Phosphomodification of GIV S1674 alters interactions with heterotrimeric G proteins to regulate cell proliferation and cell migration

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Phosphomodification of GIV S1674 alters interactions with heterotrimeric G proteins to regulate cell proliferation and cell migration

A Thesis submitted in partial satisfaction of the requirements for the degree of Master of Science

in

Biology

by

Andrew To

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2014
The Thesis of Andrew To is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego
2014
I dedicate this thesis to my mom and dad, for your love and support has given me this opportunity to excel.
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“I can do all things through Christ who strengthens me.” (Philippians 4:13)
ABSTRACT OF THE THESIS

Phosphomodification of GIV S1674 alters interactions with heterotrimeric G proteins to regulate cell proliferation and cell migration

by

Andrew To

Master of Science in Biology
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Professor Marilyn G. Farquhar, Chair
Professor Amy Kiger, Co-Chair

Changes in motogenic and mitogenic signaling pathways via epidermal growth factor receptors (EGFR) are characteristic of some cancer cells. We have shown that EGFR signaling can be fine-tuned by the Gα-interacting vesicle-associated protein (GIV/Girdin), a multidomain molecule that modifies EGFR signal transduction pathways via interactions with heterotrimeric G proteins. GIV can assemble a complex
with EGFR and Gαi3, leading to prolongation of EGFR signaling from the plasma membrane and resulting in amplification of migratory signaling. Trafficking of activated EGFR through early endosomes is regulated by interactions between Gαs and GIV to attenuate proliferation. In this study, we have pinpointed a heavily phosphorylated residue, S1674, near GIV’s GEF domain that alters interaction with both Gαi3 and Gαs upon EGF stimulation. We showed that a nonphosphorylatable mutation at GIV’s S1674 abolishes the binding of GIV to Gαi3 and Gαs. Using immunofluorescence, we found that the stay of activated EGFR in EEA1 early endosomes was prolonged, causing cells to proliferate more and migrate less than normal cells. However, in cells containing a phosphomimetic mutation, the binding of GIV to Gαi3 and Gαs is enhanced, resulting in increased migration and decreased proliferation in comparison to wild-type cells. The results of this study indicate that phosphorylation of GIV S1674 is important for modifying EGFR trafficking and signaling and for determining whether cells preferentially migrate or proliferate in response to EGFR stimulation. We hope that these findings may ultimately be useful in designing therapeutic agents capable of modifying abnormal cell growth that occurs in various cancers.
INTRODUCTION

Cell signaling by GPCRs and RTKs

Cell signaling is one of the most important bases of communication within a cell, organizing cell actions and controlling cellular activities to maintain homeostasis [1]. Much of this signaling is associated with the cell’s response to external stimuli through a mechanism known as signal transduction, where the binding of extracellular signaling molecules transmits information to specific receptors on the plasma membrane, which causes a cascade of signals inside the cell to occur [2]. Molecularly, this transduction of information across the cell membrane gives rise to a multitude of intracellular protein-protein interactions to further amplify the signal.

There are two main classes of receptors, the G Protein Coupled Receptors (GPCRs) and the Receptor Tyrosine Kinases (RTKs). The seven transmembrane GPCRs are known to be very important receptors for the human body due to their regulation of a wide spectrum of physiological processes. They initiate signaling via the cAMP and the Phosphotidylinositol pathways, both of which are key to cell survival [3]. Heterotrimeric G proteins, the key molecular switch that is involved in transmitting signals into the cells, are activated by these GPCRs. Upon binding of hormones, neurotransmitters, or various other molecules, GPCRs undergo a conformational change that causes them to act as a guanine nucleotide exchange factors (GEFs). As GEFs, they cause the G protein to release GDP in exchange for a GTP, which then allows the G protein to dissociate into the α and βγ subunits. The GTP-bound α subunit can then trigger and activate a cascade of other intracellular proteins in its downstream signaling pathways [3, 4]. Depending on the type of α
The second class of receptors that function in cell signaling are receptor tyrosine kinases (RTKs). As a transmembrane receptor, their actions of facilitating signals from the extracellular environment into the cell are important for the regulation of cellular processes such as growth, proliferation, migration, and cell survival [5, 6]. They consist of an extracellular region with a ligand-binding domain, a transmembrane helix, and an intracellular region which contains the catalytic tyrosine kinase domain [7]. Activation of the receptor by the binding of growth factors lead to the dimerization of two receptor monomers, which allows the monomers to phosphorylate each other, thereby propagating a signal across the cell membrane [2, 8]. The key difference that distinguishes RTKs from GPCRs is that with RTKs, downstream signaling is transduced by the initiation of autophosphorylation during dimerization [9]. This action creates binding sites on the intracellular region of the receptor for proteins containing a phosphotyrosine and Src Homology 2 (SH2) domain, which leads to the binding of adaptor proteins for downstream signaling.

**Epidermal Growth Factor Receptor**

Of these RTKs, the Farquhar lab has extensively studied epidermal growth factor receptors (EGFRs), which when activated by epidermal growth factor (EGF), lead to several signal transduction pathways. EGFRs play a role in signaling for cell migration and cell proliferation through two different signaling cascades, the phosphoinositide 3-kinase (PI3-kinase)/Akt pathway and the ERK pathway, respectively [6]. The migratory pathway is facilitated by activation of PI3-kinase that
activates Akt to trigger cell migration [10]. Proliferative signaling is initiated by the binding of growth factor receptor-bound protein 2 (GRB2) to EGFR at the phosphotyrosine domain, which then recruits SOS, Ras, and Raf binding proteins. Their interaction activates MEK and ultimately ERK, which mediates cell proliferation [11].

Changes in signaling via EGFRs and its pathways are some of the many aberrations characteristic of cancer cells and other diseases [12]. In cancer cells, EGFRs are often overexpressed or mutated, leading to changes in mitogenic (proliferative) or motogenic (migratory) signaling [5, 13]. Under normal conditions, down-regulation of EGFR activation is managed by endocytosis [14]. Upon activation, EGFRs are rapidly internalized through clathrin-coated pits and delivered to early endosomes where they are sorted to lysosomes for degradation (Figure 1). Some take another path and are recycled back to the cell membrane [15, 16]. Studies demonstrate that although EGFR signaling is initiated at the cell membrane, signaling is continued from endosomes until the receptor is degraded [16]. Signaling at the cell membrane mostly activates migratory signaling through the Akt pathway while proliferative signaling occurs at endosomes through the ERK pathway [15, 17]. Thus, the endocytic behavior of EGFRs regulates downstream signaling in a spatial-temporal fashion to determine the strength and duration of signals influencing cell dynamics [18, 19].

Classically it has been assumed that G proteins act exclusively downstream of GPCRs. It was unknown as to whether EGFR and G proteins had any sort of connection. Previous studies done by Bomsel and Mostov et al. have suggested that G proteins take part in membrane trafficking [4]. More importantly, they proposed that
the stimulatory subunit of G protein, $\text{G}_\alpha$s, regulated endocytic trafficking. The Farquhar lab provided further indication that this is the case when we discovered RGS-PX1, a member of the regulator of G protein signaling (RGS) that serves as a GTPase activating protein (GAP) specifically for $\text{G}_\alpha$s [20]. We showed that its presence delayed EGFR degradation, which suggests that $\text{G}_\alpha$s might be involved in regulating EGFR.

Previous studies by the Farquhar lab on EGFR used overexpression and knockdown assays to investigate the G protein’s regulation of EGFR endocytic trafficking [21]. Focusing on the stimulatory $\text{G}_\alpha$s subunit, it was revealed that heterotrimeric $\text{G}_\alpha$s-protein is located on endosomes and plays a regulatory role in EGFR trafficking. When $\text{G}_\alpha$s is overexpressed in the cell, EGFR degradation was accelerated when compared to wild-type cells. Conversely it was shown that when $\text{G}_\alpha$s was knocked down by RNA-interference, degradation of activated EGFR was delayed [21]. These studies by Zheng et al. indicated that $\text{G}_\alpha$s plays a role in regulating EGFRs at the endosomal level.

Since EGFR does not have a GEF domain to activate G proteins, the question still remained as to how EGFR’s downstream signaling is affected by manipulating the presence of G proteins.

**Discovery of GIV, a multidomain molecule**

Because $\text{G}_\alpha$ proteins take part in a variety of pathways in the cell, extensive research was done to identify proteins that interact with G proteins. Through a yeast
two-hybrid screen, the Farquhar lab identified a Gα-interacting vesicle-associated protein (GIV/Girdin) that binds to members of the Gαs and Gαi subfamilies of heterotrimeric G proteins in the Gα-binding domain (Figure 2A) [22]. Further studies done by Le-Niculescu et al. show that GIV is found on COPI transport vesicles in the Golgi region [22].

Around the same time, several labs also independently discovered GIV by virtue of its interaction with other cellular components. Together these studies showed that GIV is a large multidomain molecule (Figure 2A) that is involved in turning on or off many biochemical processes in cells [23]. Simpson and Enomoto et al. showed that GIV contains a hook domain that is responsible for binding and regulating microtubules, and a coiled-coil domain that mediates homodimerization [24, 25]. The research of Enomoto et al. also showed that GIV contains a domain that binds phosphatidylinositol 4'-monophosphate (PI4P), which facilitates interaction with the Golgi and the plasma membrane [25, 26]. Together with Anai et al., they have also identified regions in the C-terminus that regulate actin remodeling for cell migration, Akt kinase, and DNA synthesis [25-27].

**GIV and Gαi3 play a role in Akt signaling and cell migration**

Because GIV was shown to regulate cell migration by its interaction with PI3-Kinase/Akt and that it binds to Gαi3, we sought to define the role of this G protein and its interaction with GIV on downstream signaling pathways. Studies by Ghosh et al. showed that during cell migration, Gαi3 moves to the leading edge of the cell where migration is occurring [10]. When Gαi3 was depleted, cell migration was impaired as
shown by the finding that the cells were unable to close a wound. Likewise when GIV was depleted, wound healing was also impaired. This study also provided evidence that Gai3 is required for Akt signaling, which is similar to the study by Anai et al where they showed that GIV was required for Akt activation [10, 27]. Although Niculescu et al. had previously shown binding between Gai3 and GIV, the question still remained as to whether these two proteins function in the same common pathway, or do they signal through independent pathways that mediate Akt activation [22]. When Gai3, GIV or both were depleted in cells, there were no significant differences in levels of activated Akt, solidifying previous studies that GIV and Gai3 form a complex that is important for Akt signaling to promote cell migration [10, 27].

Further studies from the Farquhar lab have shown that GIV contains a unique C-terminal GEF domain that specifically activates the trimeric G-protein, Gai3 (Figure 2) [28]. Through the use of bioinformatics, mutagenetic approaches, and molecular modeling, GIV’s GEF domain was identified to contain critical residues that form the GIV-Gai3 interface. This stretch of amino acids was shown to be highly conserved across mammals. In-vivo assays showed that disruption of this interface by mutating a key amino acid in GIV (Phenylalanine, F1685 to Alanine, F1685A) abolished their interaction and impaired the role of GIV as an Akt enhancer for cell migration and actin remodeling in normal cells [10, 28].

**GIV forms a complex with Gai3 and EGFR**

Studies by Ghosh et al. demonstrated that the interaction between GIV and Gai3 plays a crucial role in regulating EGFR dynamics [17]. GIV modifies EGFR
signal transduction pathways via interactions with heterotrimeric G-proteins by assembling a Gai3-GIV-EGFR signaling complex that can lead to prolongation of EGFR signaling from the plasma membrane. This results in amplification of migratory signaling. It was also shown that in cells expressing a GEF-deficient mutant (F1685A), the signaling complex does not form, causing EGFR signaling to favor proliferative signaling over migratory signaling.

Recent work has provided evidence that phosphorylation of GIV’s GEF domain alters its interaction with G-proteins. Specifically, phosphorylation of GIV at Serine-1689 (S1689) by protein kinase C-theta (PKCθ) reduces its interaction with Gai3. This phospho-residue within the GEF domain is highly conserved. Mutation of S1689 to Aspartic acid to mimic its phosphorylation showed that GIVs interaction with Gai3 was abolished, resulting in decreased motogenic Akt signaling whereas mitogenic ERK signaling was increased [29].

**GIV interacts with Gαs to promote EGFR degradation and attenuate proliferative signaling**

Recent studies by Beas et al. provided evidence that GIV’s interaction with Gαs plays significant roles in EGFR trafficking and thus activation of downstream kinases from EEA1 endosomes [19]. Disruption of the GIV/Gαs complex by knockdown of either Gαs or GIV, led to the accumulation of EGFR in EEA1 early endosomes. It was also shown that maturation of EEA1 endosomes to late endosomes (LAMP-2) and lysosomes was delayed after Gαs knockdown as more EGFR remained at EEA1 and LAMP-2 endosomes than in WT cells.
Important in this study was that the pool of EGFR that was prolonged in EEA1 endosomes contained activated EGFR. Prolonging the residence of activated EGFR at this particular compartment of the cell caused amplification of mitogenic signaling via the ERK pathway [17]. Staining for phospho-Histone H3 (pH3) and incorporation of Bromodeoxyuridine (BrdU), both of which are indicators of cell division, showed that when EGFR is delayed in EEA1 early endosomes, ERK signaling is enhanced and cell proliferation is increased.

Although G proteins are found throughout the cell, Beas et al. demonstrated that Gαs was primarily associated with GIV at EEA1 early endosomes and not at the cell membrane where interaction with Gai3 takes place. pAKT is affected only when Gai3 and GIV interaction is disrupted [10, 17, 28]. Through these studies, we concluded that the interaction of Gαs with GIV is necessary for maturation of early endosomes so that mitogenic (ERK) signaling can be turned off (Figure 1).

**Hypothesis and Aim**

The accumulated findings revealed that binding of GIV to Gai3 activates cell migration while the binding of GIV to Gαs limits cell proliferation [10]. This provides a greater understanding of the migration-proliferation dichotomy of cells, where cells will either migrate or proliferate, but cannot do both at the same time [17]. These studies have shown that GIV’s GEF motif plays an important role in cellular dynamics. Upon EGF stimulation, GIV is able to bind and form a complex with EGFR, activate Gai, and promote migratory signaling at the plasma membrane through Akt (Figure 1) [22]. After internalization into clathrin-coated pits, EGFR is
trafficked through early endosomes where ERK signaling occurs. However, signaling from early endosomes is short-lived because GIV binds inactive $G_\alpha_s$, which promotes degradation of EGFR.

Through a mutagenesis approach, we have considerable knowledge pertaining to GIV’s interaction with $G_{i3}$ [17, 28, 29]. However, many questions still remained unanswered concerning GIV’s interaction with $G_\alpha_s$. In normal cells, GIV’s interaction with $G_{i3}$ is much stronger than to $G_\alpha_s$, which leads us to question what specifically regulates the interaction between GIV and $G_\alpha_s$. Based on the studies of GIV’s GEF activity, I believe that phosphomodifications at or near the GEF motif may regulate this interaction. Recent mass spectrometric studies in cells and tissues have identified another conserved serine residue, S1674, which is located near the GEF domain that may play an important role in its interaction with G proteins ($G_{i3}$ and $G_\alpha_s$) [30-42]. In this study, I have taken advantage of the mutagenic approach on residue S1674 and subsequently used protein interaction and trafficking assays to study GIV’s role in regulating EGFR signaling and to ultimately provide more information on migration-proliferation dichotomy. I hypothesize that a phosphomimetic mutation at S1674 will alter GIV’s interaction with both $G_{i3}$ and $G_\alpha_s$, and affect cellular dynamics of EGFR. Based on this, I focused on the following three aims: (1) to investigate how a phosphomimetic or nonphosphorylatable mutant at S1674 may alter binding to $G_{i3}$ and/or $G_\alpha_s$; (2) to determine the effects of these mutations on EGFR trafficking; and (3) to determine the physiological relevance of S1674 on cell proliferation and migration.
RESULTS

GIV-CT contains evolutionarily conserved sequences with heavily phosphorylated sites.

GIV is a modular protein consisting of various domains carrying out specific functions (Figure 2A). The extreme N-terminus is made up of the hook domain that facilitates interaction with microtubules. Following this is the coiled-coil sequence of GIV that facilitates its dimerization [25]. The next domain is the Gα-binding domain (GBD) which as the name suggests, plays a role in the interaction with Gαi and Gαs [22]. The C-terminus of GIV is the domain responsible for its interaction with actin, Akt kinase, and EGFR where it modulates growth factor signaling. Situated in the middle of this region is the guanine nucleotide exchange factor (GEF) motif that plays a key role in maintaining the interaction and activation of Gαi3 as well as interaction with Gαs [17, 19, 28, 29, 43]. Previous findings in our lab on the C-terminus of GIV have shown it to be highly conserved from fish (T. nigroviridis) to mammals (H. sapiens) [28], especially in a short stretch of amino acids known as the GEF motif (amino acid 1678-1694) (Figure 2B). Studies done in the Farquhar and Ghosh labs have identified two key residues within the GEF motif (F1685 and S1689) through site-directed mutagenesis, which affect the interaction of GIV and Gα proteins, leading to altered cell signaling [28, 29]. The C-terminus of GIV has been shown to be extensively phosphorylated in multiple high-throughput studies (phosphosite.org). To gain further insight into the role of phosphorylation in regulation of GIV’s function, we performed a mass spectrometric analysis on GIV’s C-terminus (expressed and purified from Cos7 cells). We identified multiple phosphorylated residues in GIV with
S1674 being the most heavily phosphorylated site, which has also been reported by various other groups [30-42]. Since S1674 is an evolutionarily conserved residue that resides adjacent to the GEF motif (Figure 2B), we hypothesized that phosphorylation at S1674 may affect GIV’s ability to bind G\(\alpha\)i3 and/or G\(\alpha\)s.

**Phosphorylation at S1674 regulates binding to both G\(\alpha\)i and G\(\alpha\)s.**

We first asked how the binding of G\(\alpha\) proteins to GIV is affected when GIV is phosphorylated at S1674. To this end, we generated a phosphomimetic S1674 to aspartic acid [S1674\(\rightarrow\)D (74D)] mutation using the GST-GIV-C-terminus (CT) (1660-1870) construct as a template. After the mutation was confirmed by sequencing, we expressed and purified GST-GIV-CT (WT and 74D) as well as GST alone from BL21-DE3 bacterial cells. His-tagged G\(\alpha\)i3 and G\(\alpha\)s proteins were also purified in a similar fashion and were tested for their ability to bind to GST-GIV-CT (WT and 74D) immobilized on Glutathione sepharose beads in a GST pull-down assay. Purifying the proteins from bacteria gave us the advantage that GIV displays a phosphomimetic charge only at our residue of interest and no modifications elsewhere. We found that the phosphomimetic mutant shows enhanced binding to both G\(\alpha\)i3 and G\(\alpha\)s in comparison to GIV-WT (Figure 3A and 3B).

**Nonphosphorylatable GIV mutant abolishes binding of G\(\alpha\)-proteins.**

To further explain S1674’s importance in G-protein binding, we generated phosphomimetic (S1674D, 74D) and nonphosphorylatable serine 1674 to alanine
[S1674→A (74A)] mutations on pCEFL-GST-GIV CT (1660-1870) and tested the binding of the mutants to endogenous G proteins using an \textit{in cellulo} GST-pull down assay. Equal amounts of GST-CT-WT and the mutants were transfected into Cos7 cells. Cell lysates were generated 48 h post-transfection and incubated with equilibrated Glutathione-sepharose beads. The bound proteins were analyzed by Western blotting and, as expected, we observed increased binding of both Gαi3 and Gαs to 74D in comparison to WT (Figure 4). The GIV-74A mutant, on the other hand, failed to bind either Gαi3 or Gαs, suggesting that phosphorylation of S1674 plays an important role in mediating interaction between GIV and Gα subunits.

The phosphomimetic, but not the nonphosphorylatable mutation in full-length GIV co-immunoprecipitates with both Gα-proteins.

Because we showed that binding of Gαi3 and Gαs to GIV-CT-74D was enhanced while GIV-CT-74A decreased binding to either G-protein, we next wanted to confirm that the same is true for full-length GIV. When Cos7 cell lysates expressing Myc-tagged full-length GIV and FLAG-tagged Gαi3 and Gαs were immunoprecipitated with anti-FLAG antibody and analyzed by western blotting, GIV-74D but not GIV-74A co-immunoprecipitated with Gαi3 and Gαs (Figure 5). GIV-74D once again showed enhanced binding to both G-proteins in comparison to GIV-WT. Based on these three assays (Figures 3, 4, and 5), we concluded that GIV’s binding to Gαi3 and Gαs is enhanced by its phosphorylation at S1674.
S1674A delays trafficking of EGFR.

We next asked how the phosphomimetic or nonphosphorylatable GIV mutants affect EGFR trafficking through EEA1 positive early endosomes. Previous studies in our lab have shown that depletion of either Gαs or GIV delays the trafficking of EGFR out of EEA1 early endosomes. Because a mutation at S1674 affects binding of both Gα-proteins, we hypothesized that a change in binding may affect EGFR trafficking as well. To this end, we created HeLa stable cell lines expressing GIV-WT, GIV-74D, and GIV-74A DNA. Cells plated on coverslips were stimulated with 50 nm EGF and co-stained for EGFR and the early endosome marker EEA1. As shown in Figure 6, at 0 min of EGF stimulation, EGFR localized at the PM in all three cell lines. After 5 min, some colocalization of EGFR to EEA1 was seen in GIV-74D cells, but not in GIV-WT or GIV-74A cells. At 15 min, EGFR colocalized with EEA1 endosomes in GIV-WT and both GIV mutants. However, more colocalization was seen in GIV-74A in comparison to both GIV-WT and GIV-74D. By 30 min, EGFR were not seen in EEA1 endosomes in control and GIV-74D cells. However, GIV-74A still contained EGFRs in EEA1 endosomes. These findings suggest that the nonphosphorylatable GIV mutant prolongs the stay of EGFR at endosomes and delays its trafficking to lysosomes.

S1674A prolongs EGFR autophosphorylation at EEA1 endosomes.

We next investigated whether the prolonged duration of EGFR in EEA1 endosomes in GIV-74A cells also affects receptor signaling. We treated and stained cells as in Figure 6 using the anti-EEA1 and anti-pY1068 EGFR (SH2 docking site of
Grb2) antibodies [19, 44]. Before EGFR stimulation (0 min), very little to no pEGFR was observed in either GIV-WT, GIV-74D, or GIV-74A cells (Figure 7). At 5 min after stimulation, activated EGFRs were localized in EEA1 endosomes in GIV-74D, but not GIV-WT or GIV-74A cells suggesting a faster endocytosis of EGFRs. By 15 min after stimulation, all three cell lines displayed colocalization of EGFR at EEA1 early endosomes, but the amount in GIV-74D cells was decreased in comparison to GIV-WT and GIV-74A cells. It was also noted that EEA1 endosomes varied in size at 15 min in the three cell lines. Endosomes containing pY1068 EGFR were the smallest in GIV-74D cells and largest in GIV-74A cells in comparison to GIV-WT. At 30 min, no colocalization and very little amounts of activated EGFRs remained in the cytoplasm of GIV-WT and GIV-74D cells. However, GIV-74A cells contained a higher amount of activated EGFRs over GIV-WT and GIV-74D cells. In addition, GIV-74A cells also retained some activated EGFR in EEA1 endosomes indicating prolonged signaling by EGFR from endosomes.

**Phosphomimetic mutation S1674D increases cell migration.**

Because binding of Gαi3 to GIV-74D was enhanced, and since we know that migratory Akt signaling is associated with this binding [17], we then asked how the phosphomimetic GIV-74D and nonphosphorylatable GIV-74A mutations affect cell migration. Stable cell lines were starved overnight (0.2% FBS) and the plates were scratch-wounded (0 hr). Total area of the wound covered over 48 hr was recorded by microscopy, quantitated and normalized as a percentage of that covered by GIV-WT (set to 100%).
Analysis of the area covered by cells showed that GIV-74D cells closed the wound almost 50% faster in comparison to WT (Figure 8B). In the GIV-74A mutation, however, cell migration was significantly slower as indicated by the area covered compared to its phosphomimetic counterpart and about 25% less than GIV-WT (Figure 8A). These results indicate that phosphorylation of GIV leads to increased cell migration and motogenic signaling.

**Nonphosphorylatable GIV mutant enhances cell proliferation.**

Based on the delayed trafficking and prolonged stay of active EGFR on early endosomes in the GIV-74A mutant, we reasoned that it might enhance proliferative signaling from the endosomal compartment. To test this, Hela cells stably expressing GIV-WT, 74D, and 74A were grown in low serum overnight (2% FBS) and incubated in BrdU for 30 min. After staining with anti-BrdU, there were significantly more cells that stained positive for BrdU in GIV-74A in comparison to GIV-WT (Figure 9A). We found a significantly less number of cells undergoing proliferation in 74D as compared to GIV-WT. These results are consistent with the reduced stay of activated receptor in endosomes in the case of GIV-74D cells and the prolonged stay of EGFR in early endosomes in GIV-74A cells (Figure 7). Quantification showed that GIV-74A contained 53% more cells than GIV-WT with BrdU positive staining. On the other hand, there were about 37% less cells undergoing proliferation in GIV-74D when compared with GIV-WT (Figure 9B). Thus, a phosphomimetic mutation at S1674 promotes cell migration while a phospho-deficient mutation increases cell proliferation.
Figure 1. GIV facilitates the trafficking and signaling of EGFR by binding to Gαi3 and Gαs. Upon EGF stimulation, an EGFR-GIV-Gαi3 complex forms and activates Gαi3 [1]. This prolongs the stay of EGFR on the PM for Akt signaling to occur [2]. After internalization into clathrin-coated pits, EGFR is trafficked through early endosomes where ERK1/2 signaling occurs [3 and 4]. GIV then binds inactive Gαs to promote maturation of EEA1 early endosomes and degradation of EGFR [5]. Receptors may also be recycled back to the cell membrane to replenish the amount of EGFR for further usage [6].
Figure 2. Domain architecture of GIV. (A) The N-terminal Hook domain (blue) interacts with microtubules, the coiled-coil domain (green) mediates homodimerization, the Gα-binding domain (GBD, yellow) interacts with a α-subunits of Gαi and Gαs, and the extreme C-terminus (purple) interacts with EGFR, Akt kinase, and actin. The guanine exchange factor (GEF) domain (red), which interacts with Gαi and Gαs and activates Gαi is located within the C-terminus. (B) Diagram of GIV showing the phylogenetically conserved sequence of the GEF motif (in red box) of GIV and its surrounding residues. The conserved and heavily phosphorylated Ser1674 (in orange box) is shown. Sequences obtained from the accession numbers (in brackets) were aligned using Clustal W. Conserved residues are shaded in black and similar residues in gray.
Figure 3. Phosphomimetic mutation at S1674 in GIV enhances its interaction with Gαi3 and Gαs in vitro. Equimolar amounts of purified His-tagged Gαi3 (A) or Gαs (B) were incubated with bacterially expressed and purified GST, WT or 74D GST-GIV-Cytoplasmic Tail (CT) immobilized on glutathione sepharose beads (n = 3). Bound proteins were analyzed by immunoblotting for His (Gαi3 and Gαs). Equal amounts of GST-CT was confirmed by immunoblotting for GST. Input represents 2% of the total His-tagged proteins used in the binding reactions. Both Gαi3 and Gαs showed enhanced binding to 74D GIV-CT as compared to WT GIV-CT.
Figure 4. Nonphosphorylatable GIV mutant shows impaired binding to both Gαi3 and Gαs in cellulo. Cos7 cells were transiently transfected with WT, 74D, or 74A GST-GIV-CT, and clarified cell lysates were incubated with glutathione-Sepharose beads (n = 3). Bound proteins were analyzed by immunoblotting for endogenous Gαi3 and Gαs. Equal loading of GST and GST-GIV-CT proteins was confirmed by immunoblotting for GST (pull-down) and for Gαi3, Gαs, and Actin (lysates). While GIV-74D mutant showed enhanced binding to both Gαi3 and Gαs, the GIV-74A mutant showed greatly decreased binding of GIV-CT to Gαi3 and Gαs.
Figure 5. The phosphomimetic but not the nonphosphorylatable form of full length GIV co-immunoprecipitates with Gαi3 and Gαs. Immunoprecipitation was carried out with anti-FLAG mouse antibody on equal aliquots of Cos7 lysates co-expressing FLAG-Gαi3 (A) or FLAG-Gαs (B) and full-length Myc-tagged GIV-WT, 74D, or 74A followed by incubation with protein-G sepharose beads (n = 3). The bound immune complexes were analyzed for FLAG and Myc by immunoblotting and equal loading was confirmed by immunoblotting for Tubulin (Lysates). Both FLAG-Gαi3 and FLAG-Gαs co-immunoprecipitated GIV 74D better than GIV-WT whereas binding of GIV-74A was impaired.
Figure 6. Nonphosphorylatable GIV mutant delays EGFR trafficking from early endosomes. Cells stably expressing GIV-WT, 74D, and 74A were serum starved (0.2% FBS) overnight and stimulated with 50 nM EGF for 0, 15, 30 min. Cells were fixed (3% paraformaldehyde), stained for total EGFR (green) and EEA1 (red), and examined by confocal microscopy. Before EGF stimulation (0 min), EGFRs do not localize with EEA1. By 5 min, EGFR is colocalized at EEA1 early endosomes in GIV-74D, but not in GIV-WT and GIV-74A cells. At 15 min after EGF stimulation, colocalization of EGFR with EEA1 is seen in all three cell lines (yellow, arrows). By 30 min, colocalization does not occur in GIV-WT and GIV-74D, but in GIV-74A, EGFR is still retained in EEA1-positive endosomes.
Figure 7. Nonphosphorylatable GIV mutant enhances EGFR signaling from EEA1 endosomes. Cells stably expressing GIV-WT, 74D, and 74A were treated as in Figure 5 and stained for activated (phosphorylated) pY1068-EGFR (green) and EEA1 (red). All three cell lines show no pY1068 staining at 0 min. At 5 min in GIV-74D, activated pY1068 EGFRs are colocalized with EEA1 endosomes, whereas colocalization is not seen in GIV-WT and GIV-74A. At 15 min after EGF stimulation, pY1068 colocalized with EEA1 early endosomes in GIV-WT, GIV-74D, and GIV-74A. EEA1 endosomes containing activated EGFR also varied in size. GIV-74D cells showed the smallest endosomes while GIV-74A showed the largest in comparison to GIV-WT. By 30 min, both WT and 74D cells show very few pY1068 EGFRs, none of which co-localize with EEA1. In contrast, GIV-74A cells still show pY1068 EGFRs localized on EEA1-positive endosomes 30 min post-stimulation.
Figure 8. Phosphomimetic mutation at S1674 in GIV enhances cell migration. (A) HeLa cells stably expressing GIV-WT, GIV-74D, and GIV-74A were subjected to scratch wounding and examined immediately after wounding (0 hr) and 48 hrs later (n = 3). The GIV-74D cells showed enhanced migration in scratch-wound assays in comparison to cells expressing GIV-WT. Stable expression of nonphosphorylatable GIV (GIV-74A) slowed down cell migration. (B) Bar graph showing quantification of cell migration in A. Migration index in WT cells was set to 100% and those of the mutants were calculated accordingly.
Figure 9. **GIV-74A cells show enhanced proliferation.** HeLa cells stably expressing GIV-WT, GIV-74D, and GIV-74A were grown in low serum (2% FBS) overnight, incubated in BrdU for 30 min, fixed (3% paraformaldehyde), stained for BrdU, and analyzed by confocal microscopy (n = 3). (A) The number of cells that incorporated BrdU (green) is increased in GIV-74A compared to GIV-WT and GIV-74D. GIV-74D cells had significantly less incorporated BrDU. (B) Bar graph presenting percent of cells showing BrdU incorporation. GIV-74A has a 53% increase in BrdU incorporation over GIV-WT, while GIV-74D show a 37% reduction as compared to GIV-WT.
Figure 10. Working Model. This is a schematic illustration of EGFR trafficking and cellular phenotype in cells expressing phosphomimetic GIV-74D mutation (left panel) or nonphosphorylatable GIV-74A mutation (right panel). When GIV’s C-terminus contains a phosphomimetic mutation at S1674, this enhances the binding of GIV to Gαi3, causing increased migratory signaling. To maintain enhanced migration, endocytosis of EGFR is accelerated. Once internalized, the enhanced binding of GIV to Gαs swiftly moves endosomes containing activated EGFR to degradation or rapidly recycles the receptor back to the membrane to facilitate motogenic signaling. On the other hand, when GIV contains a nonphosphorylatable mutant, an EGFR-GIV-Gαi3 complex does not form and the receptor is internalized into endosomes. Because GIV-74A abolishes the binding between GIV and Gαs, receptor degradation is delayed, causing signaling by EGFR in early endosomes to be prolonged and proliferative signaling to be promoted. Thus, phosphorylation of GIV at S1674 is essential for regulation of EGFR trafficking and signaling.
DISCUSSION

Previous findings from the Farquhar lab demonstrated that GIV and Gα proteins play an important role in compartmentalizing the signaling of EGFR [17, 19]. GIV’s ability to bind to G proteins in a yeast two-hybrid assay and later discovery of its GEF domain revealed it to be a crucial site of G protein interaction [22, 28]. As our research dug deeper into GIV’s role in signaling pathways, we found that it binds to EGFR upon stimulation to form a complex with Gai3 [17]. This provided a link between EGFR and activation of G proteins, which was previously thought to be done only by GPCRs [4].

Using EGFR as our model (Figure 1), we have shown that after EGF stimulation, activated EGFR forms a complex with GIV and Gai3 to induce Akt signaling from the plasma membrane [17]. As the cell internalizes EGFR into clathrin-coated pits, signaling of EGFR continues within early endosomes [16, 19]. The interaction between GIV and Gαs facilitates endosome maturation and downregulation of ERK signaling [19]. Through this model, the GEF motif plays a key role in facilitating G protein interaction, regulating different signaling pathways and controlling where they occur.

Noting that GIV’s C-terminus is the site of interaction with both Gai3 and Gαs, we targeted this region for further analysis. Through mass spectrometric analysis on GIV’s C-terminus, we found multiple phosphorylated residues in this area, with the major phosphorylated site being S1674 (Figure 2). Studies from other labs have also identified this heavily phosphorylated residue due to its correlation with kinase activities and activation of downstream signaling [30-42]. With this identification, my
work demonstrates that S1674, a specific residue near the GIV’s GEF domain, can alter how the cell responds to EGFR stimulation.

**GIV’s S1674 interaction with G protein determines EGFR signaling**

In order to understand this residue’s role with G proteins, we mimicked phosphorylation at S1674 (GIV-74D) and also created a nonphosphorylatable form (GIV-74A) to pinpoint how the residue interacts with G proteins. Using these unique tools, our present work provides further evidence for GIV’s essential role in compartmentalizing EGFR signaling [17]. Compartmentalizing through receptor endocytosis allows the cell to regulate the myriad of pathways in the cell, in part by shutting down signaling pathways or upregulating others to compensate for its needs.

Previous studies in our lab have provided knowledge on two important areas of the cell that facilitate migratory or proliferative signaling. It was shown that signaling at the plasma membrane is regulated by Gαi3 to induce motogenic signaling, while receptor trafficking data identified Gαs as the key player at EEA1 early endosomes where mitogenic signaling occurs [10, 17, 19, 28, 43, 45]. Based on these studies, when EGFR’s residence at either the cell membrane or EEA1 early endosome is prolonged, there will be an upregulation of EGFR signaling from those regions.

In order to further understand compartmental receptor signaling, we focused on EEA1 early endosomes by specifically looking at GIV’s binding to Gαs. The studies done *in vitro* and *in vivo* showed that a S1674D mutation causes GIV’s binding to Gαs to be greatly enhanced while a S1674A mutation causes failure of GIV to bind Gαs (Figure 2, 3, 4). This failure of binding is in keeping with the study by Beas *et al.*, 2006.
where a knockdown of either binding partner delayed EEA1 endosome maturation and enhanced proliferative signaling from that compartment [19]. We showed a similar trend in GIV-74A cells at 30 min after activation of EGFR that when GIV cannot bind Gαs, there was still colocalization of activated EGFR and EEA1 endosomes, thus showing that the stay of EGFR in early endosomes is prolonged. Another indication of the prolonged stay of EGFR may be identified by the size of EEA1 early endosomes. GIV-74A showed larger endosomes than both GIV-WT and GIV-74D, presumably to the accumulation of receptor at this compartment. This increase in size harbors more activated EGFR and thus signaling will be enhanced from EEA1 endosomes.

Moreover, we also showed that EGFR autophosphorylation is short-lived in EEA1 endosomes in GIV-74D cells. Similar to total-EGFR trafficking, colocalization of activated EGFR with EEA1 endosomes was seen at 5 min in GIV-74D cells, as indicated by staining for pY1068-EGFR. This demonstrated that a S1674D mutation might cause accelerated trafficking of EGFR to EEA1 early endosomes (Figure 10). When comparing EEA1 endosomes containing activated EGFR, GIV-74D cells had the smallest endosomes of all three cell lines, which indicated that the stay of EGFR at endosomes is reduced. In addition, both GIV-WT and GIV-74D did not show active EGFR at EEA1 endosomes at 30 min, presumably because EGFR has already passed through early endosomes. Also in GIV-74D cells, there was less activation of receptor in comparison to WT. This revealed that enhanced binding of GIV to Gαs might accelerate the maturation of EEA1 endosomes, or promote rapid recycling of EGFR out of EEA1 endosomes to the plasma membrane in order to quickly shut down signaling from endosomes. Based on these results, we concluded that a phospho-
A phosphomimetic or phospho-deficient mutation at GIV’s S1674 dictates migration versus proliferation

Our present work demonstrates that GIV’s interaction with G proteins is important for EGFR trafficking and signaling. Through this signaling, the cell maintains its survival and growth by proliferating or migrating. Using GIV and its two different mutations (GIV-74D and GIV-74A), we provided a further example of migration-proliferation dichotomy where cells will either divide or migrate, but not both at the same time [17].

As mentioned earlier, the GEF domain in GIV’s C-terminus helps form an EGFR-GIV-Gαi3 complex at the plasma membrane that allows migratory signaling through the Akt pathway to occur [10, 28, 43]. An F1685A mutation in the GEF domain abolishes GIV’s ability to bind Gαi3 and Gαs [19, 28]. In turn, this causes motogenic signaling to decrease and mitogenic signaling to increase. Using an immunofluorescent EGFR trafficking assay, Beas et al. confirmed the partnership role of GIV and Gαs by demonstrating that a knockdown of either Gαs or GIV delays the maturation of EEA1 early endosomes, which enhances proliferative signaling [19]. From these studies, we expect that in cells expressing a phosphomimetic GIV mutant that enhances binding to Gαi3 and Gαs and accelerates degradation of EGFR, a decrease in proliferative signaling would be seen with promotion of migratory signaling. By contrast, the nonphosphorylatable GIV mutant would enhance proliferation and decrease migration.
To investigate this, we used a combination of BrdU incorporation assay to denote the cells that are dividing and scratch-wound assay to reveal the effects of the mutations on cell migration (Figures 8 and 9). Pull-down assays showed increased binding to both G proteins for GIV-74D cells, which resulted in increased migration as indicated by faster wound closure. Proliferation, however, was reduced as indicated by fewer cells incorporating BrdU. This correlates well with our trafficking assay showing that GIV-74D cells showed activated EGFR to be shut off quickly, thus giving EGFR less time to signal down the ERK pathway.

Although we have yet to deduce turnover rates of EGFR degradation or mechanisms for EGFR recycling to the cell’s plasma membrane, the accelerated trafficking of EGFR through the endosome compartments may be important in understanding how it contributes to enhanced migration. As shown in the established model of Figure 1, the receptors can either be degraded in lysosomes or they can be recycled back to the plasma membrane where receptors can signal again. Since the mutation at S1674D causes the amount of activated EGFR to be greatly decreased at EEA1 early endosomes, this may be indicative of the receptor recycling back to the cell’s membrane. EGF can then bind to its receptor again, causing EGFR to form a complex with GIV and Gαi3, and thus, upregulate migratory signaling (Figure 10).

Under a nonphosphorylatable mutation, GIV-74A cells displayed heavily impaired migration. However, they preferentially proliferated due to the disruption of binding between GIV and Gαs at EEA1 endosomes. Since activated EGFR remained at early endosomes, proliferative signaling would continue to be enhanced through the ERK pathway. Adding to the aforementioned recycling pathway, the S1674A
mutation not only delays EGFR trafficking out of EEA1 early endosomes, but it may also prevent it from recycling to the cell membrane. This is why activated EGFR is still seen at the early endosomes, as it cannot leave for the cell membrane or the lysosome. These results suggest that phosphorylation or dephosphorylation at GIV’s S1674 contributes to the cells migration-proliferation dichotomy.

My current findings confirm that the interaction between Gαs and GIV are necessary to facilitate passage of activated EGFR through early endosomes. We showed that phospho-modifications near GIV’s GEF domain changes the interaction with G-proteins to effectively enhance migration (through the Akt pathway) and decrease mitogenic signaling (through the ERK pathway), while the opposite occurs under phospho-deficient modifications. The results of this study indicate that GIV can be phosphorylated at S1674, which regulates G-protein binding to modify downstream signaling of EGFR.

In order to further investigate and confirm our data, future experiments will be aimed at analyzing the cell cycle profile of stable HeLa cell lines and specifically looking at EGFR downstream signaling proteins. We expect that in GIV-74D cells, phosphorylated EGFR and downstream ERK will be reduced while Akt will be enhanced when compared to GIV-WT. The decreased ERK signaling in GIV-74D would fit our current results of accelerated EGFR trafficking through early endosomes to either lysosomes for degradation or rapid receptor recycling to the cell membrane. The opposite may be seen in GIV-74A cells, where a failure to bind Gai3 would be expected to decrease Akt signaling, increase ERK 1/2 signaling, and cause cells to proliferate more than GIV-WT, which has been confirmed by our phenotypic results.
In order to distinguish between the degradation and recycling pathway of EGFR, we will perform a pulse-chase analysis to monitor the receptor by using radioactivity to determine whether a mutation at GIV S1674 provides the trigger for receptor degradation versus recycling back to the cell’s membrane.

These concepts are important in further understanding cellular homeostasis. Depending on extracellular signals, cells rely on harmonized mechanisms of phosphorylation and dephosphorylation to maintain balance between activation of its mitogenic and motogenic signaling pathways. This allows the cells to move or divide in response to necessary environmental cues only when needed. Our work here may be related to the aspect of wound healing, where both the processes of cell migration and proliferation are important to close a wound after injury. Through cell signaling, cells can quickly act upon the injury by mobilizing epithelial cells around the wound to transverse the area of injury. Molecularly, cells can be programmed to phosphorylate GIV’s S1674 for a longer period, causing enhanced binding of GIV to Gαi3 and Gαs for migratory signaling to occur. After migration, dephosphorylation of S1674 is critical to start initiation of proliferation. Without the binding between GIV and Gαs, activated EGFR is prolonged at early endosomes, causing increasing proliferative signaling for cells to divide and create new tissue to cover the wound. This process of phosphorylation and dephosphorylation at S1674 may be repeated many times with the help of other cellular processes to repair and heal the wound. With this knowledge, I hope that these findings may ultimately be useful in the design of therapeutic agents capable of quickly and efficiently repairing wounds and modifying the abnormal cell growth that occurs in various cancers.
MATERIAL AND METHODS

Antibodies, Buffers, and Reagents

For buffers and reagents, refer to Table 1. For antibodies, refer to Table 2 and Table 3.

Plasmid Constructs and Mutagenesis

GIV constructs harboring S1674D and S1674A mutations (hereafter referred to as GIV-74D and GIV-74A) were created by site-directed mutagenesis (primers in Table 4) using either the pGEX-4T1-GIV-CT, the pCEFL-GST-GIV-CT (provided by the Ghosh lab) or the GIV-FLAG [28] constructs as a template. All mutations and the integrity of the rest of the insert was confirmed by sequencing.

Cell Culture, Transfection, and Stable Cell Line Preparation

HeLa cells used in this work were cultured according to American Type Culture Collection guidelines. They were grown in DMEM (Invitrogen) with 10% FBS (Hyclone) and penicillin-streptomycin-glutamine (Invitrogen). HeLa cell lines stably expressing GIV-WT, GIV-74D, and GIV-74A were generated by transfecting cells with the desired GIV-FLAG construct and selection in the presence of G418 (800 µg/µl) for 4 weeks. All transfections were performed using TransIT-LT1 reagent (Mirus). Stable cells lines were maintained in DMEM with 10% FBS and G418.

Protein Expression in Bacteria

Plasmids encoding His-Gαi3, His-Gαs, or GST-GIV-CT wild type and mutant
(GST-GIV-CT-74D) fusion constructs were used to express these proteins in *Escherichia coli* strain BL21 (DE3) (Invitrogen). Cells were grown in LB-Ampicillin medium at 37°C until an OD$_{600}$ ρ0.5-0.7, and protein expression was induced by adding 0.5 mM IPTG and growing cultures overnight at 25°C. For His-tagged proteins, pelleted bacteria from 1 L of culture were resuspended in 10 ml His-lysis buffer, sonicated four times for 20 sec with 20 sec between cycles and centrifuged at 12,000 × g for 20 min at 4°C to remove insoluble material. Solubilized protein was purified using a cobalt resin, eluted with 250 mM Imidazole, and dialyzed in 1x PBS. Likewise for GST-tagged proteins, bacteria were pelleted from 500 ml of culture, resuspended in 20 ml of lysis binding buffer, sonicated, and centrifuged to remove insoluble material. Affinity purification of solubilized proteins was carried out using glutathione Sepharose 4B beads (GE Healthcare).

**Immunoblotting**

Protein samples were separated on 10% SDS-PAGE and transferred for 2 h at 400 mA onto PVDF membranes (Millipore). Membranes were blocked with 5% nonfat milk in PBS. When anti-phosphoprotein antibodies were used, blocking was done with 5% BSA in PBS. Membranes were incubated with primary antibodies at 4°C overnight then washed with PBS-Tween and incubated with secondary antibodies for 1 h at room temperature. Western blots were imaged and quantitated using the Odyssey infrared imaging system. Antibodies were diluted as shown in Table 2.
**In vitro Glutathione S-Transferase (GST) Pull-Down Assays**

Equimolar amounts of purified GST-GIV-CT (amino acids 1660–1870; WT or 74D) or GST alone immobilized on glutathione-Sepharose beads were incubated with purified His-tagged Gαi3 or Gαs in binding buffer for 4 h with constant tumbling at 4°C. Beads were washed (three times) with 1 mL of binding buffer and eluted by boiling in 1x Laemmli sample buffer for 5 min. The binding between GIV-CT and His-Gα proteins was analyzed by immunoblotting.

**In cellulo GST Pull-Down Assays**

Cos7 cells were grown in 10 cm plates and transfected with pCEFL-GST-GIV-CT wild type and mutants (74D and 74A). After 48 h, cells were washed (1 time) with cold 1x PBS and harvested by scraping in 500 µl of binding buffer. Cell lysates were incubated with glutathione-Sepharose beads with constant tumbling at 4°C for 4 h. Beads were washed (three times) with 1 ml of lysis buffer, eluted in 1x Laemmli sample buffer for 5 min, and binding of endogenous Gα proteins was analyzed by immunoblotting.

**Co-Immunoprecipitation Assays**

Cos7 cells were plated in 10 cm plates and co-transfected with either FLAG-Gαi (4 µg) or FLAG-Gαs (500 ng) with pcDNA3.1, GIV-Myc WT, 74D, or 74A (6 µg with Gαi3 and 9 µg with Gαs) constructs. After 48 h, cells were washed (1 time) with cold 1x PBS and harvested by scraping into the IP buffer on ice. Cell lysates were
incubated for 10 min at 4°C and centrifuged at 12,000 × g for 10 min. Clarified cell lysates were incubated with anti-mouse FLAG-M2 antibody overnight at 4°C, followed by incubation with protein G-Sepharose beads for 1 h at 4°C. Beads were washed (three times) with IP buffer, eluted by boiling in 1x Laemmli sample buffer, and bound proteins were analyzed by SDS-PAGE and immunoblotting.

**EGF Stimulation**

For EGF stimulation experiments, 65,000 HeLa cells stably expressing either GIV-WT, GIV-74D, or GIV-74A were seeded in a 6-well plate in DMEM with 10% FBS. 24 h post seeding, cells were starved for ~18 h in DMEM with 0.2% FBS. Cells were stimulated with 50 nM EGF for 0-30 min.

**Immunofluorescence Microscopy**

For immunofluorescence analysis, cells grown on coverslips were fixed at room temperature with 3% paraformaldehyde (PFA) for 30 min, washed (3 times) with 1x PBS, blocked and permeabilized for 45 min, and then incubated for 1 h each with primary and then secondary antibodies. For staining with anti-pEGFR (pY1068), coverslips were incubated in the primary antibody overnight at 4°C. DAPI at 1:10,000 (Invitrogen) was used to stain the nucleus. Antibody dilutions used were as listed in Table 2. Images were taken using a Leica DMI400B confocal microscope.

**Wound Healing Assay**

HeLa stable cell lines (WT, 74D, and 74A) were grown on 6 well plates to
90% confluency and starved in 0.2% FBS (DMEM) overnight before the day of wounding. The wound was made by scratching the plate with a p200 pipette tip. Plates were rinsed once with 0.2% FBS (DMEM) to remove floating cells, and cells were maintained in 0.2% FBS containing DMEM throughout the experiment. Images were taken using the Canon Powershot A630 camera with lens adapter for Zeiss Axiovert 40 CFL microscope every 24 h. To quantify cell migration (expressed as percentage of wound area covered), images were analyzed using ImageJ (National Institutes of Heath) to calculate the difference between area covered at 0 min and at the end of the assay divided by the area of the wound at 0 min multiplied by 100.

**BrdU Incorporation Assay**

HeLa cells stably expressing GIV-WT, GIV-74D, and GIV-74A were grown on coverslips to 90% confluency in low serum (2%) overnight. Cells were incubated with 10 µM BrdU for 30 min at 37°C and fixed with 70% ethanol for 30 min at room temperature. Cells were then washed (three times) with 1x PBS, treated with 2 M HCl for 20 min at room temperature, followed by 0.1 M Na₂B₄O₇ (pH 8.5) for 2 min at room temperature, and washed again in 1x PBS (three times). The coverslips were incubated with anti-BrdU for 30 min at room temperature, washed (three times) with 1x PBS, and then incubated with goat anti-mouse 594 secondary antibody for 30 min at room temperature. DAPI at 1:10,000 was used to stain the nucleus. Antibody dilutions used are listed in Table 2. Images were taken using Leica DMI400B confocal microscopy. 300-400 cells from 10 randomly chosen fields were counted (DAPI stained) from three independent experiments and quantitated by dividing BrdU
positive cells against total cell and normalized to GIV-WT (100%).

**Data Analysis**

Each set of data presented in the figures is representative of at least three independent experiments. All averages, SEMs, and significance p values (Student’s t test: *P<0.05; **P<0.01; ***P<0.001) were calculated with Microsoft Excel and graphed using Graphpad Prism software (GraphPad Software, Inc.). All images were processed and figures were assembled using Photoshop and Illustrator software (Adobe).
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### Table 1. Buffers, Continued

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### Table 2. Antibodies for Western Blot (WB)

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<td>Girdin C-terminus</td>
<td>Rabbit Polyclonal IgG</td>
<td>IB 1:500</td>
<td>Santa Cruz Biotechnology</td>
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<td>GST</td>
<td>Goat IgG</td>
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<td>Mouse Monoclonal IgG2a</td>
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<td>IgG</td>
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### Table 3. Antibodies for Immunofluorescence (IF)

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<tr>
<td>EEA1</td>
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<tr>
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<td>IF 1:100</td>
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<tr>
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<td>Goat anti-Rabbit</td>
<td>IF 1:500</td>
<td>Invitrogen Life Technologies</td>
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<td></td>
<td>Monoclonal IgG</td>
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</tr>
<tr>
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<td>Goat anti-Mouse</td>
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### Table 4. Primer Sequences

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<td>GIV-S1674</td>
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<table>
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<td>GIV-S1674D</td>
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<td>Rev: 5’ aacaactcactccaggtcaccagttcttttgcgttg 3’</td>
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<td></td>
<td>Rev: 5’ aacaactcactccaggtcaccagttcttttgcgttg 3’</td>
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REFERENCES


36. Kettenbach AN, Schwegge DK, Faherty BK, Pechenick D, Pletnev AA and Gerber SA (2011) Quantitative phosphoproteomics identifies substrates and
functional modules of Aurora and Polo-like kinase activities in mitotic cells. Sci Signal 4:rs5. doi: 10.1126/scisignal.2001497


