi Gs-PKA pathway. However, the collective observations are in line with this pathway playing a major role in Golgi homeostasis (Griffiths et al., 1984; Klumperman, 2000; Martínez-Menárguez et al., 1999; Thor et al., 2009; Wieland et al., 1987) (see scheme in Figure S7). Notably, the Golgi Gs-PKA pathway regulates not only retrograde traffic but also, albeit indirectly, anterograde traffic through the Golgi, presumably by controlling the levels of the KDELR at the Golgi and hence the intensity of Gq signaling. The Golgi Gs-PKA pathway is thus central to the control of Golgi transport.

Whether or not defects of this pathway are compatible with cell or organism life, and whether they can be partially compensated for by adaptive mechanisms for survival, remains to be investigated. Homozygous Gs knockout (KO) mice die at an early embryonic stage (Farfel et al., 1999; Weinstein et al., 2002), which might be due to traffic or to intercellular communication defects. KO mice are not available for other components of the KDELR-Gs-PKA pathway such as AC9 and PDE7A1. However, the presence of numerous isoforms of these enzymes might afford a degree of adaptation in depleted cells/animals.

Also relevant is the question of how broadly the regulatory/homeostatic machinery is conserved across species. It might even be asked whether in simpler organisms such as yeast these homeostatic mechanisms are built into the core machinery, without a need for regulatory networks. It is clear that the yeast secretory apparatus is potently controlled by signaling pathways. For instance, glucose controls yeast secretion via complex signaling networks that include a GPCR and PKA (Aoh et al., 2011; Levi et al., 2010; Versele et al., 2001); osmolarity changes affect the secretory machinery via the HOG1 pathway (Piao et al., 2012; Reynolds et al., 1998); and the KDELR, as noted, is essential for secretory traffic in yeast via a mechanism that is not related to the loss of KDEL-bearing chaperones and might be mediated by a signaling network (Semenza et al., 1990). These observations suggest that homeostatic mechanisms based on signaling networks such as those described by us in mammalian cells might well exist also in yeast. It is now important to verify whether this is the case and to uncover these potential yeast mechanisms. Trimeric G proteins exist in yeast, but their physical location in endomembranes is not known. We expect that at least some of the key components, such as the G proteins or the PKA pathway (see above), will be found to be conserved.

A salient feature of the KDELR-G-based cascade is that it is cell autonomous, localizes to an internal organelle, the Golgi complex, and is activated by an internal function, traffic. The simplest way to understand this regulatory circuit is to view it as a device that controls transport, or a control system. The theory of control was developed by engineers to manage complex manmade machines, but it can be applied to biological processes as well (Iglesias and Ingalls, 2010; Stelling et al., 2004). Within this framework, membrane transport at the ER-Golgi

interface is the process that is controlled to achieve robust functioning in the face of internal drifts and/or exogenous perturbations. The Golgi control system operates by sensing the traffic input into the Golgi through the binding of the KDELR (here acting as a traffic sensor) with chaperones that escape the ER. Whether all chaperones can act as traffic signals, or specialized KDEL-bearing proteins serve this function preferentially, is currently being investigated. The KDELR sensor then activates a controller, the Gs-PKA pathway, which "calculates" the response and triggers the actuators of retrograde traffic, including the actin-based molecular machines described above. This compensates for the membrane input from the ER, and helps to maintain suitable size and composition of the organelles involved.

In addition to regulating the Golgi acutely, the system exerts a long-term control of the availability of the transport machinery components. Thus, the KDELR regulates the expression of a large number of genes, many of which code for proteins that localize both in biosynthetic and in endolysosomal organelles, such as SNAREs, adaptor complexes, GTPases, chaperones, and Golgi and lysosomal enzymes (Figure 7; Tables S2 and S3). The simplest interpretation of these findings is that a prolonged activation of the KDELR is decoded by the cell as a signal of chronic transport overload, which needs to be compensated for by expansion of the transport pathways (see scheme in Figures 7F and 7G and detailed in Figure S7A). Many other upregulated genes, however, belong to different functional classes, such as mitochondrial and energy metabolism, peroxisomal and lipid metabolism, protein degradation, and autophagy. These findings point to the presence of coordination between membrane transport and these cellular functions. Perhaps related to these observations, the KDELR has been implicated in the clearance of neurodegeneration-inducing proteins such as superoxide dismutase 1, α-synuclein, and huntingtin, possibly through modulation of autophagy (Wang et al., 2011), and has been proposed to have prosurvival properties during the unfolded protein response (Yamamoto et al., 2003). Future work will examine and define the molecular basis and the functional significance of the coordination between transport and other functions.

In terms of design, a feature of the KDELR-Gs-PKA-traffic control system is the integration of components previously known to participate in intercellular communication at the PM with components of the membrane transport machinery. In this regard, it is similar to another control system, the unfolded protein response, which relies on the crosstalk between signaling and machinery proteins (Walter and Ron, 2011). We expect that the description of further traffic control systems and of their design and molecular composition will advance our understanding of the physiopathology of membrane transport, and might provide tools to manipulate transport processes for experimental and therapeutic purposes.

EXPERIMENTAL PROCEDURES

Cell Handling and In Vivo Treatments
Transfection
Cos7 cells were transfected with plasmid vectors using TransIT-LT1 (Mirus Bio) following the manufacturer's protocol. Cos7, HeLa, and CHO cells were transfected with Lipofectamine 2000. siRNA treatments were conducted using a Dharmacon SMARTpool.

ER-to-Golgi Traffic Pulses

VSVG infection and traffic-pulse generation were carried out as previously described (Pulvirenti et al., 2008). Synchronous hGH-GFP-FM release from the ER was performed as previously described (Gordon et al., 2010).

Cell Microinjection

Cos7 and HeLa cells were microinjected as described (Cancino et al., 2007).

Shiga Toxin Uptake and Retrograde Transport

HeLa cells were incubated with Cy3-labeled B fragment of Shiga toxin (1 μg/ml) for 2 hr at 20°C in FBS-free DMEM-HEPES medium to allow toxin internalization and incubated for 4 hr to allow the toxin to arrive at the ER. R8-Gs (10 μM) and 8-Br-cAMP (100 μM) were added during the last 2 hr.

Other Treatments

All of the drug, BODIPY568- or BODIPY633-conjugated peptide, and R8-peptide treatments were performed in fetal calf serum-free DMEM-HEPES medium.

Confocal Microscopy

Images were acquired using either a Leica SP5 or Zeiss LSM710 with a 63× oil-immersion objective (1.4 NA).

Quantitative Fluorescence Image Analysis

The images for quantitative analysis were used as originals (8-bit depth gray-scale images, separated channels) without any processing or adjustment. Quantitative analysis was performed using MetaMorph software (Universal Imaging) as previously described (Cancino et al., 2007). The PKAcat Golgi:total ratio decreased with PKAcat detachment from the Golgi, reflecting PKA activation. KDELR Golgi:total ratio decreased with Golgi-to-ER retrograde transport and reflected Golgi-to-ER relocation of the KDELR.

Image Processing

For figure presentation only, the images were channel separated, and each channel is shown as a grayscale image. The contrast was inverted, and the levels were adjusted to facilitate the observation of dim structures.

Antibody Microarrays

HeLa cells infected with VSVG and cells under the 40°C block and during a traffic pulse were lysed in lysis buffer (50 mM HEPES [pH 7.4], 100 mM NaCl, 0.5% NP-40, 30 mM NaF, 2 mM Na_3O_4V, 60 mM β-glycerophosphate, 5 mM EDTA, 5 mM EGTA, and protease inhibitor cocktail [Roche]). Quantification and data analysis were performed at Kinexus. The resultant changes are expressed as percentages of change with respect to the control (CFC) and as Z factor. Changes $\geq$ 50% CFC and Z $\geq$ 0.8 were considered to be real changes.

mRNA Microarray Analysis of Gene Expression

HeLa cells were transfected to transiently express KDELR-GFP by 72 hr. Wild-type cells (control) and wild-type cells incubated with 1 μM KDEL-BODIPY633 peptide for 6 hr were processed in triplicate to extract the total mRNA. Microarray measurements were performed using Affymetrix human gene arrays (HGA 1.0 ST) at the Coriell Institute. ANOVA was performed, and the genes with an assigned ratio up to 1.5 and below −1.5 were used to select upregulated and downregulated genes, respectively.

Gene set enrichment analysis was performed over a ranked list using Broad Institute tools (http://www.broadinstitute.org/gsea/index.jsp). Volcano plot analysis was carried out by plotting the negative log of the p value on the y axis (base 10), with the x axis as the log (base 2) of the fold change between the two selected conditions.

Reagents and other methods are described in detail in Supplemental Experimental Procedures.

ACCESSION NUMBERS

The microarray data reported in this paper have been deposited in the Gene Expression Omnibus database under accession number GSE48937.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.06.018.

REFERENCES

Aoe, T., Cukierman, E., Lee, A., Cassel, D., Peters, P.J., and Hsu, V.W. (1997). The KDEL receptor, ERD2, regulates intracellular traffic by recruiting a GTPase-activating protein for ARF1. EMBO J. 16, 7305–7316.

Aoh, Q.L., Graves, L.M., and Duncan, M.C. (2011). Glucose regulates clathrin adaptors at the trans-Golgi network and endosomes. Mol. Biol. Cell 22, 3671–3683.

Arber, S., Barbayannis, F.A., Hanser, H., Schneider, C., Stanyon, C.A., Bernard, O., and Caroni, P. (1998). Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature 393, 805–809.

Bejarano, E., Cabrera, M., Vega, L., Hidalgo, J., and Velasco, A. (2006). Golgi structural stability and biogenesis depend on associated PKA activity. J. Cell Sci. 119, 3764–3775.

Bonfanti, L., Mironov, A.A., Jr., Martínez-Menárguez, J.A., Martella, O., Fusella, A., Baldassarre, M., Buccione, R., Geuze, H.J., Mironov, A.A., and Luini, A. (1998). Procollagen traverses the Golgi stack without leaving the lumen of cisternae: evidence for cisternal maturation. Cell 95, 993–1003.

Cabrera, M., Muñiz, M., Hidalgo, J., Vega, L., Martín, M.E., and Velasco, A. (2003). The retrieval function of the KDEL receptor requires PKA phosphorylation of its C-terminus. Mol. Biol. Cell 14, 4114–4125.

Cancino, J., Torrealba, C., Soza, A., Yuseff, M.I., Gravotta, D., Henklein, P., Rodriguez-Boulan, E., and González, A. (2007). Antibody to AP1B adaptor

blocks biosynthetic and recycling routes of basolateral proteins at recycling endosomes. Mol. Biol. Cell 18, 4872–4884.

Chia, J., Goh, G., Racine, V., Ng, S., Kumar, P., and Bard, F. (2012). RNAi screening reveals a large signaling network controlling the Golgi apparatus in human cells. Mol. Syst. Biol. 8, 629.

Cole, N.B., Ellenberg, J., Song, J., DiEuliis, D., and Lippincott-Schwartz, J. (1998). Retrograde transport of Golgi-localized proteins to the ER. J. Cell Biol. 140, 1–15.

Cooper, D.M. (2003). Regulation and organization of adenylyl cyclases and cAMP. Biochem. J. 375, 517–529.

Csikász-Nagy, A., Battogtokh, D., Chen, K.C., Novák, B., and Tyson, J.J. (2006). Analysis of a generic model of eukaryotic cell-cycle regulation. Biophys. J. 90, 4361–4379.

Curwin, A.J., von Blume, J., and Malhotra, V. (2012). Cofilin-mediated sorting and export of specific cargo from the Golgi apparatus in yeast. Mol. Biol. Cell 23, 2327–2338.

De Matteis, M.A., Santini, G., Kahn, R.A., Di Tullio, G., and Luini, A. (1993). Receptor and protein kinase C-mediated regulation of ARF binding to the Golgi complex. Nature 364, 818–821.

Durán, J.M., Valderrama, F., Castel, S., Magdalena, J., Tomás, M., Hosoya, H., Renau-Piqueras, J., Malhotra, V., and Egea, G. (2003). Myosin motors and not actin comets are mediators of the actin-based Golgi-to-endoplasmic reticulum protein transport. Mol. Biol. Cell 14, 445–459.

D'Ursi, A.M., Giusti, L., Albrizio, S., Porchia, F., Esposito, C., Caliendo, G., Gargini, C., Novellino, E., Lucacchini, A., Rovero, P., and Mazzoni, M.R. (2006). A membrane-permeable peptide containing the last 21 residues of the Gα(s) carboxyl terminus inhibits G(s)-coupled receptor signaling in intact cells: correlations between peptide structure and biological activity. Mol. Pharmacol. 69, 727–736.

Farfel, Z., Bourne, H.R., and Iiri, T. (1999). The expanding spectrum of G protein diseases. N. Engl. J. Med. 340, 1012–1020.

Feldman, D.S., Zamah, A.M., Pierce, K.L., Miller, W.E., Kelly, F., Rapacciuolo, A., Rockman, H.A., Koch, W.J., and Luttrell, L.M. (2002). Selective inhibition of heterotrimeric Gs signaling. Targeting the receptor-G protein interface using a peptide minigene encoding the Gα(s) carboxyl terminus. J. Biol. Chem. 277, 28631–28640.

Fox, R.M., Hanlon, C.D., and Andrew, D.J. (2010). The CrebA/Creb3-like transcription factors are major and direct regulators of secretory capacity. J. Cell Biol. 191, 479–492.

Francis, S.H., Blount, M.A., and Corbin, J.D. (2011). Mammalian cyclic nucleotide phosphodiesterases: molecular mechanisms and physiological functions. Physiol. Rev. 91, 651–690.

García, M.A., Campillos, M., Marina, A., Valdivieso, F., and Vázquez, J. (1999). Transcription factor AP-2 activity is modulated by protein kinase A-mediated phosphorylation. FEBS Lett. 444, 27–31.

Giannotta, M., Ruggiero, C., Grossi, M., Cancino, J., Capitani, M., Pulvirenti, T., Consoli, G.M., Geraci, C., Fanelli, F., Luini, A., and Sallese, M. (2012). The KDEL receptor couples to Gαq/11 to activate Src kinases and regulate transport through the Golgi. EMBO J. 31, 2869–2881.

Gilchrist, A., Li, A., and Hamm, H.E. (2002). Gα COOH-terminal minigene vectors dissect heterotrimeric G protein signaling. Sci. STKE 2002, pl1.

Gong, H., Sengupta, D., Linstedt, A.D., and Schwartz, R. (2008). Simulated de novo assembly of Golgi compartments by selective cargo capture during vesicle budding and targeted vesicle fusion. Biophys. J. 95, 1674–1688.

Gordon, D.E., Bond, L.M., Sahlender, D.A., and Peden, A.A. (2010). A targeted siRNA screen to identify SNAREs required for constitutive secretion in mammalian cells. Traffic 11, 1191–1204.

Griffiths, G., Warren, G., Quinn, P., Mathieu-Costello, O., and Hoppeler, H. (1984). Density of newly synthesized plasma membrane proteins in intracellular membranes. I. Stereological studies. J. Cell Biol. 98, 2133–2141.

Griffiths, G., Ericsson, M., Krijnse-Locker, J., Nilsson, T., Goud, B., Söling, H.D., Tang, B.L., Wong, S.H., and Hong, W. (1994). Localization of the Lys,

Asp, Glu, Leu tetrapeptide receptor to the Golgi complex and the intermediate compartment in mammalian cells. J. Cell Biol. 127, 1557–1574.

Hai, T., and Hartman, M.G. (2001). The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis. Gene 273, 1–11.

Han, P., Sonati, P., Rubin, C., and Michaeli, T. (2006). PDE7A1, a cAMP-specific phosphodiesterase, inhibits cAMP-dependent protein kinase by a direct interaction with C. J. Biol. Chem. 281, 15050–15057.

Heinrich, R., and Rapoport, T.A. (2005). Generation of nonidentical compartments in vesicular transport systems. J. Cell Biol. 168, 271–280.

Heitz, F., Morris, M.C., and Divita, G. (2009). Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. Br. J. Pharmacol. 157, 195–206.

Hirschberg, K., Miller, C.M., Ellenberg, J., Presley, J.F., Siggia, E.D., Phair, R.D., and Lippincott-Schwartz, J. (1998). Kinetic analysis of secretory protein traffic and characterization of Golgi to plasma membrane transport intermediates in living cells. J. Cell Biol. 143, 1485–1503.

Hsu, V.W., Shah, N., and Klausner, R.D. (1992). A brefeldin A-like phenotype is induced by the overexpression of a human ERD-2-like protein, ELP-1. Cell 69, 625–635.

Iglesias, P.A., and Ingalls, B.P. (2010). Control Theory and Systems Biology. (Cambridge, MA: MIT Press).

Kitano, H. (2007). Towards a theory of biological robustness. Mol. Syst. Biol. 3, 137.

Klumperman, J. (2000). Transport between ER and Golgi. Curr. Opin. Cell Biol. 12, 445–449.

Levi, S.K., Bhattacharyya, D., Strack, R.L., Austin, J.R., II, and Glick, B.S. (2010). The yeast GRASP Grh1 colocalizes with COPII and is dispensable for organizing the secretory pathway. Traffic 11, 1168–1179.

Lewis, M.J., and Pelham, H.R. (1990). A human homologue of the yeast HDEL receptor. Nature 348, 162–163.

Lewis, M.J., and Pelham, H.R. (1992). Ligand-induced redistribution of a human KDEL receptor from the Golgi complex to the endoplasmic reticulum. Cell 68, 353–364.

Maier, O., Ehmsen, E., and Westermann, P. (1995). Trimeric G protein α subunits of the Gs and Gi families localized at the Golgi membrane. Biochem. Biophys. Res. Commun. 208, 135–143.

Majoul, I., Straub, M., Hell, S.W., Duden, R., and Söling, H.D. (2001). KDEL-cargo regulates interactions between proteins involved in COPI vesicle traffic: measurements in living cells using FRET. Dev. Cell 1, 139–153.

Martin, M.E., Hidalgo, J., Rosa, J.L., Crottet, P., and Velasco, A. (2000). Effect of protein kinase A activity on the association of ADP-ribosylation factor 1 to Golgi membranes. J. Biol. Chem. 275, 19050–19059.

Martínez-Menárguez, J.A., Geuze, H.J., Slot, J.W., and Klumperman, J. (1999). Vesicular tubular clusters between the ER and Golgi mediate concentration of soluble secretory proteins by exclusion from COPI-coated vesicles. Cell 98, 81–90.

Mavillard, F., Hidalgo, J., Megias, D., Levitsky, K.L., and Velasco, A. (2010). PKA-mediated Golgi remodeling during cAMP signal transmission. Traffic 11, 90–109.

Mayr, B., and Montminy, M. (2001). Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat. Rev. Mol. Cell Biol. 2, 599–609.

Mazzoni, M.R., Taddei, S., Giusti, L., Rovero, P., Galoppini, C., D'Ursi, A., Albrizio, S., Triolo, A., Novellino, E., Greco, G., et al. (2000). A Gα(s) carboxyl-terminal peptide prevents G(s) activation by the A(2A) adenosine receptor. Mol. Pharmacol. 58, 226–236.

Meberg, P.J., Ono, S., Minamide, L.S., Takahashi, M., and Bamburg, J.R. (1998). Actin depolymerizing factor and cofilin phosphorylation dynamics: response to signals that regulate neurite extension. Cell Motil. Cytoskeleton 39, 172–190.

Meinkoth, J.L., Alberts, A.S., Went, W., Fantozzi, D., Taylor, S.S., Hagiwara, M., Montminy, M., and Feramisco, J.R. (1993). Signal transduction through the cAMP-dependent protein kinase. Mol. Cell. Biochem. *127-128*, 179–186.

Mironov, A.A., Beznoussenko, G.V., Nicoziani, P., Martella, O., Trucco, A., Kweon, H.S., Di Giandomenico, D., Polishchuk, R.S., Fusella, A., Lupetti, P., et al. (2001). Small cargo proteins and large aggregates can traverse the Golgi by a common mechanism without leaving the lumen of cisternae. J. Cell Biol. *155*, 1225–1238.

Muñiz, M., Alonso, M., Hidalgo, J., and Velasco, A. (1996). A regulatory role for cAMP-dependent protein kinase in protein traffic along the exocytic route. J. Biol. Chem. *271*, 30935–30941.

Muñiz, M., Martín, M.E., Hidalgo, J., and Velasco, A. (1997). Protein kinase A activity is required for the budding of constitutive transport vesicles from the *trans*-Golgi network. Proc. Natl. Acad. Sci. USA *94*, 14461–14466.

Nadella, K.S., Saji, M., Jacob, N.K., Pavel, E., Ringel, M.D., and Kirschner, L.S. (2009). Regulation of actin function by protein kinase A-mediated phosphorylation of Limk1. EMBO Rep. *10*, 599–605.

Nigg, E.A., Hilz, H., Eppenberger, H.M., and Dutly, F. (1985a). Rapid and reversible translocation of the catalytic subunit of cAMP-dependent protein kinase type II from the Golgi complex to the nucleus. EMBO J. *4*, 2801–2806.

Nigg, E.A., Schäfer, G., Hilz, H., and Eppenberger, H.M. (1985b). Cyclic-AMP-dependent protein kinase type II is associated with the Golgi complex and with centrosomes. Cell *41*, 1039–1051.

Pagano, R.E., Martin, O.C., Kang, H.C., and Haugland, R.P. (1991). A novel fluorescent ceramide analogue for studying membrane traffic in animal cells: accumulation at the Golgi apparatus results in altered spectral properties of the sphingolipid precursor. J. Cell Biol. *113*, 1267–1279.

Pepperkok, R., Scheel, J., Horstmann, H., Hauri, H.P., Griffiths, G., and Kreis, T.E. (1993). β-COP is essential for biosynthetic membrane transport from the endoplasmic reticulum to the Golgi complex in vivo. Cell *74*, 71–82.

Piao, H., MacLean Freed, J., and Mayinger, P. (2012). Metabolic activation of the HOG MAP kinase pathway by Snf1/AMPK regulates lipid signaling at the Golgi. Traffic *13*, 1522–1531.

Presley, J.F., Ward, T.H., Pfeifer, A.C., Siggia, E.D., Phair, R.D., and Lippincott-Schwartz, J. (2002). Dissection of COPI and Arf1 dynamics in vivo and role in Golgi membrane transport. Nature *417*, 187–193.

Pulvirenti, T., Giannotta, M., Capestrano, M., Capitani, M., Pisanu, A., Polishchuk, R.S., San Pietro, E., Beznoussenko, G.V., Mironov, A.A., Turacchio, G., et al. (2008). A traffic-activated Golgi-based signalling circuit coordinates the secretory pathway. Nat. Cell Biol. *10*, 912–922.

Rehfuss, R.P., Walton, K.M., Loriaux, M.M., and Goodman, R.H. (1991). The cAMP-regulated enhancer-binding protein ATF-1 activates transcription in response to cAMP-dependent protein kinase A. J. Biol. Chem. *266*, 18431–18434.

Reiling, J.H., Olive, A.J., Sanyal, S., Carette, J.E., Brummelkamp, T.R., Ploegh, H.L., Starnbach, M.N., and Sabatini, D.M. (2013). A CREB3-ARF4 signalling pathway mediates the response to Golgi stress and susceptibility to pathogens. Nat. Cell Biol. *15*, 1473–1485.

Reynolds, T.B., Hopkins, B.D., Lyons, M.R., and Graham, T.R. (1998). The high osmolarity glycerol response (HOG) MAP kinase pathway controls localization of a yeast Golgi glycosyltransferase. J. Cell Biol. *143*, 935–946.

Rohlff, C., Ahmad, S., Borellini, F., Lei, J., and Glazer, R.I. (1997). Modulation of transcription factor Sp1 by cAMP-dependent protein kinase. J. Biol. Chem. *272*, 21137–21141.

Romero, N., Dumur, C.I., Martinez, H., García, I.A., Monetta, P., Slavin, I., Sampieri, L., Koritschoner, N., Mironov, A.A., De Matteis, M.A., and Alvarez, C. (2013). Rab1b overexpression modifies Golgi size and gene expression in HeLa cells and modulates the thyrotrophin response in thyroid cells in culture. Mol. Biol. Cell *24*, 617–632.

Rothman, J.E., and Wieland, F.T. (1996). Protein sorting by transport vesicles. Science *272*, 227–234.

Rozenfeld, R., and Devi, L.A. (2011). Exploring a role for heteromerization in GPCR signalling specificity. Biochem. J. *433*, 11–18.

Salvarezza, S.B., Deborde, S., Schreiner, R., Campagne, F., Kessels, M.M., Qualmann, B., Caceres, A., Kreitzer, G., and Rodriguez-Boulan, E. (2009). LIM kinase 1 and cofilin regulate actin filament population required for dynamin-dependent apical carrier fission from the *trans*-Golgi network. Mol. Biol. Cell *20*, 438–451.

Saudek, V. (2012). Cystinosin, MPDU1, SWEETs and KDELR belong to a well-defined protein family with putative function of cargo receptors involved in vesicle trafficking. PLoS ONE *7*, e30876.

Sells, M.A., Boyd, J.T., and Chernoff, J. (1999). p21-activated kinase 1 (Pak1) regulates cell motility in mammalian fibroblasts. J. Cell Biol. *145*, 837–849.

Semenza, J.C., Hardwick, K.G., Dean, N., and Pelham, H.R. (1990). ERD2, a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway. Cell *61*, 1349–1357.

Sengupta, D., and Linstedt, A.D. (2011). Control of organelle size: the Golgi complex. Annu. Rev. Cell Dev. Biol. *27*, 57–77.

Stamnes, M. (2002). Regulating the actin cytoskeleton during vesicular transport. Curr. Opin. Cell Biol. *14*, 428–433.

Stelling, J., Sauer, U., Szallasi, Z., Doyle, F.J., III, and Doyle, J. (2004). Robustness of cellular functions. Cell *118*, 675–685.

Sunahara, R.K., Dessauer, C.W., and Gilman, A.G. (1996). Complexity and diversity of mammalian adenylyl cyclases. Annu. Rev. Pharmacol. Toxicol. *36*, 461–480.

Thor, F., Gautschi, M., Geiger, R., and Helenius, A. (2009). Bulk flow revisited: transport of a soluble protein in the secretory pathway. Traffic *10*, 1819–1830.

Townsley, F.M., Wilson, D.W., and Pelham, H.R. (1993). Mutational analysis of the human KDEL receptor: distinct structural requirements for Golgi retention, ligand binding and retrograde transport. EMBO J. *12*, 2821–2829.

Trucco, A., Polishchuk, R.S., Martella, O., Di Pentima, A., Fusella, A., Di Giandomenico, D., San Pietro, E., Beznoussenko, G.V., Polishchuk, E.V., Baldassarre, M., et al. (2004). Secretory traffic triggers the formation of tubular continuities across Golgi sub-compartments. Nat. Cell Biol. *6*, 1071–1081.

Valderrama, F., Durán, J.M., Babià, T., Barth, H., Renau-Piqueras, J., and Egea, G. (2001). Actin microfilaments facilitate the retrograde transport from the Golgi complex to the endoplasmic reticulum in mammalian cells. Traffic *2*, 717–726.

Verdurmen, W.P., and Brock, R. (2011). Biological responses towards cationic peptides and drug carriers. Trends Pharmacol. Sci. *32*, 116–124.

Versele, M., Lemaire, K., and Thevelein, J.M. (2001). Sex and sugar in yeast: two distinct GPCR systems. EMBO Rep. *2*, 574–579.

Vilardaga, J.P., Agnati, L.F., Fuxe, K., and Ciruela, F. (2010). G-protein-coupled receptor heteromer dynamics. J. Cell Sci. *123*, 4215–4220.

von Blume, J., Duran, J.M., Forlanelli, E., Alleaume, A.M., Egorov, M., Polishchuk, R., Molina, H., and Malhotra, V. (2009). Actin remodeling by ADF/cofilin is required for cargo sorting at the *trans*-Golgi network. J. Cell Biol. *187*, 1055–1069.

Walter, P., and Ron, D. (2011). The unfolded protein response: from stress pathway to homeostatic regulation. Science *334*, 1081–1086.

Wang, P., Li, B., Zhou, L., Fei, E., and Wang, G. (2011). The KDEL receptor induces autophagy to promote the clearance of neurodegenerative disease-related proteins. Neuroscience *190*, 43–55.

Weinstein, L.S., Chen, M., and Liu, J. (2002). Gs(α) mutations and imprinting defects in human disease. Ann. N Y Acad. Sci. *968*, 173–197.

Wieland, F.T., Gleason, M.L., Serafini, T.A., and Rothman, J.E. (1987). The rate of bulk flow from the endoplasmic reticulum to the cell surface. Cell *50*, 289–300.

Yamamoto, K., Hamada, H., Shinkai, H., Kohno, Y., Koseki, H., and Aoe, T. (2003). The KDEL receptor modulates the endoplasmic reticulum stress response through mitogen-activated protein kinase signaling cascades. J. Biol. Chem. *278*, 34525–34532.

Yang, N., Higuchi, O., Ohashi, K., Nagata, K., Wada, A., Kangawa, K., Nishida, E., and Mizuno, K. (1998). Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. Nature *393*, 809–812.

Yee, D.C., Shlykov, M.A., Västermark, A., Reddy, V.S., Arora, S., Sun, E.I., and Saier, M.H., Jr. (2013). The transporter-opsin-G protein-coupled receptor (TOG) superfamily. FEBS J. *280*, 5780–5800.

Zaccolo, M., and Pozzan, T. (2002). Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. Science *295*, 1711–1715.

Zhai, Y., Heijne, W.H., Smith, D.W., and Saier, M.H., Jr. (2001). Homologues of archaeal rhodopsins in plants, animals and fungi: structural and functional predications for a putative fungal chaperone protein. Biochim. Biophys. Acta *1511*, 206–223.

Zhang, X., Odom, D.T., Koo, S.H., Conkright, M.D., Canettieri, G., Best, J., Chen, H., Jenner, R., Herbolsheimer, E., Jacobsen, E., et al. (2005). Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. Proc. Natl. Acad. Sci. USA *102*, 4459–4464.