Activation state-dependent interaction between Gαq subunits and the Fhit tumor suppressor

Hao Zuo¹, Grace PW Chan¹, Jing Zhu¹, Wendy WS Yeung¹, Anthony SL Chan¹, Hermann Ammer² and Yung H Wong¹,³*

Abstract

Background: The FHIT tumor suppressor gene is arguably the most commonly altered gene in cancer since it is inactivated in about 60% of human tumors. The Fhit protein is a member of the ubiquitous histidine triad proteins which hydrolyze dinucleoside polyphosphates such as Ap3A. Despite the fact that Fhit functions as a tumor suppressor, the pathway through which Fhit inhibits growth of cancer cells remains largely unknown. Phosphorylation by Src tyrosine kinases provides a linkage between Fhit and growth factor signaling. Since many G proteins can regulate cell proliferation through multiple signaling components including Src, we explored the relationship between Gα subunits and Fhit.

Results: Several members of the Gαq subfamily (Gα16, Gα14, and Gαq) were found to co-immunoprecipitate with Fhit in their GTP-bound active state in HEK293 cells. The binding of activated Gαq members to Fhit appeared to be direct and was detectable in native DLD-1 colon carcinoma cells. The use of Gα16/z chimeras further enabled the mapping of the Fhit-interacting domain to the α2-β4 region of Gα16. However, Gαq/Fhit did not affect either Ap3A binding and hydrolysis by Fhit, or the ability of Gαq/16 to regulate downstream effectors including phospholipase Cβ, Ras, ERK, STAT3, and IKK. Functional mutants of Fhit including the H96D, Y114F, L25W and L25W/I10W showed comparable abilities to associate with Gαq. Despite the lack of functional regulation of Gq signaling by Fhit, stimulation of Gq-coupled receptors in HEK293 and H1299 cells stably overexpressing Fhit led to reduced cell proliferation, as opposed to an enhanced cell proliferation typically seen with parental cells.

Conclusions: Activated Gαq members interact with Fhit through their α2-β4 region which may result in enhancement of the growth inhibitory effect of Fhit, thus providing a possible avenue for G protein-coupled receptors to modulate tumor suppression.

Keywords: Fhit, G protein, Phospholipase Cβ, Src, Tumor suppression

Background

The chromosomal localization of FHIT (Fragile Histidine Triad) in the common fragile region of the human genome suggests a positive correlation between the loss or inactivation of the FHIT gene and carcinogenesis. As predicted for a tumor suppressor, the Fhit protein is absent or markedly reduced in most human cancers [1]. The role of FHIT in tumor suppression is perhaps best exemplified by studies performed with FHIT-deficient mice. Transgenic mice carrying one or two inactivated Fhit alleles are viable and long-lived, but they show increased rates of spontaneous and carcinogen-induced cancers [2,3]. Encouragingly, the development of carcinogen-induced tumors in these mice can be prevented by administration of Fhit-expressing viral vectors [4]. Moreover, Fhit overexpression enhances the susceptibility of many types of cancer cells to exogenous inducers of apoptosis. Fhit is one of the HIT (histidine triad) superfamily members, which share an HxHxHxx motif (where x is a hydrophobic residue) for nucleotide binding. Human Fhit can hydrolyze dinucleoside polyphosphates, preferably Ap3A (to AMP and ADP). Despite numerous attempts to elucidate the function of Fhit in tumor suppression, the
biological action of Fhit remains elusive. Current evidence based on Fhit mutants with impaired substrate binding (L258 and I108/W258 mutants) or hydrolytic activity (H196D mutant) supports the notion that the formation and stability of the Fhit-Ap3A complex is crucial in growth inhibition and apoptosis [5-7]. There is also evidence to suggest that the intracellular concentration of Ap3A [8] or its abundance relative to other dinucleotide polyphosphates [9] may be correlated with Fhit-mediated apoptosis. The hypothesis that the Fhit-Ap3A complex could be an important signaling molecule is an interesting possibility, but it has yet to be confirmed biochemically.

A number of important cancer-related genes and pathways have recently been linked to Fhit. In colon cancer cell lines, Fhit inhibits cell growth by attenuating the signaling mediated by NFkB [10]. Fhit also inhibits the activity of Akt, a key effector in the phosphatidylinositol 3-OH kinase (PI3K) pathway [11], and serves as a physiological target of the Src tyrosine kinase [12]. Src is a crucial cytoplasmic tyrosine kinase downstream of several growth factor receptors, including those of the EGF receptor family, which are often overexpressed and activated in human breast and ovarian carcinomas. Indeed, activation of EGF receptor family members induces Fhit degradation via the proteasome pathway which purportedly depends on Src-mediated Fhit phosphorylation at Tyr114 [13]. However, biochemical data suggest that phosphorylation favors the formation and persistence of the Fhit-Ap3A complex [14]. Additionally, the mitochondrial Fhit can sensitize cells to apoptosis by binding and stabilizing ferredoxin reductase [15], which is important for the production of reactive oxygen species, and by enhancing mitochondrial Ca2+-uptake capacity [16]. These reports help us to better understanding the mechanism of tumor suppression by Fhit, but it remains unclear as to how one can restore Fhit levels in the tumor cells for cancer treatment.

Many signaling pathways operated by growth factors are similarly modulated by the heterotrimeric G proteins, which are critical players in many aspects of cellular function including cell proliferation, differentiation and apoptosis. These signaling pathways include the mitogen-activated protein kinases (MAPKs) [17], PI3K/Akt [18], tyrosine kinases [19], and transcription factors such as STAT3 and NFkB [20,21]. Ga subunits of heterotrimeric G protein are classified into four subfamilies (Gaα, Gaβ, Gaγ, and Ga12) [22]. It is noteworthy that some Ga subunits can directly activate tyrosine kinases such as Bruton’s tyrosine kinase (Btk) [19]. Interestingly, Src has also been shown to be activated by members from all four subfamilies of G proteins [23-26] and this may provide a link to regulate Fhit phosphorylation. Constitutively activating mutations of the Ga subunits that lock these signaling molecules in their GTP-bound active state have been found to be associated with several types of tumor [27]. Sustained stimulation of the Gαq and G12 pathways often leads to mitogenesis in various cell types [28]. As a continuing effort to understand the functions of G proteins in cell growth and proliferation, we have explored the notion that G proteins can modulate Fhit. Surprisingly, we discovered that several α subunits of Gα family members can associate with Fhit only in their active state.

Results
Constitutively active Gqα mutants stimulate Fhit phosphorylation at Tyr114 through Src
Src is known to be activated by Gαq subunits [20,25] and thus it is conceivable that stimulation of Gαq-coupled receptors may lead to Fhit phosphorylation. To facilitate the detection of Fhit phosphorylation, we raised an anti-phospho-Fhit Tyr114 antiserum which can detect Src-induced Fhit Tyr114 phosphorylation with high sensitivity (Figure 1A); overexpression of Src was sufficient to induce Fhit phosphorylation in transfected HEK293 cells due to the increase in activated Src (P-Src in Figure 1A). We then began the study by examining the ability of the Gqα-coupled type 2 bradykinin receptor (BK2R) to stimulate Fhit phosphorylation by using a previously characterized HEK293 cell line stably expressing BK2R (293/BK2R cells) [29]. 293/BK2R cells transiently expressing Flag-Fhit were stimulated with or without 100 nM bradykinin for various durations and then assayed for Fhit phosphorylation. Bradykinin-induced Fhit phosphorylation was hardly detected at short treatment times (data not shown) but was reproducibly observed albeit weakly with cells treated for 24 h (~2.5-fold of basal; Figure 1B, DMSO control). As shown in Figure 1B, bradykinin-induced Fhit Tyr114 phosphorylation was significantly suppressed by pretreatment of the cells with Src inhibitors (10 μM PP1 or 25 μM PP2). As HEK293 cells endogenously express the Gαq-coupled muscarinic M3 receptor [30], we examined whether receptor activation can induce Src-mediated Tyr114 phosphorylation of endogenous Fhit. In contrast to 293/BK2R cells overexpressing Flag-Fhit, we could not detect carbachol-induced phosphorylation of endogenous Fhit in native HEK293 cells unless the cells were treated with 100 μM Na3VO4, a tyrosine phosphatase inhibitor (Figure 1C); this suggests that phosphorylated Fhit may undergo dephosphorylation and thereby making its detection extremely difficult when the level of phospho-Fhit is limiting. Nevertheless, the carbachol-induced phosphorylation of endogenous Fhit was sensitive to Src inhibition by PP1 (Figure 1C). In order to confirm that Gαq signals can lead to Fhit phosphorylation, we made use of constitutively active mutants of Gαq subunits as well as Fhit Y114F, a previously characterized non-phosphorable mutant [12,13]. The constitutively
active $G_{\alpha_q}$ mutants harbor a point mutation at a conserved arginine or glutamine (e.g., $G_{\alpha_q}$R183C or $G_{\alpha_q}$Q209L) which abolishes the GTPase activity of the $G_{\alpha}$ subunit and maintains them in the GTP-bound active state. Transient co-expression of constitutively active $G_{\alpha_q}$ mutants with Fhit should lead to increased phosphorylation of wild-type Fhit but not Fhit Y114F. Interestingly, co-expression of constitutively active mutants of $G_{\alpha_q}$ or $G_{\alpha_t}$ (another member of the $G_{\alpha_t}$ family) with Fhit resulted in increased levels of the latter (Additional file 1), a phenomenon similar to that seen with bradykinin-treated 293/BK$_2$R cells (cf lanes 1 and 2 of the Flag-Fhit immunoblot in Figure 1B). After adjusting the expression level of Fhit between the various transfectants, Fhit phosphorylation was clearly detected in cells co-expressing the constitutively active $G_{\alpha_q}$RC or $G_{\alpha_t}$QL (Figure 1D). Transfectants co-expressing the wild-type $G_{\alpha_q}$ subunits exhibited little or no Fhit phosphorylation while no phospho-Fhit could be detected in cells co-expressing Fhit Y114F (Figure 1D).

As tyrosine kinases such as Btk can be directly activated by $G_{\alpha_q}$ [19], we examined whether Src can form complexes with Fhit and/or $G_{\alpha_q}$. Because activated $G_{\alpha_16}$ ($GNA15$, another member of the $G_{\alpha_t}$ subfamily with 85% sequence identity to its mouse isofrom $G_{\alpha_15}$ [31]) has previously been shown to stimulate Src phosphorylation at Tyr$^{416}$ [21], we transfected HEK293 cell with different combinations of Flag-Fhit, Src, $G_{\alpha_16}$ and $G_{\alpha_16}$QL and then subjected the cell lysates to co-immunoprecipitation assays using an anti-Flag affinity gel (Figure 1E). Both Src and $G_{\alpha_16}$QL were detected in the immunoprecipitates of Flag-Fhit when all three proteins were co-expressed simultaneously (Figure 1E, lane 4); note that the Src-specific band (marked by an asterisk) ran just above a non-specific IgG band. Control experiments omitting either Src or $G_{\alpha_16}$QL demonstrated that both proteins were able to interact with Flag-Fhit independently or endogenous levels of interacting proteins (including Src and $G_{\alpha_t}$ subunits) were not limiting (cf lanes 1 and 6 in Figure 1E). Compared to $G_{\alpha_16}$QL, wild-type $G_{\alpha_16}$ exhibited a much weaker ability to associate with Flag-Fhit (cf lanes 3 and 5 versus 4 and 6 in Figure 1E). Yet again, co-expression of $G_{\alpha_16}$QL but not wild-type $G_{\alpha_16}$ or Src, increased the levels of Fhit in the transfectants (Figure 1E, lanes 4 and 6). Taken together, these results suggest that Fhit may associate with $G_{\alpha_t}$ subunits in a GTP-bound state-dependent and Src-independent manner.

**Several $G_{\alpha_t}$ members interact with Fhit in an activity-dependent manner**

The preceding experiments suggest that members of the $G_{\alpha_t}$ subfamily may interact with Fhit upon binding GTP. To assess if this interaction is specific to $G_{\alpha_t}$ subunits, we performed co-immunoprecipitation assays using Flag-Fhit and various $G_{\alpha_t}$ subunits. HEK293 cells were co-transfected...
with Flag-Fhit or Flag-vector in combination with a selected Ga subunit in its wild-type or constitutively active form. The expressions of Flag-Fhit and Ga subunits between different groups were adjusted to comparable levels prior to co-immunoprecipitation with an anti-Flag affinity gel or anti-Ga antisera. Constitutively active mutants of Gaq, Ga11, Ga14, Ga16, Gs, Gi2, and Gi13, but not their wild-type counterparts, formed complexes with Flag-Fhit as predicted (Figure 2A). However, despite being a member of the Gaq subfamily, the constitutively active mutant of Ga11 failed to interact with Flag-Fhit (Figure 2A). Representative members (Gaq, Ga12, and Ga13) from each of the remaining Ga subfamilies were also subjected to co-immunoprecipitation assays with Flag-Fhit. As shown in Figure 2A, both wild-type and
constitutively active $\text{G}_\alpha_s$ and $\text{G}_\alpha_{13}$ were pulled down by Flag-Fhit, but not by the vector control, suggesting that $\text{G}_\alpha_s$ and $\text{G}_\alpha_{13}$ were capable of forming complexes with Flag-Fhit irrespective of their activation status. Neither wild-type nor constitutively active $\text{G}_\alpha_i2$ or $\text{G}_\alpha_z$ was co-immunoprecitated with Flag-Fhit, indicating that both $\text{G}_\alpha_i2$ and $\text{G}_\alpha_z$ behaved like $\text{G}_\alpha_{11}$ and could not associate with Fhit. To ascertain that Fhit can truly interact with activated members of $\text{G}_\alpha_q$, we examined the association between $\text{G}_\alpha_{16}^{QL}$ and Fhit by reciprocal co-immunoprecipitation using an anti-$\text{G}_\alpha_{16}$ antiserum to pull down Fhit from lysates of HEK293 cells expressing wild-type $\text{G}_\alpha_{16}$ or $\text{G}_\alpha_{16}^{QL}$; Fhit was indeed co-immunoprecipitated along with $\text{G}_\alpha_{16}^{QL}$, but not with wild-type $\text{G}_\alpha_{16}$ (Figure 2B).

To further confirm their interaction in a native system, we screened for cell lines that endogenously express Fhit at a detectable level. Out of eight cell lines examined, DLD-1 colon carcinoma cells have relatively high levels of endogenous Fhit (data not shown) and they were used to examine the interaction between endogenous Fhit and $\text{G}_\alpha_q$. Cell lysates were incubated with non-hydrolysable GDP$\beta$S or GTP$\gamma$S (100 $\mu$M each at 4°C for 30 min) to shift the endogenous G proteins to the basal or activated state, respectively. Cell lysates were subsequently subjected to co-immunoprecipitation with anti-Fhit antisera and protein A sepharose. As compared to the controls, more $\text{G}_\alpha_q$ was detected in the Fhit immunoprecipitate following GTP$\gamma$S treatment (Figure 2C). This result suggests that activated $\text{G}_\alpha_q$ subunits can interact with Fhit in a native cellular environment.

Since other signaling components along the G protein pathway may also be involved in the Fhit/G$\alpha_q$ interaction, possible association of Fhit with $\text{G}_\beta\gamma$ regulators of G protein signaling (RGS proteins), and monomeric GTPases were examined by co-immunoprecipitation assays. Many effectors such as adenylyl cyclase and phospholipase C$\beta$ (PLC$\beta$) can be simultaneously regulated by $\text{G}_\alpha$ and $\text{G}_\beta\gamma$ subunits. It is thus worth investigating whether Fhit can also associate with $\text{G}_\beta_1 \text{G}_\gamma_2$, a $\text{G}_\beta\gamma$ complex which is known to bind various effectors including tyrosine kinases [32]. We co-expressed Flag-tagged $\text{G}_\beta_1$ and HA-tagged $\text{G}_\gamma_2$ with untagged Fhit. The Flag-$\text{G}_\beta_1$ subunit was clearly capable of forming a complex with HA- $\text{G}_\gamma_2$, yet it was unable to co-immunoprecipitate Fhit (Figure 2D). As shown in Figure 2E, both RGS19 (also known as $\text{G}_\alpha$-interacting protein, GAIP) and RGS16 did not co-immunoprecipitate with Flag-Fhit. RGS4, RGS10, and RGS20 also failed to interact with Fhit (data not shown). It should be noted that, under identical experimental conditions, RGS19 and Ras can interact efficiently with their known partners [33,34]. Monomeric small GTPases contain the same core domains for GTP-binding as the heterotrimeric $\text{G}_\alpha$ subunits. Hence, the ability of Flag-Fhit to form a complex with selected small GTPases was examined. Neither Ras nor Rap1A, which belong to the Ras family of the small GTPase superfamily, could be co-immunoprecipitated by Flag-Fhit (Figure 2E), suggesting that small GTPases cannot form complexes with Fhit protein. These observations further support the notion that $\text{G}_\alpha_q$/Fhit interactions are specific and not shared by other signaling components along the G protein pathway.

**Activated $\text{G}_\alpha_{16}$ interacts with Fhit directly through its $\alpha_2\beta_4$ region**

To investigate whether Fhit is able to directly interact with activated $\text{G}_\alpha_q$ members, we performed pull-down assays using purified GST, GST-tagged Fhit (GST-Fhit) and His-tagged $\text{G}_\alpha_{16}$ (His-$\text{G}_\alpha_{16}$). The purity of both GST-Fhit and His-$\text{G}_\alpha_{16}$ proteins was estimated to be...
greater than 90% by Coomassie blue staining (Figure 3A). Equal amounts of recombinant His-α16 and GST-Fhit (or GST) were incubated at 4°C for 30 min in the presence of 100 μM GDPβS or GTPγS in order to stabilize His-α16 in the inactive or active conformation. Although a small amount of His-α16 appeared to be non-specifically associated with the glutathione sepharose (Figure 3B, lanes 1 and 2 of right panel), GTPγS-His-α16 was clearly pulled down by GST-Fhit (Figure 3B, lane 4 of right panel). In contrast, GDPβS-His-α16 failed to associate with GST-Fhit. Collectively, these results suggest that Fhit can selectively associate with activated Gαq members except Gα11, and both purified Gα16 and endogenous Gαq can interact with Fhit in their active states. Such activation state-dependent interactions are reminiscent of Gα/effector regulations.

In order to understand the molecular basis of the interaction between Gαq and Fhit, we mapped the Fhit-interacting regions on Gα16 by using a series of chimeras in which discrete regions of Gα16 were swapped with Gαz (a member of Gαi subfamily). These chimeras have
been previously used to successfully determine the receptor and effector interacting domains of $\alpha_{16}$ and $\alpha_z$ [35,36]. $\alpha_{16}/\alpha_z$ chimeras were preferred because of the lack of endogenous expression of either $\alpha_{16}$ or $\alpha_z$ in HEK293 cells. The differential ability of $\alpha_{16}^{QL}$ and $\alpha_z^{QL}$ to interact with Fhit (Figure 2A) permits identification of Fhit-interacting regions on $\alpha_{16}$ through gain of function analyses. Since the effector interacting domain is likely to reside in the carboxyl half of the $\alpha$ subunit [36,37], we have selected chimeras composed of $\alpha_z$ backbones with their C-terminal regions increasingly replaced by $\alpha_{16}$ sequences all the way up to the $\beta_2$ domain (Figure 4A); mirror images of selected chimeras were also included. Among the various chimeras examined, constitutively active N188QL and N210QL (N-terminal 188 or 210 amino acids from $\alpha_z$, respectively) were more efficiently pulled down by the anti-Flag affinity gel than their corresponding wild-types; both chimeras were as effective as, if not better than, $\alpha_{16}^{QL}$ (Figure 4B). Constitutively active C128QL (C-terminal 128 amino acids from $\alpha_z$) also showed higher affinity with Fhit than its wild-type (Figure 4B). In contrast, N246QL, N266QL and C164QL failed to associate with Flag-Fhit and behaved like the negative control $\alpha_z^{QL}$ (Figure 4B). These results demonstrate that the residues between 210 and 246 of $\alpha_{16}$, which represent the regions from $\alpha_2$ to $\beta_4$, are required for interaction with Fhit. Based on the structures of active $\alpha_q$ in the

**Figure 6** Fhit does not affect Gq-mediated PLCβ activation. A. HEK293 cells were co-transfected with wild-type or the constitutively active mutant of $\alpha_{16}$ or $\alpha_q$ in combination with pcDNA3 (Vector), Fhit and different Fhit mutants in the pcDNA3 vector: Fhit-Y114F (Y114F), Fhit-L25W (L25W), Fhit-I10W/L25W (I10W/L25W) and Fhit-H96D (H96D). Transfectants were then labeled and lysed for determining the IP$_3$ in cell lysates as described in Methods. PLC activity was calculated as the amount of IP$_3$ formed divided by the corresponding level of total inositol. B. HeLa cells were transfected with control siRNA, Fhit siRNA or Fhit cDNA. One day later, expression levels of Fhit were detected by Western blot. C. HeLa cells in B were seeded into black-walled 96-well plates and the Ca$^{2+}$ responses of these cells with histamine treatment (0.1, 1, or 10 μM) were detected by the FLIPR device. Here shows the fluorescence signals of 0.1 μM histamine-induced Ca$^{2+}$ response (FLU) in the control (dotted), Fhit-knocked down (dashed) or Fhit-overexpressing (solid) HeLa cells. D. The maximal fluorescence signals of the Ca$^{2+}$ responses (FLU) induced by 0.1, 1, or 10 μM histamine in the control, Fhit-knocked down and Fhit-overexpressing HeLa cells were illustrated as white, gray and black, respectively.
complex with p63RhoGEF and RhoA [PDB: 2RGN_A] as well as inactive Gαq with Gβγ complex [PDB: 3AH8], molecular modeling of Gα16 predicted that the α2-β4 domain interacts with Gβγ in the inactive state but becomes exposed to the outer surface in the active state (Additional file 2).

We have also attempted to determine the Gαq-interacting region on Fhit by constructing a series of Fhit truncation mutants with deletions at either the C- or N-terminus (Additional file 3). However, deletion at either terminus apparently impaired the stability of these mutants because their expressions were hardly detectable unless the transfected cells were treated with the proteasome inhibitor MG132 (Additional file 3). The inadequate expression of these truncation mutants precluded co-immunoprecipitation assays. Nevertheless, expressions of two mutants were enhanced upon co-expression of GαqQL, but not Gαq (Additional file 3). This suggests that interaction with activated Gαq may stabilize Fhit.

**Formation of the Gαq/Fhit complex is independent of Fhit’s ability to bind Ap3A or be phosphorylated at Tyr**

In an attempt to unveil the biological function of the Gαq/Fhit interaction, we asked if such association is affected by Fhit phosphorylation at Tyr114 or Fhit’s ability to bind Ap3A. Previous studies have shown that Fhit undergoes degradation upon phosphorylation by Src kinase at Tyr114 [13] and activated Gαq can stimulate tyrosine kinases [25]. Many signaling molecules regulate their binding to protein partners through tyrosine phosphorylation. To test if this holds true for Fhit, we employed the Fhit Y114F mutant in co-immunoprecipitation assays. Since Flag-Fhit Y114F appeared to interact with constitutively active GαqRC to an extent similar to Flag-Fhit (Figure 5A), it suggests that phosphorylation of Fhit Tyr114 is not a prerequisite for the formation of Gαq/Fhit complexes.

Ap3A is the substrate of Fhit, and binding of Ap3A to Fhit can affect the conformation of Fhit and hence its ability to associate with other proteins. 110W/L25W and L25W are Fhit mutants that exhibit 30- and 7-fold increase of Kms, respectively [7]. Apparently, these mutants have a lower affinity to associate Ap3A although they can still hydrolyze Ap3A. On the other hand, H96D, the Ap3A hydrolytic dead mutant of Fhit does not hydrolyze Ap3A and stabilizes the Ap3A/Fhit conformation [6]. Therefore, the associations between Gαq and these mutants were assessed. As shown in Figure 5A, all three mutants effectively co-immunoprecipitated GαqRC but not wild-type Gαq; their interactions with GαqRC were essentially similar to that observed with Flag-Fhit. Hence, the binding of Ap3A to Fhit has little or no effect on the formation of Gαq/Fhit complexes.

Since many activated Gα subunits can regulate the enzymatic activity of their effectors, constitutively active Gαq may modulate the hydrolase activity of Fhit. To test this possibility, we used purified GST-Fhit and His-Gα16 proteins. The hydrolysis of Ap3A to AMP and ADP was monitored by HPLC as described previously [38]. Upon incubation with 1 μg GST-Fhit at 37°C for 10 min, 100 μM Ap3A was completely hydrolyzed to AMP and ADP (Figure 5B). No hydrolysis was detected when Ap3A was incubated with GST alone or with heat denatured GST-Fhit (Figure 5B). We then optimized the assay in order to cater for the detection of possible stimulatory effect on the hydrolyase activity of Fhit. Upon reducing the amount of GST-Fhit in the reaction to 0.5 μg, approximately half of the Ap3A was hydrolyzed to AMP and ADP (Figure 5C). To mimic the constitutively active Gα16QL, the recombinant His-Gα16 protein was loaded with 100 μM GTPγS. His-Gα16 protein loaded with GDPβS was used as a negative control. As shown in Figure 5C, the presence of GTPγS-bound or GDPβS-bound His-Gα16 did not affect the ability of GST-Fhit to hydrolyze Ap3A. The extent of Ap3A
tions. These results suggest that activated Gαq could not be detected under our experimental condi-

pressive effect by altering the functions of these Gα subunits. To test this postulation, we determined the ef-
sffect of Fhit on the ability of Gαq and Gα16 to regulate a panel of known effectors. We first examined the ability of GαqRC and Gα16QL to stimulate PLCβ in the absence or presence of Fhit overexpression. HEK293 cells were co-
transfected with various combinations of Fhit, Fhit mutants, and wild-type or constitutively active mutants of Gαq and Gα16. As predicted, both GαqRC and Gα16QL were capable of stimulating the endogenous PLCβ and in-
duction of the IP3 formation (Figure 6A). Co-expression of Fhit or its mutants neither stimulated nor inhibited the ability of GαqRC and Gα16QL to activate PLCβ (Figure 6A).

We have also examined whether Fhit affects the ability of endogenous Gαq-coupled histamine receptors to stimulate PLCβ activity in HeLa cells. As there are conflicting results on the Fhit expression level in HeLa cells; Dunnett’s t test, P < 0.05). Immunoblots shown represent one of at least three sets; all other sets yielded similar results.

As members of the Gαq family are known to regulate mitogenic pathways [39], Fhit may exert its tumor suppressive effect by altering the functions of these Gα subunits. To test this postulation, we determined the effect of Fhit on the ability of Gαq and Gα16 to regulate a panel of known effectors. We first examined the ability of GαqRC and Gα16QL to stimulate PLCβ in the absence or presence of Fhit overexpression. HEK293 cells were co-
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Figure 9 Stimulation of Gαq-coupled receptors inhibits cell growth in Fhit-expressing cells. A. 293/Fhit and 293/vector cells, or H1299/Fhit and H1299/vector cells, which stably expressed Flag-tagged Fhit and the Flag tag alone (vector), respectively, were established as described in Methods. The expressions of Fhit were confirmed by Western blot. B. 293/vector or 293/Fhit cells were seeded into 96-well plates and 24 h later, the cells were treated with or without 100 μM carbachol in the growth medium (Day 0). MTT assay were performed on Day 0, 2, 4 and 6 to examine the relative viable cell number. Absorbance values on Day 0 were set as 1 for the respective group of cells.* Significantly different from that of the 293/vector cells without carbachol treatment on the same day; # carbachol treatment significantly inhibited the growth of 293/Fhit cells; Dunnett’s t test, p < 0.05, n = 8. C. H1299/vector or H1299/Fhit cells were seeded into 96-well plates and 24 h later, the cells were treated with or without 100 nM bombesin (Day 0). MTT assays were performed on Day 0 and 4. The values of MTT assay on Day 0 were set as 1 for the respective group of cells. * Bombesin significantly increased the growth of H1299/vector cells while it significantly inhibited the growth of H1299/Fhit cells; Dunnett’s t test, p < 0.05, n = 8.

hydrolysis by GST-Fhit was essentially identical under all three conditions (Figure 5D). Neither guanine nucleo-
tides interfered with the detection of the substrate or product; GDPβS was eluted after Ap4A while GTPγS could not be detected under our experimental conditions. These results suggest that activated Gα16 does not regulate the hydrolyase activity of Fhit. However, it remains possible that activated Gα16 can indirectly modulate the enzymatic activity of Fhit in a cellular environment.

Fhit does not alter the signaling function of Gαq
As members of the Gαq family are known to regulate mitogenic pathways [39], Fhit may exert its tumor suppressive effect by altering the functions of these Gα subunits. To test this postulation, we determined the effect of Fhit on the ability of Gαq and Gα16 to regulate a panel of known effectors. We first examined the ability of GαqRC and Gα16QL to stimulate PLCβ in the absence or presence of Fhit overexpression. HEK293 cells were co-
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down of Fhit by siRNA or overexpression of Fhit in HeLa cells (Figure 6B), intracellular Ca$^{2+}$ mobilization was measured by a FLIPR device with 0.1, 1 or 10 μM histamine as agonist. Figure 6C showed typical Ca$^{2+}$ signals induced by 0.1 μM histamine. There was no significant difference among the maximal Ca$^{2+}$ responses induced by different concentrations of histamine in control, Fhit-deficient or Fhit-overexpressing cells (Figure 6D). These observations suggest that Fhit does not affect the Gαq/16/PLCβ pathway.

Apart from PLCβ, Gαq subunits are known to interact with TPR1 which associates with activated Ras [34,44]. This raises the question whether Fhit could interfere with Gα16QL/TPR1/Ras signaling. If Fhit and TPR1 compete for the same region on Gα16, Fhit will displace and prevent TPR1 from binding to Gα16QL. In co-immunoprecipitation assays, the ability of Flag-TPR1 to pull down Gα16QL was not affected by the co-expression of untagged-Fhit (Figure 7A, lane 2 versus lane 4), suggesting that TPR1 and Fhit do not compete for the same region on Gα16QL. Interestingly, Fhit was clearly present in the immunoprecipitates of lysates prepared from Flag-TPR1/Fhit/ Gα16QL transfectants (lane 4), whereas it was weakly detected from those of Flag-TPR1/Fhit (lane 5) and Flag-TPR1/Fhit/Gα16 (lane 3) transfectants. This might occur if Gα16QL could simultaneously bind to both Fhit and Flag-TPR1, thus forming a TPR1/Gα16QL/Fhit complex that can be immunoprecipitated by the anti-Flag antibody. The presence of such a complex implies that Fhit may be involved in regulating Gα16QL-mediated Ras activation. Ras activation assay was employed to investigate the effect of Fhit on Gα16QL-induced Ras activity. In agreement with a previous report [44], Gα16QL significantly induced Ras activation as compared to the vector control and wild-type Gα16 (Figure 7B). However, there was no significant elevation or attenuation of Ras activity when cells were co-transfected with Fhit (Figure 7B).

In addition to PLCβ and Ras signaling, other cytoplasmic signaling molecules known to be regulated by Gαq and Gα16 were examined in the presence or absence of Fhit expression. Phosphorylation states of various signaling molecules including ERK, STAT3 and IKK were examined using phospho-specific antibodies. Gαq,RC significantly stimulated the phosphorylations of ERK and IKK and such responses were unaffected by the presence of Fhit (Figure 8A). Similar results were obtained with Gα16QL (data not shown). Likewise, Gα16QL significantly stimulated STAT3 phosphorylation at both Tyr705 and Ser727 and these responses were not affected by the co-expression of Fhit (Figure 8B); similar results were obtained with Gαq,RC (data not shown).

Since phosphorylation of IKK results in activation of NFκB transcription, Gα16QL-stimulated NFκB transcriptional activity was also evaluated. As shown in Figure 8C, Gα16QL significantly induced NFκB luciferase activity as compared to pcDNA3 and Gα16 control. Consistent with the phosphorylation profiles of IKK, expression of Fhit did not affect the Gα16QL-stimulated NFκB transcriptional activity.

Gαq activation enhanced the growth inhibitory effect of Fhit

As Fhit is a tumor suppressor, we asked whether the growth inhibitory effect of Fhit could be affected upon activation of Gαq-coupled receptors. HEK293 and H1299 cells were chosen for this part of the study because they endogenously express Gαq-coupled muscarinic M1 and gastrin-releasing peptide receptors (GRPRs), respectively. We established 293/Fhit cells and H1299/Fhit cells stably expressing Fhit (Figure 9A). Prolonged stimulation of Gαq-coupled receptors is often associated with mitogenesis [28], and thus treatment of 293/vector cells with 100 μM carbachol for 4 days or more significantly stimulated cell growth (Figure 9B). In contrast, carbachol significantly inhibited the growth of 293/Fhit cells (Figure 9B); it should also be noted that 293/Fhit cells exhibited reduced growth rate as compared to the 293/vector cells (Figure 9B). A similar effect was observed in H1299 cells. Bombesin has previously been shown to stimulate the proliferation of non-small lung cancer cells including H1299 cells [45,46]. In the present study, activation of GRPR by 100 nM bombesin for 4 days significantly increased the growth of H1299/vector cells but it suppressed the growth of H1299/Fhit cells (Figure 9C). These data suggest that mitogenic responses elicited by Gαq activation are re-directed into growth suppressive signals when the level of Fhit is elevated. This switching of functional outcome is consistent with the notion that the tumor suppressive action of Fhit is correlated to its expression level [47].

Discussion

Receptors coupled to members of the Gαq subfamily mediate a wide range of diverse cellular responses, ranging from cell growth and proliferation to cell differentiation [39]. Established models indicate that the actions of Gαq-linked receptors are mediated by inositol lipid signaling, but growing evidence suggests that these pathways alone cannot account for all of the responses. Instead, the extensive list of diverse cellular events involving Gαq-linked signals suggests that Gαq subfamily members have multifaceted roles in signal transduction which are not yet fully appreciated. The present study has demonstrated that activated Gαq subunits can directly interact with Fhit, a tumor suppressor widely implicated in many types of cancer [1]. This is especially interesting in view of the ability of Gαq subunits to modulate cell growth and proliferation through regulating critical signaling pathways [48].
The interaction between Ga subunits and Fhit exhibits a high degree of selectivity as demonstrated by the lack of association of Fhit with Gβγ, monomeric GTPases, and RGS proteins. Among the four subfamilies of Ga subunits, at least three can interact with Fhit. Although Ga12 is often regarded as a representative member of the Gi subfamily, its inability to interact with Fhit does not necessarily indicate that the other eight Ga members cannot be partners of Fhit. Likewise, one cannot exclude the possibility that some specific combinations of Gβγ can interact with Fhit unless all viable permutations have been tested. Since both the wild-type and constitutively active mutants of Ga, and Ga13 associate with Fhit equally well, such interactions may not be subjected to dynamic cell signaling regulations. Far more interesting is the activation state-dependent interaction between Gaq subunits and Fhit. Activation of Gaq subunits by agonist-bound receptor is expected to drive the formation of Gaq/Fhit complexes. Our data suggest that Fhit can indeed interact with activated Gaq in a native cellular environment (Figure 2C) and it can directly associate with activated Ga16 in vitro (Figure 3B). It is noteworthy that the Ga subunits are attached to the inner leaflet of the plasma membrane through fatty acylation and thus Fhit needs to be present at the plasma membrane in order to interact with Ga subunits productively. Analysis of Fhit protein expression in subcellular fractions of normal rat tissue suggests that it is localized at the plasma membrane and the nucleus [49]. Hence Fhit can be in close proximity to Gaq subunits for efficient interactions.

The inability of Ga14 to interact with Fhit is rather surprising. The ubiquitously expressed Ga14 exhibits 90% sequence homology to Gaq and is thus more closely related to Gaq than the primarily hematopoietic Ga14 and Ga16 [22], and yet the latter two could interact with Fhit as effectively as Ga14. No report has indicated any major difference between Ga11 and Gaq both in terms of receptor coupling and effector regulation [39]. The ability of Fhit to distinguish Ga11 from Gaq as well as Ga14 and Ga16 thus represents a unique feature of Fhit, but no immediate clue can be drawn as to why it does not form a complex with Ga11.

The use of Ga16/2 chimeras has enabled us to identify the α2-β4 region of Ga16 as an Fhit-interaction domain (Figure 4). This region has been shown to interact with Gβγ complex in the GDP bound Gaq but it becomes available for effector interaction when Gαq adopts the active GTP-bound conformation (Additional file 2). In different Gaq members, this region associates with various effectors such as p63RhoGEF [50] and PLCβ [51]. The binding of Fhit to the α2-β4 region may thus account for the preference of Fhit for constitutively active Gaq mutants that are dissociated from the Gβγ dimers.

Figure 10 Distinct regulations of Fhit by Gaq- and EGF-dependent pathways. Agonist binding to Gq-coupled receptor leads to Gaq activation and dissociation with Gβγ complex. Activated Gaq can interact with Fhit and stabilize it, which results in increased Fhit level and consequent enhancement of the growth suppressive effect of Fhit. On the other hand, activation of the EGF receptor stimulates Src-mediated phosphorylation of Fhit at the Tyr114 site. The phosphorylated Fhit undergoes degradation which leads to a decrease in the Fhit protein level as well as the tumor suppressive effect of Fhit. Although activated Gaq also stimulates Src-mediated Fhit Tyr114 phosphorylation, the overall Fhit protein amount is increased rather than decreased, indicating that either an additional signal is required for the induction of Fhit degradation (which is concomitantly generated by EGF but not by activated Gaq; indicated as a dashed line) or activated Gaq can up-regulate Fhit via stabilization. The interaction of Gaq with Fhit opens a host of possibilities in terms of their biochemical and cellular consequences. Given the known functions of Ga subunits as signal transducers and that only activated Gaq can interact with Fhit, perhaps the most logical prediction is that Fhit acts as an effector of Gaq. If this hypothesis is correct, then activated Gaq subunits may affect the localization, stability, or function of Fhit. However, there is a lack of effect of GaqQL on the Ap3A hydrolyase activity of Fhit. Because Fhit binds and hydrolyzes Ap3A in vitro [38], any model of Fhit function should take this into account. The ability of GST-Fhit to hydrolyze Ap3A into AMP and ADP was, however, unaffected by either GDPβS- or GTPγS-bound His-Gα16. Moreover, Fhit mutants with impaired affinity for Ap3A (L25W and I10W/H89D) formed complexes with activated Gaq subunits as effectively as wild-type Fhit (Figure 5A). These results suggest that activated Gaq subunits have little effect, if any, on the enzymatic activity of Fhit. However, it should be noted that because the catalytic mechanism of Fhit requires leaving-group exit and water entry at the substrate-exposed surface of the dimeric enzyme, polypeptides that bind to the Fhit-Ap3A complex are expected to stabilize the complex and retard turnover [6]. Subtle changes in the Km and/or Kcat of Ap3A hydrolysis by Fhit will require detailed kinetic studies.

Equally disappointing is that the formation of the Gaq/Fhit complex was unable to interfere with any of the
known signaling pathways triggered by G\(\alpha_q\). The canonical effector molecules of activated G\(\alpha_q\) subunits are the various isoforms of PLC\(\beta\). Despite the fact that PLC\(\beta\) also binds to the Flt-interacting α2-β4 region of G\(\alpha_q\) [51], overexpression of wild-type Flt or its mutants did not affect G\(\alpha_q\)RC- or G\(\alpha_{16}\)QL-induced PLC\(\beta\) activity (Figure 6A). Activated G\(\alpha_q\) may have a higher affinity and preference for PLC\(\beta\), resulting in the almost instantaneous formation of IP\(3\) and mobilization of intracellular Ca\(^{2+}\) (agonist-induced Ca\(^{2+}\) mobilization peaks within 10–15 s; Figure 6C). The co-localization of G\(\alpha_q\) and PLC\(\beta\) in lipid rafts [52] helps to ensure the efficiency of the G\(\alpha_q/PLC\beta\) pathway. Flt and other effectors may bind to the activated G\(\alpha_q\) when the latter becomes dissociated from PLC\(\beta\). In this scenario, Flt would not be able to compromise PLC\(\beta\) signaling effectively. However, it should be noted that overexpression of p63RhoGEF can inhibit G\(\alpha_{16}\)QL-induced PLC\(\beta\) activity albeit only partially [53] and the presence of Flt in lipid rafts remains to be confirmed. Flt can apparently associate with the G\(\alpha_{16}\)QL/TPR1 complex since it is detected in the G\(\alpha_{16}\)QL/TPR1 immunoprecipitates but not in the absence of G\(\alpha_{16}\)QL (Figure 7A). The possible existence of an Flt/G\(\alpha_{16}\)QL/TPR1 complex suggests that Flt binds to G\(\alpha_{16}\)QL on a region distinct from that of TPR1, and this is in agreement with our mapping of the Flt-interaction domain by using the G\(\alpha_{16}\)QL chimeras (Figure 4) and the fact that TPR1 interacts with the β3 domain of G\(\alpha_{16}\) [36]. The lack of effect of Flt on G\(\alpha_{16}\)QL-induced Ras activation further suggests that co-expression of Flt would not affect the activities of signaling molecules downstream of Ras. This is indeed true for ERK, STAT3, IKK, and NFκB (Figure 8).

Although the interaction of activated G\(\alpha_q\) and Flt is independent of the ability of Flt to become phosphorylated or to bind and hydrolyze Ap3A, activation of G\(\alpha_q\) could apparently increase Flt Tyr\(^{114}\) phosphorylation through Src (Figure 1B), stabilize Flt (Figure 1B and Additional file 1, Additional file 3) and enhance the cell growth inhibition effect of Flt (Figure 9). G\(\alpha\) signals often lead to increased cell growth [28], but by forming a complex with Flt which can stabilize Flt, activation of G\(\alpha_q\) may result in reduced cell growth (Figure 9). Given that activation of EGF receptors triggers the degradation of Flt [13], and despite the demonstrated ability of activated G\(\alpha_q\) to stimulate Flt phosphorylation (Figure 1B-C), it is rather puzzling to observe that activated G\(\alpha_q\) can apparently increase the levels of Flt (Figure 1B and Additional file 1) and stabilize the truncation mutants of Flt (Additional file 3). The divergent regulatory outcome of phosphorylated Flt may be attributed to the differing signaling capacities of EGF- and G\(\alpha_q\)-dependent pathways, which could lead to conditional proterminal degradation of Flt (Figure 10). An alternative explanation is that Flt becomes less susceptible to degradation upon binding activated G\(\alpha_q\), and this might lead to an elevated level of Flt (Figure 10). Increased Flt levels can lead to the suppression of cell proliferation (Figure 9B and C; [4]), while the knock down of Flt by siRNA increases the viability of DLD-1 cells [10]. If activation of G\(\alpha_q\) can elevate the level of Flt, this might account for the ability of G\(\alpha_q\)-coupled receptors to inhibit cell proliferation (Figure 9B and C; [48]). Further investigations are required to elucidate the mechanism by which activated G\(\alpha_q\) regulates the level of Flt. We are currently pursuing the notion that G\(\alpha_q\) stimulates the translation of Flt as we have preliminary data to suggest that the up-regulation of Flt is blocked by cycloheximide. Since the expression level of Flt may determine its functional outcome [47], it is tremendously important that quantification of Flt should be carefully determined in any cellular system to be employed. It should also be noted that Flt expression can enhance the effects of the p53 tumor suppressor [54] by modulating p53-regulated genes [55]. Hence, the functional relevance of G\(\alpha_q/Flt\) interaction should be revisited in experimental systems with different p53 status.

Conclusions

The present study provides multiple indications that several members of the G\(\alpha_q\) family can bind to the tumor suppressor Flt in their GTP-bound active state. The Flt-interaction domain on the G\(\alpha\) subunit was identified as the α2-β4 region which would be occluded by the G\(\beta\)β dimer in the GDP-bound inactive heterotrimeric G\(\alpha\) protein, thus accounting for the preference of Flt to bind activated forms G\(\alpha_q\) subunits. Neither the hydrolase activity of Flt nor the signaling capacity of activated G\(\alpha_q\) was affected by the formation of activated G\(\alpha_q/Flt\) complexes. In cells with elevated levels of Flt, activation of G\(\alpha_q\)-coupled receptors led to growth suppression rather than stimulation. Consistent with the tumor suppressive function of Flt, these observations suggest that the formation of G\(\alpha_q/Flt\) complex may modulate cell proliferation.

Methods

Reagents

Human cDNAs of various G\(\alpha\) subunits were obtained from Guthrie Research Institute (Sayre, PA). Wild-type Flt in pCMV-SPORT6 was purchased from Invitrogen (Carlsbad, CA). pRcCMV-Flt Y114F was a generous gift from Dr. K. Huebner (Comprehensive Cancer Center and Department of Molecular Virology, Immunology, and Medical Genetics, Ohio State University). L25W, I10W/L25W, and H96D mutants of Flt were kindly provided by Dr. C. Brenner (Department of Genetics and
Biochemistry, Dartmouth Medical School). Cell culture reagents, including LipofectAMINE PLUS reagents were purchased from Invitrogen (Carlsbad, CA). Anti-Gα16 and anti-Gα14 were obtained from Gramsch Laboratories (Schwabhausen, Germany). Anti-Fhit antibody was from Invitrogen (Carlsbad, CA). Anti-Gq/11 α-subunit antibody was purchased from Calbiochem (San Diego, CA). Anti-α-tubulin antibody, anti-HA antibody, anti-Flag antibody and anti-Flag affinity gel were from Sigma-Aldrich (St. Louis, MO). Antiserum against Gαs, Gαi2 and Gα13 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GST antibody was from Abcam (Cambridge, UK). Anti-phospho-Fhit-Tyr114 antibody was raised in rabbits against a synthetic peptide corresponding to AA 106–122 of human Fhit containing the phosphorylated tyrosine residue and an additional N-terminally cysteine residue for coupling (C-DFHRNDS[ pY]EE LQKHDK). Antibodies were affininity-purified using the immunizing phospho-peptide coupled to SulfoLink® Agarose beads from Thermo Scientific (Rockford, IL) and subsequently cross-absorbed against the non-phosphorylated peptide. Specificity of antibodies was verified by Western blot using cell lysates prepared from HEK293 cells transiently transfected with cDNAs of Fhit or Fhit and Src. Other antibodies were purchased from Cell Signaling Technology (Darners, MA). GDPβS and GTPγS were from Calbiochem (San Diego, CA). Protein G-agarose and diethiobis[succinimidylpropionate] (DSP) cross-linker were from Pierce Biotechnology (Rockford, IL). ECL kit and Glutathione Sepharose™ 4 Fast Flow beads were from Amersham Biosciences (Piscataway, NJ). Ni-NTA Agarose was obtained from Qiagen (Valencia, CA). Ras activation kit was a product of Upstate-Millipore (Billerica, MA).

**Construction of G protein chimeras and truncation mutants of Fhit**

The Ga chimeras (except C128) were constructed as described previously [36] by PCR method using human Gα16 and Gαs cDNAs. Briefly, the N-terminal 188, 210, 246 and 266 amino acids or the C-terminal 128 and 164 amino acids of Gα16 were swapped to the corresponding regions of Gαs to generate N188, N210, N246, N266, C128 and C164. Primers were designed to cover the overlapping regions of the chimeras, so that 5’ and 3’ fragments can be annealed together to obtain the full length chimeras by PCR. Then the full length PCR products were subcloned into the pcDNA3 vector. All chimeras were confirmed by dideoxynucleotide sequencing. Primer sequence for constructing C128 is 5’- GTG CCT GGA GGA GAA CAA CCA GAC AAG TCG GAT GGC AG-3’.

Flag-tagged Fhit truncation mutants, F131N, F95N, F50C and F27C, were constructed by PCR method using the human Fhit cDNA as a template. The primers were designed based on the secondary structure of Fhit. The outer forward and reverse primers of Fhit are 5’- CGA AGC TTA TGG ACT ACA AAG ACG ATG AGT ACA AGT CGT TCA GAT TTG GCC AAC ATC TC-3’ and 5’- CCT CGA GTC ACT GAA AGT AGA CCC GCA GAG CTG C-3’, respectively. The reverse primers of F131N and F95N are 5’- CCT CGA GTC ATG ATC TCC AAG AGG CAG GAA AGT C-3’ and 5’- CCT CGA GTC AAA CGT GCT TCA CAG TCT GTG CGG C-3’, respectively. The forward primers of F50C and F27C are 5’- CGA AGC TTA TGG ACT ACA AAG ACG ATG AGT ACA AGT CGT TCA GAT TTG GCC AAC ATC TC-3’ and 5’- CGA AGC TTA TGG ACT ACA AAG ACG ATG AGT ACA AGT CGT TCA GAT TTG GCC AAC ATC TC-3’, respectively. All truncation mutants were confirmed by dideoxynucleotide sequencing.

**Cell culture and co-immunoprecipitation**

HEK293, DLD-1, HeLa and H1299 cells were obtained from the American Type Culture Collection (CRL-1573, Rockville, MD). They were maintained in Eagle’s minimum essential medium (HEK293), RPMI-1640 medium (DLD-1 and H1299) or ATCC-formulated Eagle’s minimum essential medium (HeLa) at 5% CO₂, 37°C with 10% fetal bovine serum, 50 units/mL penicillin and 50 µg/mL streptomycin.

For co-immunoprecipitation experiments, HEK293 cells were grown to 80% confluency in 100 mm tissue culture plates and then co-transfected with various combinations of cDNAs (3 µg/plate) using 15 µL PLUS and LipofectAMINE reagents in MEM. Serum was replenished 3 h after transfection. Cross-linking was performed one day after transfection; transfected HEK293 cells were washed with PBS twice and then treated with 0.5 mM DSP in PBS for 10 min at room temperature. Cells were then washed with PBS twice and maintained in quenching solution containing 50 mM glycine in PBS, pH 7.4, for 5 min. Cells were subsequently lysed in ice-cold RIPA buffer (25 mM HEPES at pH 7.4, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM dithiothreitol, 200 µM Na₃VO₄, 4 µg/mL aprotinin, 100 µM phenylmethylsulfonl fluoride, and 2 µg/mL leupeptin). Cell lysates were gently rocked with a primary antiserum at 4°C overnight, and then incubated in 30 µL protein G-agarose (50% slurry) at 4°C for 2 h. Alternatively, the cell lysates were incubated in 30 µL anti-Flag affinity agarose gel (50% slurry) at 4°C for 4 h. Immunoprecipitates were washed with ice-cold RIPA buffer (400 µL) for four times, resuspended in 50 µL RIPA buffer and 10 µL 6x sample buffer and then boiled for 5 min. Target proteins in the immunoprecipitates were analyzed by Western blots. Signal intensities of the immunoreactive bands were quantified using Image J software, version 1.38x (National Institutes of Health, USA).
Expression and purification of recombinant Go16 and Fhit proteins, and GST pull-down
Fhit and Go16 were subcloned into pGEX-4T-1 and pET21a(+) expression vectors, respectively, and transformed into E. coli BL21 strain. 750 ml bacterial cultures were grown at 37°C until the OD600 reached 0.6-0.8. The cultures were cooled down at 4°C for 20 min and 0.2 mM IPTG was added. The cultures were then grown at 18°C overnight (for GST-Fhit) or 30°C for 15 h (for His-Go16).

Cells were harvested by centrifugation for 15 min at 6,000 rpm and resuspended in 30 ml ice-cold lysis buffer for GST-tagged Fhit (50 mM Tris, pH 7.5, 500 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfon fluoride, 2 μg/ml leupeptin) and lysed by three rounds of sonication. After addition of Triton X-100 to a final concentration of 1%, the lysate was incubated at 4°C for 10 min. Cell debris was removed by centrifugation at 18,000 rpm for 20 min. The cleared supernatant was then incubated with Glutathione Sepharose™ 4 Fast Flow beads at 4°C for 1.5 h with gentle rotation. The beads were spun down at 4,000 rpm for 1 min and washed four times with wash buffer (lysis buffer with 150 mM NaCl and 10% glycerol). The beads were then loaded into a chromatography column and GST-Fhit was eluted washing buffer containing 20 mM glutathione. Similar procedure was used for the purification of His-tagged Go16 except that Ni-NTA Agarose and a different lysis buffer was employed (PBS, pH 8.0, 300 mM NaCl, 10 mM imidazole, 1 mM dithiothreitol, 1 mM phenylmethylsulfon fluoride, 2 μg/ml leupeptin and 20 mM 2-mercaptoethanol). His-Go16 was eluted in washing buffer containing a discontinuous gradient of imidazole (from 30 mM to 250 mM). Proteins eluted at fractions 6 and 7 were pulled. Purified GST or GST-Fhit were mixed with Go16 (2 μg each) in 500 μl pull-down buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 40 mM Na2SO4 and 5 mM MgCl2) in combination with 1 μM GDPβS or GTPγS, and then the mixture was incubated at 4°C for 30 min. Glutathione sepharose was then added and the mixture was further incubated at 4°C for 2 h. After being washed with pull-down buffer twice, the beads were resuspended in sample buffer and subjected to Western blot analysis.

Assay for diadenosine triphosphate hydrolysis by recombinant Fhit
100 μM of Ap3A was incubated with or without recombinant GST-Fhit protein or GST protein in 50 mM HEPES-NaOH, pH 6.8, containing 0.5 mM MnCl2 for 10 min at 37°C in a total volume of 100 μl. Reactions were stopped by heat inactivation (95°C, 10 min). 50 μl of nucleotide standards and assay solutions were then analyzed by HPLC with a Mono Q column, eluted with a gradient from 50 to 600 mM ammonium bicarbonate, pH 8.5, at a flow rate of 1 ml/min. Absorbance of nucleotides were detected at 254 nm. For reactions that required His-Go16 incubations, 0.5 μg His-Go16 was pre-incubated with either GDPβS or GTPγS (100 μM each) at 30°C for 30 min in GTP binding activation buffer (50 mM Hepes, pH 8, 10 mM MgCl2, 1 mM dithiothreitol, 1 mM EDTA, and 100 mM NaCl) prior to incubation with Fhit/Ap3A for 10 min. The extent of Ap3A hydrolysis by 0.5 μg GST-Fhit was measured in the absence or presence of His-Go16 and was expressed as percentage of Ap3A hydrolyzed during the reaction based on the areas under the peaks of Ap3A before and after the hydrolysis reaction [38].

Ras activation assay
HEK293 cells were co-transfected with 200 ng Ga, 200 ng Flag-Fhit and 100 ng Ras cDNAs. After 1 day, transfectants were serum starved for 4 h. Cells were then washed twice with ice-cold PBS and lysed with the Mg2+-lysis buffer (MLB; 125 mM HEPES at pH 7.5, 750 mM NaCl, 5% Nonidet P-40, 50 mM MgCl2, 5 mM EDTA, 10% glycerol, and appropriate protease inhibitors). Clarified cell lysates were immunoprecipitated with 20 μl Raf-1 RBD agarose for 45 min and subsequently washed three times with 400 μl ice-cold MLB. Eluted protein samples in 50 μL MLB and 10 μL 6x sampling dye were then resolved in SDS gels and analyzed using specific anti-Ras antibody.

Inositol phosphates accumulation assay
HEK293 cells were seeded on a 12-well plate at 2 × 10^5 cells/well one day prior to transfection. Various cDNAs at a concentration of 0.5 μg/well were transiently transfected into the cells using Lipofectamine PLUS® reagents. One day after transfection, cells were labeled with inositol-free Dubecco’s modified Eagle’s medium (DMEM; 750 μL) containing 5% FBS and 2.5 μCi/mL myo-[3H]inositol overnight. The labeled cells were then washed once with IP3 assay medium (20 mM HEPES, 5 mM LiCl, serum-free DMEM) and then incubated with 500 μl IP3 assay medium at 37°C for 1 h. Reactions were stopped by replacing the assay medium with 750 μl ice-cold 20 mM formic acid and the lysates were kept in 4°C for 30 min before the separation of [3H]inositol phosphates from other labeled species by sequential ion-exchange chromatography as described previously [56].

Transfection of HeLa cells with Fhit siRNA
Previously validated siRNA against Fhit (Fhit si1 sequence; [57]) was used for the knockdown of Fhit. HeLa cells (1 × 10^6 cells) cultured in 10-cm plates were transfected with siFhit (50 nM; Ribobio, Guangzhou, China) or a negative universal control med GC siRNA by Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad,
After 24 h incubation, 2 x 10⁶ cells per well were seeded into 96-well plates for Ca²⁺ measurement or 5 x 10⁵ cells per well into 6-well plates and lysed for Western blotting.

Western blotting analysis
Protein samples were resolved on 12% SDS-polyacrylamide gels and transferred to Osmonics nitrocellulose membrane. Resolved proteins were detected by their specific primary antibodies and horseradish peroxidase-conjugated secondary antisera. The immunoblots were visualized by chemiluminescence with the ECL kit from Amersham, and the images detected in X-ray films were quantified by densitometric scanning using the Eagle Eye II still video system (Stratagene, La Jolla, CA, USA).

Measurement of intracellular Ca²⁺ by FLIPR
The intracellular Ca²⁺ was measured by using an optimized Fluorometric Imaging Plate Reader (FLIPR) protocol [58]. HeLa cells were seeded into clear-bottomed black-walled 96-well plates. The growth medium was replaced by 200 μL labeling medium containing 1:1 (v/v) ATCC-MEM medium: Hank’s balanced salt solution, 2.5% (v/v) fetal calf serum, 20 mmol/L HEPES, pH 7.4, 2.5 mmol/L probenecid and 2 μmol/L Fluo-4 AM. Histamine was prepared as a 5× solution in Hank’s balanced salt solution into another polypropylene 96-well plate. After 1 h labeling, cell and drug plates were placed in a FLIPR (Molecular Devices, Sunnyvale, CA, USA). Immediately after the addition of 50 μL of drug solution into the cell medium, changes in fluorescence were monitored over 120 s following excitation at a wavelength of 488 nm and detection at 510–560 nm.

Luciferase assay
The growth medium of serum-starved transfectants was removed and replaced by 25 μL of lysis buffer provided in the Luciferase Reporter Gene Assay kit (Roche Applied Science). The 96-well microplate was shaken on ice for 30 min. The luciferase activity was determined by a microplate luminometer LB96V (EG&G Berthold, Germany). Injector M connected to lysis buffer and injector P connected to the luciferin substrate were set to inject 25 μL of each component into each well. A 1.6 sec delay time followed by a 2 sec measuring time period was assigned to infector M whereas injector P was measured for 10 s after introduction of luciferin into the well. Results were collected by WinGlow version 1.24 and expressed as relative luminescence units (RLU). Statistical calculation was performed using KyPlot version 2.0.

Establishment of stable cell lines
HEK293 or H1299 cells stably expressing Flag-tagged Fhit, or the pFlag-CMV2 vector were established by LipofectAMINE-mediated transfection along with excess pcDNA3 (9:1 ratio), followed by G418 selection for 2 weeks. The resultant cell lines were named as 293/Fhit and 293/vector, or H1299/Fhit and H1299/vector, respectively.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric assay
Cells (5,000 cells/well of HEK293 or 2,000 cells/well of H1299) were seeded in 96-well plates and incubated in the absence or presence of agonists (100 μM carbachol or 100 nM bombesin) for various durations. After removing the growth medium, 100 μL MTT labeling reagent (0.5 mg/ml; Roche Applied Science) in serum-free medium was added. The plate was incubated for 4 h at 37°C prior to the addition of 100 μL solubilization buffer (10% SDS in 0.01 M HCl). The plate was incubated overnight at 37°C. The absorbance reading was taken at the wavelength of 570 nm, with the reference value taken at the wavelength of 630 nm.

Additional files

Additional file 1: Fhit expression is increased by activated Gaq.
Additional file 2: The structures of active and inactive Gaq.
Additional file 3: Co-expression of constitutively activated mutant of Gaq increases the stability of Fhit truncation mutants.
Additional file 4: Expression of endogenous Fhit in HeLa cells.

Abbreviations
PLCβ: Phospholipase Cβ; IP3: Inositol 1,4,5-trisphosphate; ApA: Diadenosine 5′,5‴-P′,P‴-triphosphate; GDPβS: Guanosine 5′-O-(2-thiodiphosphate); GTPγS: Guanosine 5′-O-(3-thiotriphosphate); Raf-1 RBD: Ras-binding domain of Raf-1; RGS: Regulator of G protein signaling; TPR1: Tetra-tricopeptide repeat 1; IP: Immunoprecipitation; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
HZ and WWSY performed the experiments, participated equally in the design of the study and wrote the manuscript. JZ and WWSY carried out some of the experiments. HA raised and characterized the anti-phospho-Fhit Tyr114 antisemur. ASLC participated in the design of experiments. YHW participated in the design of the study and the writing of the manuscript. All authors read and approved the final manuscript.

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Author details
1Division of Life Sciences, Biotechnology Research Institute, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong. 2Department of Veterinary Sciences, University of Munich, Munich, Germany. 3State Key Laboratory of Molecular Neuroscience, Molecular Neuroscience Center, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong.
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