**Gαs promotes EEA1 endosome maturation and shuts down proliferative signaling through interaction with GIV (Girdin)**

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**ABSTRACT** The organization of the endocytic system into biochemically distinct subcompartments allows for spatial and temporal control of the strength and duration of signaling. Recent work has established that Akt cell survival signaling via the epidermal growth factor receptor (EGFR) occurs from APPL early endosomes that mature into early EEA1 endosomes. Less is known about receptor signaling from EEA1 endosomes. We show here that EGF-induced, proliferative signaling occurs from EEA1 endosomes and is regulated by the heterotrimeric G protein Gαs through interaction with the signal transducing protein GIV (also known as Girdin). When Gαs or GIV is depleted, activated EGFR and its adaptors accumulate in EEA1 endosomes, and EGFR signaling is prolonged, EGFR down-regulation is delayed, and cell proliferation is greatly enhanced. Our findings define EEA1 endosomes as major sites for proliferative signaling and establish that Gαs and GIV regulate EEA1 but not APPL endosome maturation and determine the duration and strength of proliferative signaling from this compartment.

**INTRODUCTION** Cells respond to their environment through binding of extracellular signals to plasma membrane (PM) receptors that transduce information to the cell interior and activate elaborate signaling networks via sequential protein–protein interactions. The extent and duration of receptor signal transduction are tightly regulated by endocytic trafficking, during which receptors are removed from the cell surface by endocytosis, pass through the endosomal system, and are either recycled or delivered to lysosomes, where signaling is down-regulated and the receptors are degraded (Sorkin and von Zastrow, 2009; Scita and Di Fiore, 2010; Platta and Stenmark, 2011). Specifically, in the case of the epidermal growth factor receptor (EGFR), it was shown that binding of EGF to its receptor activates EGFR signaling at the PM and initiates internalization and trafficking of the receptor to peripheral APPL/Rab5–positive early endosomes and then to early EEA1 endosomes (Zoncu et al., 2009), from which the receptor may either recycle or be sorted and targeted to lysosomes for degradation. APPL endosomes mature into EEA1 endosomes by a gradual switch in the phosphatidylinositol phosphate content from phosphatidylinositol (4,5)-bisphosphate to phosphatidylinositol (3,4,5)-trisphosphate (Shin et al., 2005), which leads to dissociation of APPL1 and acquisition of the Rab effector EEA1 (Zoncu et al., 2009). EEA1 endosomes gradually acquire ESCRT components, lose Rab5 and EEA1, and acquire Rab7 to become late or multivesicular endosomes (Rink et al., 2005; Poteryaev et al., 2010), which eventually become lysosomes by acquisition of lysosomal proteins (e.g., LAMPS, lysosomal enzymes; Huotari and Helenius, 2011). Thus the EGFR passes through a series of biochemically distinct...
endolysosomal subcompartments (APPL, EEA1, late multivesicular endosomes, lysosomes) with unique compositions and functions.

It is now recognized that signaling not only occurs at the cell surface, but it also continues in transit through the endosomal system (Di Guglielmo et al., 1994) and that endosomes can initiate and sustain unique growth factor receptor signaling and cellular responses from those initiated at the PM (Miazyńska et al., 2004; Murphy et al., 2009; Scita and Di Fiore, 2010; Platta and Stenmark, 2011). For example, APPL endosomes, sometimes referred to as “signaling endosomes,” were shown to be the site of generation of unique signals that specifically stimulate Akt cell survival signaling via APPL1 (Varsano et al., 2006; Schenck et al., 2008). According to the classic model, it is assumed or implied that EEA1 endosomes function principally to down-regulate receptor signaling by sorting and sequestering EGFR within intraluminal vesicles of multivesicular endosomes (MVEs). The questions of whether signaling can be initiated and sustained at EEA1 endosomes and, if so, how the cellular responses are regulated at this location are controversial (Sorkin and von Zastrow, 2009). Answering these questions would yield information common to other growth factor receptors (e.g., platelet-derived growth factor receptor, nerve growth factor receptor) that follow similar trafficking itineraries (Wang et al., 2004; Howe and Mobley, 2005; Varsano et al., 2006; Murphy et al., 2009).

We recently discovered that the multidomain signal transducing protein GIV influences EGFR trafficking and signaling. Upon EGF stimulation, GIV directly binds activated EGFR and assembles an EGFR/GIV/Gas signaling complex that prolongs PM-based signaling and enhances cell migration (Ghosh et al., 2010). We found earlier that GIV also binds Gas in yeast two-hybrid assays (Le-Niculescu et al., 2005), and Gas localizes to Rab5 endosomes and facilitates down-regulation of EGFR (Zheng et al., 2004). We therefore asked whether EGFR trafficking and signaling at endosomes are regulated by functional coupling between Gas and GIV and, if so, which endosomal subcompartments are affected. Here we provide evidence that interaction between Gas and GIV regulates proliferative signaling at EEA1 but not APPL endosomes and that sequential interaction between GIV and either Gas or Gas at spatially distinct compartments (PM vs. EEA1 endosomes) determines the cellular response to growth factor.

RESULTS

EGFR traffics efficiently through APPL endosomes but not EEA1 endosomes in the absence of Gases

First we set out to determine which step in EGF-induced receptor trafficking is regulated by Gas. To learn whether Gas regulates trafficking through APPL endosomes, we treated HeLa cells with control or Gas small interfering RNA (siRNA; Zheng et al., 2004) to deplete endogenous Gases (>90%) (see Figure 3, A, C, and E, later in the paper), starved overnight (0.2% fetal bovine serum [FBS]), stimulated with 50 nM (300 ng/ml) Texas red-EGF (TR-EGF), and stained for APPL1. At 5 min after TR-EGF stimulation some TR-EGF localized to peripheral APPL endosomes in both control and Gas-depleted cells (Figure 1, A and D); at 10 min most of the TR-EGF localized to scattered intracellular vesicles and some remained in peripheral APPL endosomes (Figure 1, B and E). At 30 min little TR-EGF could be detected in APPL endosomes in either control or Gas-depleted cells (Figure 1, C and F); however, much more total TR-EGF remained in Gas-depleted cells, and it was concentrated in tightly clustered juxtanuclear endosomes. These results indicate that TR-EGF traffics through APPL endosomes efficiently in both control and Gas-depleted cells and accumulates in a post-APPL early endosome compartment in Gas-depleted but not control cells.

We next asked whether EGFR trafficking through EEA1 endosomes is compromised in the absence of Gases. Gas-depleted HeLa cells and controls were stimulated with 300 ng/ml EGF and stained for EGFR and EEA1. Before EGF stimulation (0 min), EGFR was localized at the PM and to some cytoplasmic vesicles in both control and Gas-depleted cells (Figure 2, A and D). At 10 min after EGF stimulation EGFR had reached EEA1 endosomes scattered throughout the cytoplasm in both controls and Gas-depleted cells (Figure 2, B and E). At 30 min few receptors were seen in EEA1 endosomes in controls (Figure 2C), whereas in Gas-depleted cells many EGF receptors remained in EEA1 endosomes, which were clustered in the juxtanuclear region (Figure 2F). Findings were not dependent on the amount of growth factor added, as similar results were obtained after stimulation with a low concentration (1.5 ng/ml) of EGF (unpublished data). The residence time of TR-EGF in EEA1 endosomes was also prolonged after Gas depletion (Supplemental Figure S1, A–F), and maturation of EEA1 endosomes to late endosomes and lysosomes was delayed, as dramatically more TR-EGF remained associated with EEA1 endosomes.
endosomes or LAMP-2–positive endolysosomes at 90 min after Gαs depletion (Supplemental Figure S1H) than in controls (Supplemental Figure S1G).

To establish the morphology of the clustered EEA1 endosomes in which EGFR accumulates at 30 min after Gαs depletion, we carried out immunogold labeling for EGFR at the electron microscopic (EM) level. It has been established (Sachse et al., 2002) that after internalization via clathrin-mediated endocytosis, EGFRs are delivered to early vacuolar endosomes, which mature into MVEs that with time show increasing numbers of intraluminal vesicles and a content of increasing density and heterogeneity (endolysosomes). In controls at 30 min after EGF stimulation, EGFR was found primarily inside dense MVEs packed with internal vesicles (Supplemental Figure S1, I and J) or in endolysosomes with heterogeneous lamellar and vesicular content (Supplemental Figure S1K). In contrast in Gαs-depleted cells, EGFR was mainly localized to either the limiting membrane of vacuolar endosomes lacking internal vesicles (Supplemental Figure S1, L and M) or to the intraluminal vesicles of MVEs, which had fewer internal vesicles than controls (Supplemental Figure S1N); relatively few receptors were detected inside endolysosomes densely packed with internal vesicles.

The immunofluorescence and immunogold results validate that in the absence of Gαs, both EGFR and EGF accumulate in EEA1 endosomes, and maturation of EEA1 endosomes to late endosomes and lysosomes is delayed. We conclude that the G protein facilitates receptor trafficking through early endosomes and timely maturation of EEA1 endosomes to endolysosomes.

Gαs depletion increases and prolongs EGFR autophosphorylation, src homology 2 adaptor recruitment, and activation of downstream kinases from EEA1 endosomes

Next we asked whether the prolonged stay of EGFR in EEA1 endosomes after Gαs depletion affects receptor signaling. Control and Gαs-depleted cells were starved, stimulated with 50 nM EGF, and stained for EEA1 and activated (autophosphorylated) EGFR using antibodies specific for pY1068, the phosphosite bound by the src homology 2 (SH2) adaptor, Grb2 (Lowenstein et al., 1992). Before stimulation with EGF, very little activated EGFR was observed at the PM or endosomes in either control or Gαs-depleted cells (Figure 2, G and J). By 10 min after stimulation, activated EGFRs were associated with EEA1 endosomes in both control and Gαs-depleted cells, but the amount seen in Gαs-depleted cells (Figure 2K) was increased over controls (Figure 2H). At 30 min, activated EGFRs were barely detectable in EEA1 endosomes in controls (Figure 2I), whereas in Gαs-depleted cells abundant activated receptors remained in juxtanuclear EEA1 endosomes (Figure 2L). Thus EGFR activation (autophosphorylation) is greatly enhanced, and the stay of activated EGFR in EEA1 endosomes is prolonged after Gαs depletion. Similar findings were obtained when cells were stained with antibodies specific for pY1045, the phosphosite on activated EGFR that binds c-Cbl (unpublished data).
Increased autophosphorylation of EGFR in Gαs-depleted cells was confirmed by quantitative immunoblotting using Odyssey infrared imaging. In controls, autophosphorylation of EGFR at both pY1068 and pY1045 peaked at 5 min but was reduced by 15 min after ligand stimulation (Figure 3A), whereas in Gαs-depleted cells EGFR autophosphorylation at these sites was increased greater than threefold at both 5 and 15 min after EGF stimulation. Cell lysates from control (lanes 1–3) or Gαs-depleted (lanes 4–6) HeLa cells treated as in Figure 2, A–F, were stimulated with 50 nM EGF for 5 or 15 min, immunoblotted for total EGFR (tEGFR), activated EGFR (pY1068- and pY1045), Gαs, and actin, and quantified using Odyssey imaging software, version 2.1. Bands were normalized to actin at each time point, averaged, and plotted as the fold increase in phosphorylation vs. control ± SEM. (C, D) Gαs-depleted cells show greater than twofold more pERK1/2 at 5 (lane 5) and 15 min (lane 6) after stimulation than controls (lanes 2 and 3; n = 4, *p < 0.05). (E and F) Gαs-depleted cells show 1.6-fold more pAkt than controls (lanes 2 and 3) at 5 min (lane 5) but not at 15 min (lane 6) after stimulation (n = 4, *p < 0.001). Control (lanes 1–3) or Gαs-depleted (lanes 4–6) HeLa cells treated as in A were immunoblotted for pERK1/2, tERK1/2, pAkt, tAkt, Gαs, and actin and quantified as in B.

The effects of Gαs depletion were not due to off-target effects of the siRNA, because expression of siRNA-resistant (sr), wild-type (wt) human Gαs in Gαs siRNA-treated cells selectively reversed the effects of Gαs depletion on autophosphorylation of EGFR (Supplemental Figure S3). Moreover, the effects of Gαs depletion differed from those of Gα3 depletion: In Gα3-depleted cells EGFR activation at pY1068 and pY1045 was increased immediately (5 min) after EGF stimulation, but by 15 min EGF activation was reduced and similar to controls (Supplemental Figure S4, A and B).

Collectively these results indicate that 1) Gαs depletion increases and prolongs EGFR activation, adaptor recruitment, and downstream MAP kinase signaling at 15 min after EGF stimulation when the receptor is localized in EEA1 early endosomes (see Figure 2, E and F), and 2) the effects are specific for Gαs.

**Gαs depletion leads to enhanced cell proliferation**

Next we asked whether the delay in endosome maturation and enhanced EGFR signaling from EEA1 endosomes in Gαs-depleted cells results in increased cell proliferation, using two established indicators of mitosis—phospho-histone H3 (P-H3; Hans and Dimitrov, 2001; Ghosh et al., 2010) and incorporation of bromodeoxyuridine (BrdU; Schenck et al., 2008). We found that the levels of P-H3 determined by both immunofluorescence (IF; Figure 4A) and quantitative immunoblotting (Figure 4, B and C) were significantly increased (>1.47-fold) after Gαs depletion. Similarly, the levels of incorporated BrdU as determined by IF (Figure 4D) and flow cytometry analysis (Figure 4, E and F) were also significantly increased (>1.6-fold). Thus the results confirm that there are increased numbers of proliferating cells after Gαs depletion. Overall our findings indicate that Gαs facilitates EEA1 endosome maturation, limits EGFR signaling from EEA1 endosomes, and inhibits cell proliferation.

**Gαs regulates the membrane association of EEA1**

Next we investigated the mechanism by which maturation of endosomes is delayed in the absence of Gαs, Rab5 and its effector EEA1 associate with membranes of EEA1 endosomes and then dissociate when they mature into late endosomes (Rink et al., 2005; Poteryaev et al., 2010). To learn whether Gαs affects the membrane association of Rab5, EEA1, and other endosome markers, we staured control and Gαs-depleted HeLa cells, stimulated them with EGF, and assessed the distribution of endosome markers between membrane (120,000 × g pellet) and cytosolic (120,000 × g supernatant) fractions. There was no change in the
We previously showed that GIV prolongs EGFR signaling and the inactive Gαs state of Gαs is required for EGFR signal down-regulation and loss of EEA1 from membranes.

Inactive Gαs is required for EGFR signal down-regulation and loss of EEA1 from membranes

To learn whether the effects of Gαs on EGFR and EEA1 depend on the activation status of the G protein, we expressed siRNA-resistant (sr) mutants mimicking the active (Graziano and Gilman, 1989) or inactive state of Gαs (Lee et al., 1992) in Gαs-depleted cells. We found that the inactive Gαs-G226A (GA) but not the constitutively active Gαs-Q227L (QL) mutant reversed the effects of Gαs depletion on EGFR autophosphorylation (Figure 6, A and B) and reversed the membrane association of EEA1 (Figure 6, C and D). On the basis of these data, we conclude that inactive Gαs promotes EEA1 endosome maturation and facilitates timely EGFR endocytic trafficking, down-regulation, and degradation.

Inactive Gαs directly binds GIV

We previously showed that GIV prolongs EGFR signaling and the association of the receptor with the PM, but once the receptor is internalized, it facilitates EGFR trafficking through endosomes and promotes EGFR degradation (Ghosh et al., 2010). We reasoned that the effects of GIV on endosome dynamics might be mediated through interaction with Gαs. To investigate this possibility, we first asked whether GIV interacts with Gαs in vitro pull-down assays and whether binding depends on the activation state of the G protein. Purified glutathione S-transferase (GST)–Gαs or GST alone preloaded with GDP (to mimic the inactive state) or GDP/AlF4− (to mimic the active state; Garcia-Marcos et al., 2009) was incubated with HeLa or Cos7 cell lysates and analyzed for bound proteins. Inactive but not active GST-Gαs bound endogenous GIV from both Cos7 (Figure 7A) and HeLa cell lysates. In addition, endogenous GIV coimmunoprecipitated with Gαs-GFP in the presence of GDP but not in the presence of GDP/AlF4− (Figure 7, B and C). We further found that purified, recombinant GST-Gαs-GDP specifically bound the purified C-terminus (CT) of GIV (amino acids 1623–1870; Figure 7D), indicating that interaction between GIV and inactive Gαs is direct.

GIV binds directly to Gαi3-GDP via a defined guanine nucleotide exchange factor (GEF) motif within its C-terminus, and a point mutation within this motif (GIV-F1685A) virtually abolishes binding (Garcia-Marcos et al., 2009). To determine whether GIV binds to Gαi3 also, we first carried out pull-down assays with GST-Gαi3-GDP on cell lysates from Cos7 cells expressing FLAG-tagged GIV-wt or the FLAG-GIV-F1685A (FA) mutant. We found that FLAG-GIV-FA binding to GST-Gαi3-GDP is greatly reduced compared with FLAG-GIV-wt (Figure 7E), indicating that interaction between GIV and Gαi3-GDP bind to the same motif in the C-terminus of GIV. In contrast to Gαs, Gαi3 activity was not affected by recombinant GIV in vitro (unpublished data); however, these results do not rule out a possible effect of endogenous GIV on Gαi3 activity in vivo (e.g., due to phosphorylation; Poppleton et al., 1996; Lin et al., 2011).
GIV and Gαs regulate the membrane association of EEA1

Next we asked whether GIV might also regulate the membrane association of EEA1. Indeed, the amount of EEA1 on membranes doubled after GIV depletion (Figure 9, G and H), indicating that GIV, like Gαs, facilitates the loss of EEA1 from endosomal membranes. Our findings that GIV binds inactive Gαs and both GIV and Gαs facilitate the loss of EEA1 from membranes suggest that GIV and Gαs function in a common pathway that facilitates the dissociation of EEA1 from endosomal membranes. If so, the effects of depletion of both proteins on the membrane association of EEA1 should resemble that after depletion of either protein alone. This proved to be the case, as there was no significant difference between the results obtained after silencing both Gαs and GIV versus silencing Gαs alone (Figure 9, I and J), indicating that the effects of Gαs and GIV on EEA1 are not additive. These data implicate GIV and Gαs in a common pathway mediating the loss of EEA1 from membranes, which is a key step in EEA1 endosome maturation.

Overall our results indicate that both GIV and Gαs facilitate maturation of EEA1 endosomes and down-regulation of EGFR signaling from this compartment.

**DISCUSSION**

In this study we provide novel insights into the regulation of proliferative signaling via EGFR from EEA1 signaling endosomes. We find that in the absence of Gαs, EGFRs pass efficiently through APPL endosomes but their stay in EEA1 endosomes is prolonged and is associated with increased EGFR autophosphorylation, SH2 adaptor recruitment, activation of ERK1/2, and increased cell proliferation. Mechanistically, we find that inactive Gαs facilitates maturation of EEA1 endosomes and limits EGFR proliferative signaling and cell proliferation via direct interaction with GIV. Previously we showed that Gαs promotes ligand-induced degradation of EGFR (Zheng et al., 2004) and that Gαs binds to GIV in yeast two-hybrid assays (Le-Niculescu et al., 2005). Our present findings establish that EEA1 endosomes are the site of the Gαs effect on EGFR trafficking and that Gαs and GIV functionally interact to facilitate endosome maturation, EGFR degradation, and down-regulation of EGFR proliferative signaling.

**GIV sequentially interacts with G proteins to compartmentalize EGFR signaling**

Our present work and previous findings (Ghosh et al., 2010) define a critical role for interaction between GIV and G proteins in the spatial and temporal compartmentalization of EGFR signaling. Evidence that GIV and G proteins compartmentalize signaling initially arose from studies linking GIV and Gαi3 to EGF-induced cell migration (Ghosh et al., 2008). Later we found that GIV's C-terminus harbors a...
GIV motif that binds and activates Gai3 (Garcia-Marcos et al., 2009) and upon EGF stimulation assembles an EGFR/GIV/Gxi3 complex at the PM that prolongs association of EGFR with the PM and enhances migratory Akt signaling and cell migration (Ghosh et al., 2010; Garcia-Marcos et al., 2012). Importantly, a point mutation (GIV-F1685A) within the GEF motif that disrupts GIV’s ability to bind and activate Gai3 not only inhibited Akt activation and cell migration (Garcia-Marcos et al., 2009), but also it caused increased EGFR internalization, led to accumulation of EGFR in endosomes, and resulted in increased proliferative ERK1/2 signaling (Ghosh et al., 2010). These effects of GIV on signal compartmentalization were attributed to the ability of GIV to bind and activate Gai3 (Ghosh et al., 2010). Our present work shows that the same GIV-F1685A point mutation that disrupts interaction between GIV and Gai3 also disrupts GIV’s interaction with Gxs. This, together with our finding that Gxs affects later signaling from endosomes, whereas Gai3 affects early EGF signaling from the PM (Figure 3 and Supplemental Figure S4), suggests that the effects of GIV-F1685A on EGFR signaling at endosomes result from disrupting GIV’s interaction with Gxs. Our working model (Figure 10) is that after EGF stimulation, GIV initially binds activated EGFR and activates Gai3 and Akt to regulate EGFR signaling and cell migration at the PM (Ghosh et al., 2010), and later GIV binds Gxs at EE1 endosomes to facilitate EE1 endosome maturation and down-regulation of proliferative signaling from EE1 endosomes as shown in the present work. Based on this model, interference with GIV’s ability to bind to either G protein would alter the trafficking and signaling dynamics of EGFR and other growth factor receptors (e.g., platelet-derived growth factor receptor, nerve growth factor receptor) that follow similar trafficking itineraries (Wang et al., 2004; Howe and Moley, 2005; Varsano et al., 2006; Murphy et al., 2009).

Interaction of Gxs with GIV shuts down EGFR proliferative signaling from EE1 endosomes

Our present work demonstrates that EE1 endosomes are important signaling compartments that support and sustain proliferative signaling by EGFR. It has been clear for some time that activation of distinct signaling pathways by EGFR (e.g., Akt vs. ERK1/2, Vieira et al., 1996; Haugh and Meyer, 2002; Sigismund et al., 2008; Ghosh et al., 2010) and other receptors (Sorkin and von Zastrow, 2009; Scita and Di Fiore, 2010; Platta and Stenmark, 2011) requires their internalization and trafficking to endosomes. What has remained unclear is the contribution of individual endosomal subcompartments to signaling. Traditionally EE1 endosomes have been viewed principally as sites at which EGFR are concentrated and sorted for degradation in downstream compartments (late endosomes, lysosomes; Sorkin and von Zastrow, 2009; Huotari and Helenius, 2011). Recently it has been shown that EGFRs traffic first to biochemically distinct APPL “signaling” endosomes (Zoncu et al., 2009), where EGFRs initiate and sustain cell survival signaling via activation of Akt and phosphorylation of glycogen synthase kinase 3 (Schneck et al., 2008), after which APPL endosomes are converted to EE1 endosomes, where signal attenuation is assumed to occur (Varsano et al., 2006; Zoncu et al., 2009). This work implied that APPL endosomes represent the endosome subcompartment where signaling is initiated and sustained, whereas EE1 endosomes function to attenuate and down-regulate signaling. Our findings provide strong evidence that proliferative signaling occurs from EE1 endosomes and that the residence time of activated EGFR in EE1 endosomes and duration of proliferative signaling is controlled by Gxs and its interaction with GIV.

We found that dissociation of EE1 from endosomal membranes, a key step in the maturation of EE1 endosomes (Rink et al., 2005), is facilitated by functional interaction between Gxs and GIV.
endosomes. Efficient receptor sorting but not for the association of Hrs with Gβs does not affect the membrane association of Hrs (Figure 5), indicating that interaction of Gβs with Hrs may be required for efficient receptor sorting (Babst, 2011). However, we also found that interaction of Gβs with Hrs (Zheng et al., 2004) and GIV is critically disrupted in early tumors expressing GIV-ΔCT. Akt activation and cell migration by EGFR are inhibited due to interference with binding to GIV, whereas EE1 endosome maturation is delayed, proligative signaling is enhanced, and cell proliferation is increased due to interference with GIV binding. We also showed that GIV is overexpressed in late-stage metastatic tumors and migratory signaling by EGFR and cell migration is enhanced (Ghosh et al., 2010). We propose that GIV overexpression in metastatic tumors enhances cell migration by preferentially promoting migratory signaling from the PM via interaction with Gαi3 and then limiting endosome-based proliferative signaling via interaction with Gxs.

We conclude that the ability of GIV to sequentially interact with Gαi3 and Gxs at the PM and endosomes compartmentalizes EGFR signaling and thereby determines the cell’s response to the growth factor.

**MATERIALS AND METHODS**

### Antibodies and constructs

Silencer Negative Control #1 siRNA (Ghosh et al., 2008) was obtained from Ambion (Austin, TX), Gxs (Zheng et al., 2004) and GIV (Ghosh et al., 2010) siRNAs from Dharmacon (Lafayette, CO), and Gαi3 siRNA (Ghosh et al., 2008) from Santa Cruz Biotechnology (Santa Cruz, CA).

Affinity-purified rabbit anti-APPL1 and anti-Gxs immunoglobulin G (IgG) were gifts from Joseph Testa (Fox Chase Cancer Center, Philadelphia, PA) and Allen M. Spiegel (Albert Einstein College of Medicine, New York, NY). Mouse anti-Rab5 monoclonal antibody (mAb; clone 4F11-D9-C4) was a gift from Angela Wandler-Ness (University of New Mexico, Albuquerque, NM). Rabbit antibodies against Girdin/GIV, pan-Gβ, Grb2, Hrs, and EGFR (sc-03) were purchased from Santa Cruz Biotechnology, Gαi3 and Gxs from Calbiochem (La Jolla, CA), and those against phospho–histone H3 (P-H3, Ser-10) from Millipore (Billerica, MA). Rabbit antibodies against pY1045-EGFR, pY1068-EGFR, pAkt (Ser-473), total Akt, and pERK1/2, and EE1 were purchased from Cell Signaling Technology (Beverly, MA).

Mouse mAbs were purchased as follows: GFP (Clontech, Mountain View, CA), LAMP-2 (Developmental Studies Hybridoma.

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**FIGURE 7:** Inactive Gxs directly binds GIV. (A) Endogenous GIV and Gβi (used as a positive control) preferentially bind inactive GST-Gxs-GDP (lane 3, upper panels). Little binding to active GST-Gxs-GTP-AR/β3 (lane 5) or GST alone (lanes 2 and 4) is observed. Purified GST-Gxs (lanes 3 and 5) and GST alone (lanes 2 and 4) were preloaded with GDP (lanes 2 and 3) or GDP/AR/β3 (lanes 4 and 5) and incubated overnight with ~1 mg Cos7 cell lysate. Lane 1, 5% input. Recombinant proteins were visualized by Poncet S staining (lower panel), and bound proteins were analyzed by immunoblotting for GIV and Gβi (top). (B) Endogenous GIV coimmunoprecipitates with Gxs-GFP-GDP (B, lane 2) but not Gxs-GFP-GTP-AR/β3 (B, lane 3) or GFP alone (C, lanes 2 and 3), indicating that GIV preferentially interacts with GDP-bound Gxs. Cos7 cells expressing Gxs-GFP (B) or GFP alone (C) were lysed in the presence of GDP (lanes 1 and 2) or GDP/AR/β3 (lanes 3 and 4) and incubated with anti-GIP IgG, and bound proteins were analyzed by immunoblotting for GFP and endogenous GIV. Lanes 1 and 4, 1% input. (C) GST-Gxs-GDP (lane 4) but not GST-Gxs-GTP-AR/β3 (lanes 5) or GST alone (lanes 2 and 3) binds His-GIV-CT (amino acids 1623-1870), indicating that GIV-CT binds directly to inactive Gxs. Purified GST (lanes 2 and 3) or GST-Gxs (lanes 4 and 5) was preloaded with GDP (lanes 2 and 4) or GDP/AR/β3 (lanes 3 and 5) as in A and incubated with purified His-GIV-CT. Lane 1, 10% input. (D) GST-Gxs-GDP binds FLAG-GIV-wt (lane 3), but binding of the mutant FLAG-GIV-FA (lane 5) is greatly decreased, indicating that the GEF motif in the C-terminus of GIV binds inactive Gxs. Purified GST (lanes 2 and 4) or GST-Gxs (lanes 3 and 5) was preloaded with GDP and incubated with lysates from Cos7 cells expressing either FLAG-GIV-wt (lanes 1–3) or FLAG-GIV-FA (lanes 4–6). Lanes 1 and 6, 1% input. Recombinant proteins were visualized by Poncet S staining (bottom), and bound proteins were analyzed by immunoblotting (top) for FLAG (FLAG-GIV) and Gβi.

(Received 23 September 2010; accepted 24 October 2010.)
Gαs-GFP in pCDNA3.1 was a gift from Mark Rasenick (University of Illinois at Chicago, Chicago, IL). Human WT and constitutively active Q227L (QL) Gαs (long and short isoforms) in pCDNA3.1 were purchased from Guthrie cDNA Resource Center (Missouri University of Science and Technology, Rolla, MO). pCDNA3.1 Gαs-G226A (GA) mutant was made using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA). siRNA-resistant Gαs WT, QL, and GA mutant constructs were made by introducing silent substitutions in the Gαs cDNA within the region of homology to the siRNA Gαs oligo (Zheng et al., 2004). N-terminally tagged FLAG-Rab5-QL was subcloned into p3XFLAG-CMV (Sigma-Aldrich). Human GIV was N-terminally tagged with CFP and cloned into the pAP4 vector. Gαs-WT (human, short) was cloned into pGEX-KG and purified as described previously (Ghosh et al., 2008). All primers are available upon request.

Cell culture, transfection, and EGF stimulation
HeLa and Cos7 cells (American Type Culture Collection, Manassas, VA) were grown in DME (Invitrogen) supplemented with 10% FBS (HyClone, Logan, UT) and penicillin–streptomycin–glutamine (Invitrogen).

For transfection of siRNA oligos, HeLa cells were seeded (5.5 × 10⁴ cells/35 mm dish or 5.5 × 10⁵ cells/10 cm dish), and 24 h later they were transfected with 20 nM final siRNA for 14 h using Oligofectamine (Invitrogen) according to the manufacturer’s instructions.

For reversal of the effects of Gαs depletion, 1 × 10⁵ HeLa cells were seeded/well (six-well dish) or 5.5 × 10⁶/10 cm dish, transfected with 20 nM siRNA oligo using Oligofectamine overnight as described, and then transfected with 2 μg/well or 10 μg/dish plasmid DNA (pCDNA3.1 alone or pCDNA3.1 siRNA-resistant Gαs-WT, QL, or GA mutants) for 8 h using FuGENE (Roche, Indianapolis, IN) or TransIT-LT1 (Minus Bio, Madison, WI) according to the manufacturer’s instructions.

For expression of FLAG-Rab5-QL and CFP-GIV, HeLa cells were transfected first with control and Gαs siRNA for 14 h using Oligofectamine and then cotransfected with 1 μg of FLAG-Rab5-QL and 2 μg of CFP-GIV for 8 h using TransIT-LT1.

For expression of GFP or Gαs-GFP, 10-cm dishes of Cos7 cells were transfected with 2 μg GFP or 10 μg Gαs-GFP for 14 h using TransIT-LT1.

For EGF stimulation experiments, 48 h after siRNA transfection the serum concentration was reduced from 10 to 0.2% overnight before stimulation with 50 nM EGF (mouse submaxillary gland; Invitrogen), 300 ng/ml Texas-red EGF (Invitrogen), or 300 ng/ml Alexa 488 EGF (Invitrogen) in DME alone.

**Immunofluorescence and immunoelectron microscopy**

For immunofluorescence analysis, cells grown on coverslips were fixed in 3% paraformaldehyde (PFA) for 30–60 min at room temperature (RT), quenched (50 mM NH₄Cl), blocked (10% normal goat serum), permeabilized (0.1% Triton X-100 [TX-100]) in phosphate-buffered saline [PBS]), incubated in primary antibodies (1 h at RT) or overnight at 4°C (for phosphospecific Gαs) and then secondary IgGs (1 h at RT), and mounted on slides in 1% propyl-gallate (Sigma-Aldrich) in 1:1 glycerol/PBS. Antibody dilutions were as follows: EEA1, 1:200; pY1068-EGFR, 1:150; GIV, 1:180; pAP4, 1:200; phospho-histone H3, 1:150; EGFR (#528), 1:300; EEA1, 1:200; BrdU, 1:5; LAMP-2, 1:400; goat anti-mouse or anti-rabbit Alexa 488 or Alexa 594, 1:500. 4″,6-Diamidino-2-phenylindole (DAPI) (Invitrogen) was used at 1:3000. Confocal imaging was carried out on an
FIGURE 9: GIV depletion increases the membrane association of EEA1 and prolongs and enhances EGFR signaling from EEA1 endosomes. (A–F) Before EGF stimulation (0 min), little pY1068 staining for activated receptors (green) is observed at the PM or EEA1 endosomes (red) in either control (A) or GIV-depleted (D) cells. At 10 min after stimulation some activated EGFRs are associated with EEA1 endosomes in both control (B) and GIV-depleted (E) cells (yellow, arrowheads). By 30 min, activated EGFRs are barely detectable in EEA1 endosomes in control (C), whereas GIV-depleted cells show a striking accumulation of activated EGFRs in EEA1 endosomes (F; yellow, arrowheads). GIV-depleted HeLa cells and controls were serum starved, stimulated with EGF, and stained for pY1068 and EEA1 (red). Bar, 10 μm. (G, H) After GIV depletion, 24% of the total EEA1 is associated with membrane fractions, ~11% in controls. The distribution of EEA1, GIV, Grxs, and actin in membrane (120,000 × g pellet, P100) and cytosolic (120,000 × g supernatant, S100) fractions prepared from control (lanes 1 and 2) or GIV-depleted (lanes 3 and 4) HeLa cells was assessed by immunoblotting. EEA1 bands such as those in A were quantified from three different experiments and averaged, and the percentage of EEA1 on membrane fractions calculated and plotted as in Figure 5B (*p < 0.01). (I, J) Grx and GIV cooperatively facilitate the loss of EEA1 from membranes. The amount of EEA1 on membranes after depletion of both Grxs and GIV (lanes 5 and 6) is similar to that seen after depletion of Grxs alone (lanes 7 and 8). HeLa cells were depleted of GIV, Grxs, or both GIV and Grxs and fractions prepared and immunoblotted as in A. Results are shown as the mean ± SEM (p = 0.02; n/s, no significant difference).

inverted IX81 microscope (Olympus, Tokyo, Japan) equipped with 405-, 488-, 560-, and 640-nm laser lines, UltraView Vox Spinning Disk Confocal (PerkinElmer, Waltham, MA), a 60x oil (differential interference contrast) lens, an electron-multiplying charge-coupled device (CCD) Hamamatsu 14-bit camera (Hamamatsu, Hamamatsu, Japan), and Velocity software (PerkinElmer; University of California, San Diego, School of Medicine Light Microscopy Facility). For immunoelectron microscopic studies, cells were fixed 4 h in 4% PFA, followed by 12 h in 1% PFA in 0.1 M phosphate buffer, pelleted in 10% gelatin, cryoprotected in sucrose, and snap frozen in liquid nitrogen. Ultrathin cryosections (70–80 nm) were cut as previously described (Zheng et al., 2004). For immunogold labeling of EGFR, sections were incubated sequentially with sheep anti-EGFR IgG (2 h), rabbit anti-goat IgG (bridging antibody; 1 h), and goat anti-rabbit IgG-gold conjugates (1 h) and then contrasted (10 min in 0.4% uranyl acetate and 1.8% methyl cellulose on ice). Imaging was carried out using a JEOL 1200 EX II electron microscope equipped with an Orius CCD Gatan camera and Gatan digital micrograph software (Gatan, Pleasanton, CA; University of California, San Diego, Cellular and Molecular Medicine Electron Microscopy Facility).

Whole-cell lysis and immunoblotting
Cells were harvested, suspended in 2.5× Laemmli sample buffer, and boiled for 15 min. Samples were separated by 10% SDS–PAGE or 15% SDS–PAGE (for P-H3 analysis) and transferred to PVDF-FL membranes (Millipore). Membranes were blocked (5% BSA, 0.1% Tween-20 in PBS) and incubated with primary antibodies (4°C overnight) and then with secondary antibodies (30 min at RT). Bands were imaged and quantified by two-color detection with the Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE). Antibody dilutions were as follows: pY1068- and pY1045-EGFR, pAkt, tAkt, Girdin/GIV, tERK1/2, Hrs, and c-Cbl, 1:250; Grxα3, tEGFR, EEA1, Rab5, and APPL1, 1:500; Grb2, pERK1/2, actin, GFP, P-H3, and tubulin, 1:1000 to 1:2000; and goat anti-rabbit Alexa Fluor 680 and goat anti-mouse IRDye 800 F(ab′)2, 1:15,000.

Immunoprecipitation
To immunoprecipitate endogenous EGFR, control or Grxs deleted cells were harvested, lysed in buffer A (0.4% TX-100, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2, 5 mM Mg acetate, 125 mM K acetate, 1 mM dithiothreitol [DTT], and 20 mM N-ethylmaleimide supplemented with Complete Protease Inhibitor cocktail [Roche] and Phosphatase Inhibitor Cocktail 2 [Sigma-Aldrich] inhibitors), incubated on ice (1 h) with vortexing every 10 min, and cleared by centrifugation (10,000 × g for 10 min). Cell lysates were incubated overnight at 4°C with either control or anti-EGFR mAb #528. Protein G-Sepharose beads (GE Health Sciences) were added and incubated an additional 60 min. Beads were washed, suspended, and boiled in Laemmli sample buffer, and bound proteins were analyzed by immunoblotting.

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MVEs (5), which facilitates EGFR down-regulation and shuts off and promotes dissociation of EEA1 and endosome maturation to (3) and then to EEA1 endosomes (4), where GIV binds inactive G

FIGURE 10: Working model. GIV spatially regulates the trafficking and signaling of EGFR via sequential interactions with Gni3 and Gox. Upon EGF stimulation, GIV binds EGFR and assembles an EGFR/GIV/ Gni3 complex at the PM that activates Gni3 (1), prolongs the association of EGFR with the PM, and enhances PM-based Akt signaling (2). On internalization EGFR traffics to APPL endosomes (3) and then to EEA1 endosomes (4), where GIV binds inactive Gox, and promotes dissociation of EEA1 and endosome maturation to MVEs (5), which facilitates EGFR down-regulation and shuts off proliferative signaling from endosomes.

To immunoprecipitate GFP or Gox-GFP, Cos7 cells were transfected for 48 h with GoxGFP or GFP alone, harvested, suspended in buffer A supplemented with 1 mM DTT, 10 mM MgCl2, and 30 μM GDP ± 10 mM NaF and 10 μM AlCl3, incubated on ice, and cleared by centrifugation as described. Cell lysates were incubated overnight at 4°C with anti-GFP mAb. Bound proteins were recovered and analyzed as described.

Cell proliferation assays
For P-H3 analyses, whole-cell lysates were prepared as described and analyzed for P-H3 by immunoblotting (1:2000) or by immunofluorescence (1:150) as described previously (Lehtonen et al., 2008; Ghosh et al., 2010).

For BrdU (Sigma-Aldrich) incorporation experiments, control or Gox-depleted HeLa cells were incubated in 10 μM BrdU for 30 min at 37°C in DME supplemented with 10% FBS. Immunofluorescence and flow cytometry analysis of incorporated BrdU were carried out according to the manufacturer’s instructions (Sigma-Aldrich). Briefly, cells were trypsinized, suspended in PBS, and fixed in 100% ethanol (30 min, RT). Samples were incubated in 2 N HCl (20 min, RT), followed by 0.1 M sodium borate (5 min) and mouse anti-BrdU (1:8 dilution; BD Biosciences, San Diego, CA) for 30 min, followed by goat anti-mouse Alexa Fluor 488 for 30 min. RNA was digested, and DNA content was stained by incubation in 100 μg/ml RNase and 5 μg/ml propidium iodide (PI) for 20 min at 37°C. Samples were filtered (10-μm Nitex nylon mesh; Sefar America, Depew, NY), loaded onto an LSRII flow cytometer (BD Biosciences), and analyzed using FlowJo software (TreeStar, Ashland, OR). Cells untreated or exposed only to anti-BrdU antibody or PI were used for background measurements.

Preparation of membrane and cytosolic fractions
Cells were harvested and suspended in 3 mM imidazole buffer in 250 mM sucrose, with protease and phosphatase inhibitors, and homogenized by passage (30x) through a 22-gauge needle as previously described (Felberbaum-Corti et al., 2005). Postnuclear supernatants (prepared by centrifugation of homogenates at 1200 x g for 10 min) were centrifuged for 1 h at 120,000 x g, the cytosolic fraction (120,000 x g supernatant) was collected, and the membrane fraction (120,000 x g pellet) was resuspended in one-half the volume of homogenization buffer. Equal-volume samples of cytosolic and membrane fractions were resuspended in 2x sample buffer and analyzed by immunoblotting.

In vitro protein-binding assays
A total of 20 μg of purified GST-Goxs or GST alone was immobilized on glutathione–Sepharose (GE Healthcare) in buffer B (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.4% [vol/vol] NP-40, 10 mM MgCl2, 5 mM EDTA, 2 mM DTT, protease and phosphatase inhibitors, 30 μM GDP, 12 mM DTT, ± 30 μM AlCl3, and 10 mM NaF) as described (Garcia-Marcos et al., 2009). Immobilized GST-Goxs or GST was incubated overnight at 4°C with purified histidine-tagged GIV-CT (1623-1870) or HeLa or Cos7 cell lysates prepared in buffer A as for immunoprecipitation. Beads were boiled in 2x sample buffer and analyzed by immunoblotting.

Statistical and image analysis
Each experiment presented in the figures is representative of at least three independent experiments. All averages, SEMs, and significance p values (t test) were calculated and graphed using Excel (Microsoft, Redmond, WA). Quantification of IF images was carried out using Velocity software. All images were processed and figures assembled using Photoshop software (Adobe, San Jose, CA).

ACKNOWLEDGMENTS
We thank Gordon N. Gill for anti-EGFR antibodies, Gill and Pradip Ghosh for scientific advice and thoughtful comments during preparation of the manuscript, and Steve Dowdy for use of his LSRII flow cytometer. This work was supported by National Institutes of Health Grant R01-CA100768 to M.G.F. M.G.M. was supported by a fellowship from the Susan G. Komen Foundation. Light microscopy facilities were supported in part by University of California, San Diego, Neuroscience Microscopy Shared Facility Grant P30 NS047101.

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