Zuo and Wong *BMC Cancer* (2015) 15:775
DOI 10.1186/s12885-015-1802-z

BMC Cancer

RESEARCH ARTICLE Open Access

Association of activated $G\alpha_q$ to the tumor suppressor Fhit is enhanced by phospholipase Cβ

Hao Zuo[1,3] and Yung H. Wong[1,2*]

Abstract

Background: G proteins are known to modulate various growth signals and are implicated in the regulation of tumorigenesis. The tumor suppressor Fhit is a newly identified interaction partner of G_q proteins that typically stimulate the phospholipase C pathway. Activated $G\alpha_q$ subunits have been shown to interact directly with Fhit, up-regulate Fhit expression and enhance its suppressive effect on cell growth and migration. Other signaling molecules may be involved in modulating $G\alpha_q$/Fhit interaction.

Methods: To test the relationship of PLCβ with the interaction between $G\alpha_q$ and Fhit, co-immunoprecipication assay was performed on HEK293 cells co-transfected with different combinations of Flag-Fhit, $G\alpha_{16}$, $G\alpha_{16}$QL, pcDNA3 vector, and PLCβ isoforms. Possible associations of Fhit with other effectors of $G\alpha_q$ were also demonstrated by co-immunoprecipitation. The regions of $G\alpha_q$ for Fhit interaction and PLCβ stimulation were further evaluated by inositol phosphates accumulation assay using a series of $G\alpha_{16/z}$ chimeras with discrete regions of $G\alpha_{16}$ replaced by those of $G\alpha_z$.

Results: PLCβ1, 2 and 3 interacted with Fhit regardless of the expression of $G\alpha_q$. Expression of PLCβ increased the affinities of Fhit for both wild-type and activated $G\alpha_q$. Swapping of the Fhit-interacting α2-β4 region of $G\alpha_q$ with $G\alpha_i$ eliminated the association of $G\alpha_q$ with Fhit without affecting the ability of the mutant to stimulate PLCβ. Other effectors of $G\alpha_q$ including RGS2 and p63RhoGEF were unable to interact with Fhit.

Conclusions: PLCβ may participate in the regulation of Fhit by G_q in a unique way. PLCβ interacts with Fhit and increases the interaction between $G\alpha_q$ and Fhit. The $G\alpha_q$/PLCβ/Fhit complex formation points to a novel signaling pathway that may negatively regulate tumor cell growth.

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

Background

The fragile *FHIT* gene at the chromosomal fragile site FRA3B is often regarded as an early target of DNA damage in precancerous cells. Its gene product, the ubiquitously expressed Fhit (Fragile Histidine Triad) protein, is a member of the HIT (histidine triad) superfamily with three signature histidines in the conserved nucleoside binding motif. Fhit binds and hydrolyzes various dinucleoside polyphosphates (such as Ap_3A, Ap_4A, Ap_3G and Cp_3G) into two nucleotides where one is a nucleoside monophosphate [1]. The preferred substrate of Fhit is Ap_3A (diadenosine 5′,5‴-P1,P3-triphosphate) which is hydrolyzed to AMP and ADP. Interestingly, Fhit acts as a tumor suppressor and its down-regulation is associated with different tumors including lung cancers [2]. Re-expression of Fhit in Fhit-deficient tumor cells can notably suppress tumor development [3–5]. Several theories of tumor suppression have been proposed for Fhit with the overarching idea of Fhit acting as a genome "caretaker" [6, 7]. Reduced Fhit expression has indeed been shown to increase DNA replication stress and genome alterations as a result of a decreased intracellular thymidine triphosphate (dTTP) level [8]. Moreover, Fhit is apparently involved in suppressing lung tumor cell migration/invasion by down-regulating the expression of matrix metalloproteinase 2/9 [9]. Despite

* Correspondence: boyung@ust.hk
[1]Division of Life Sciences, and the Biotechnology Research Institute, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China
[2]State Key Laboratory of Molecular Neuroscience, and the Molecular Neuroscience Center, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong
Full list of author information is available at the end of the article

considerable efforts, the precise mechanism by which Fhit exerts its tumor suppressive function remains elusive. The dinucleoside polyphosphate hydrolase activity of Fhit seemingly plays a trivial role in tumor suppression [10].

A number of studies have revealed unsuspecting binding partners of Fhit that may provide linkages to processes that contribute to tumor eradication such as cellular oxidation and apoptosis. Fhit-interacting molecules include β-catenin [11], ferredoxin reductase [12], Src tyrosine kinase [13] and ubiquitin conjugating enzyme 9 [14]. More recently, we have demonstrated that Fhit can distinguish between inactive and active signal transducing Gα subunits of the G_q family [15]. This finding is intriguing as it may link Fhit to $G\alpha_q$-dependent signals that modulate a variety of cellular events. Fhit-mediated suppression of epithelial-mesenchymal transition in bronchial cells involves the epidermal growth factor receptor (EGFR), Src, and extracellular signal-regulated kinase (ERK) [16] that have all been shown to be activated or transactivated by $G\alpha_q$ [17, 18]. In human colon cancer cell lines, Fhit inhibits cell proliferation by attenuating the nuclear factor κB (NFκB) pathway [19] which can be stimulated by G_q-coupled receptors [20]. It is also noteworthy that sustained activation of the G_q pathway often leads to mitogenesis in a variety of cell types [21] with disparate mechanisms of regulating cell cycle progression [22]. The opposing roles of Fhit and $G\alpha_q$ tend to suggest that they may exert counteracting actions on each other. However, Fhit neither inhibits nor enhances $G\alpha_q$-induced signals [15] whereas its own expression becomes translationally up-regulated by activated $G\alpha_q$ [23]. Given the links between G_q signaling and mitogenesis as well as those between Fhit and tumor suppression are well-established, it seems reasonable to expect that the binding of Fhit to activated $G\alpha_q$ would impart functional consequences. Since the canonical signaling pathway of all $G\alpha_q$ subfamily members ($G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$ and $G\alpha_{15/16}$) is the activation of phospholipase Cβ (PLCβ), we further explored the influence of PLCβ on the formation of Fhit/activated $G\alpha_q$ complexes. Here, we report that different isoforms of PLCβ can also associate with Fhit in the absence of $G\alpha_q$ activation.

Methods

Reagents

The human cDNAs of various Gα subunits were obtained from Guthrie Research Institute (Sayre, PA). Wild-type Fhit in pCMV-SPORT6 was purchased from Invitrogen (Carlsbad, CA). $G\alpha_{16/z}$ chimeras were constructed by overlapping PCR which swapped the corresponding regions of $G\alpha_{16}$ with $G\alpha_z$ as described previously [15]. Cell culture and Lipofectamine PLUS reagents, and anti-Fhit antibody were purchased from Invitrogen (Carlsbad, CA). Anti-$G\alpha_{16}$ was obtained from Gramsch Laboratories (Schwabhausen, Germany). Anti-$G\alpha_{q/11}$ antibody was purchased from Calbiochem (San Diego, CA). Anti-α-tubulin, anti-HA, and anti-Flag antibodies as well as anti-HA affinity gel were from Sigma-Aldrich (St. Louis, MO). Antisera against PLCβ1/2/3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Other antibodies were purchased from Cell Signaling Technology (Danvers, MA). Protein G-agarose was from Thermo Fisher Scientific (Rockford, IL). ECL kit was from Amersham Biosciences (Piscataway, NJ).

Cell culture and Co-immunoprecipitation

HEK293 cells were obtained from the American Type Culture Collection (CRL-1573, Rockville, MD). They were maintained in Eagle's minimum essential medium at 5 % CO_2, 37 °C with 10 % fetal bovine serum, 50 units/mL penicillin and 50 μg/mL streptomycin. Transfection was performed according to the manual of Lipofectamine® transfection reagent. One day later, cells were 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 Na_3VO_4, 4 μg/mL aprotinin, 100 μM phenylmethylsulfonyl fluoride, and 2 μg/mL leupeptin). Cell lysates were incubated with a primary antiserum with rotation at 4 °C overnight, and then incubated in 30 μL protein G-agarose (50 % slurry) at 4 °C for 4 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 6× 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 1.38× (National Institutes of Health, USA).

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).

Inositol phosphates accumulation assay

HEK293 cells were seeded at a density of 2×10^5 cells/well into 12-well plates. Various cDNAs at a concentration of 0.5 μg/well were transiently transfected into the

cells using Lipofectamine® transfection 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*-[^{3}H]inositol overnight. The labeled cells were then washed once with IP_3 assay medium (20 mM HEPES, 5 mM LiCl, serum-free DMEM) and then incubated with 500 μl IP_3 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 [^{3}H]inositol phosphates from other labeled species by sequential ion-exchange chromatography as described previously [24].

Statistical analysis

Data were expressed as the mean ± S.E. of at least three independent sets of experiments. The probability of an observed difference being a coincidence was evaluated by Dunnett *t* test. Differences at values of $P < 0.05$ were considered significant (* $P < 0.05$).

Results

We have previously shown that Fhit directly interacts with activated members of the $G\alpha_q$ family ($G\alpha_q$, $G\alpha_{14}$, and $G\alpha_{16}$) via their α2-β4 region without affecting $G\alpha_q$-induced PLCβ activation [15]. As PLCβ also interacts with the α2 region of the activated $G\alpha_q$ [25], we asked whether PLCβ can compete with Fhit for the activated $G\alpha_q$ in co-immunoprecipitation assays. HEK293 cells were co-transfected with Flag-tagged Fhit and wild-type or activated $G\alpha_{16}$ ($G\alpha_{16}$QL) with or without PLCβ1, PLCβ2 or PLCβ3. Because activated $G\alpha_q$ signaling always increase the expression levels of Fhit [23], we adjusted the Fhit cDNA amount for transfection to obtain similar Fhit expression levels. In order to facilitate the assessment of expression and to minimize interference by endogenous $G\alpha_q$ subunits, we have opted for using $G\alpha_{16}$ as a representative $G\alpha_q$ member. In vector transfected cells, Fhit pulled down detectably more $G\alpha_{16}$QL than wild-type $G\alpha_{16}$ (Fig. 1), as reported previously [15]. Overexpression of PLCβ1, PLCβ2 or PLCβ3 increased the affinities of both wild-type and activated $G\alpha_{16}$ for Fhit (*cf* lanes 1, 2 and lanes 4, 5 in the three panels of the second row of Fig. 1). Thus PLCβs did not appear to compete with Fhit for activated $G\alpha_{16}$. Instead, the presence of PLCβs apparently enhanced or stabilized the association of Fhit and $G\alpha_{16}$. More interestingly, all three isoforms of PLCβ were also detected in the Fhit-immunoprecipitates (Fig. 1). To test if Fhit can form complexes with PLCβs, HEK293 cells were transfected with vector or PLCβ1-3 in combination with Flag-Fhit or Flag vector. All three isoforms of PLCβ were able to co-immunoprecipitate with Fhit (Fig. 2a). Since HEK293 cells endogenously express PLCβ1 (Fig. 2a, upper left panel), Flag-Fhit might be able to pull down endogenous PLCβ1. Longer exposure of the anti-PLCβ1 blot indeed

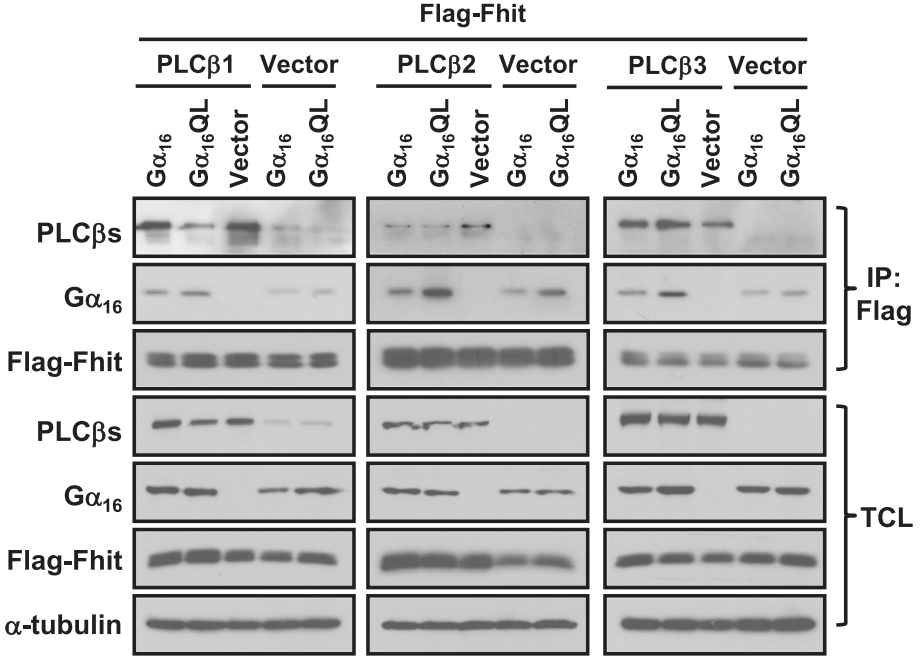

Fig. 1 PLCβs enhance the interaction between Fhit and $G\alpha_q$. HEK293 cells were co-transfected with different combinations of Flag-Fhit, $G\alpha_{16}$, $G\alpha_{16}$QL, pcDNA3 vector, PLCβ1, PLCβ2 or PLCβ3. One day after transfection, cell lysates were prepared and immunoprecipitated with anti-Flag affinity gel. PLCβ1, 2, 3, $G\alpha_{16}$, Fhit and α-tubulin of the co-immunoprecipitation (IP) assay and total cell lysate (TCL) were determined by Western blotting

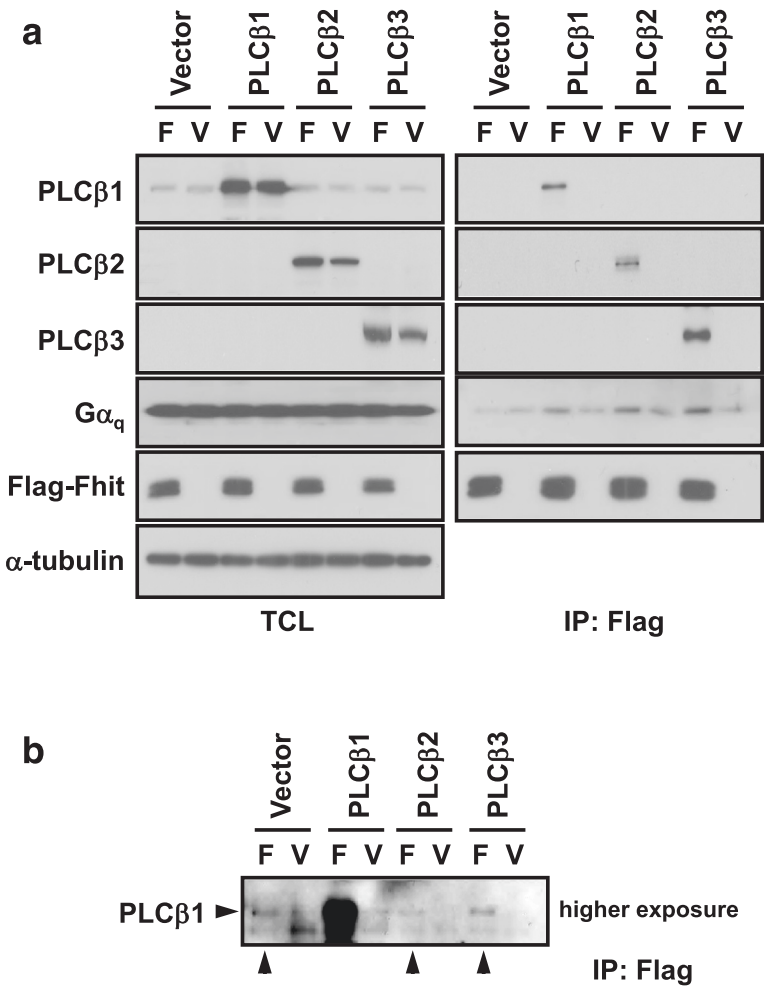

Fig. 2 Fhit interacts with PLCβs. **a** HEK293 cells were transfected with pcDNA3 vector, PLCβ1, PLCβ2 or PLCβ3 in combination with Flag-Fhit (F) or pFlag-CMV2 (V) vector. Following expression for 1 day, cells were lysed and subjected to co-immunoprecipitation assay with anti-Flag affinity gel. The levels of Fhit and PLCβs were examined by Western blotting. **b** In the co-immunoprecipitation assay in a, a longer exposure of the anti-PLCβ1 blot showed that endogenous PLCβ1 (indicated by a horizontal arrow) was pulled down by Fhit (indicated by vertical arrows)

revealed the presence of endogenous PLCβ1 in the immunoprecipitates of Fhit (Fig. 2b). Moreover, as compared to the Flag vector control, Fhit could pull down endogenous wild-type $G\alpha_q$ when one of the PLCβ isoforms was overexpressed (*cf* lane 1 and lanes 3, 5, and 7 of the fourth row on the right in Fig. 2a). These findings indicate that PLCβs may interact with Fhit and increase the association between Fhit and $G\alpha_q$. We have previously reported that increased cell proliferation by $G\alpha_q$ activation is suppressed in the presence of Fhit [15]. Not surprisingly, we also found that PLCβ3 overexpression alone was sufficient to trigger a higher cell growth rate and Fhit co-expression significantly decreased PLCβ3-induced cell proliferation (data not shown). Therefore, Fhit appears to be capable of suppressing G_q-PLCβ mediated cell proliferation.

If PLCβ binds to Fhit via activated $G\alpha_q$, other proteins known to associate with $G\alpha_q$ may also co-immunoprecipitate with Fhit. Besides PLCβs, activated $G\alpha_q$ also interacts with other proteins such as RGS2 [26] and p63RhoGEF [27]. Unlike PLCβs, Fhit did not interact with HA-tagged RGS2 in the co-immunoprecipitation assay using anti-Flag or anti-HA affinity gel (Fig. 3a). Similarly, in transfected HEK293 cells expressing various combinations of Flag-Fhit, myc-tagged p63RhoGEF, $G\alpha_q$ and constitutively active $G\alpha_q$RC, myc-p63RhoGEF pulled down $G\alpha_q$RC but not Fhit (Fig. 3b). Moreover, the expression of Fhit did not affect the interaction between $G\alpha_q$RC and p63RhoGEF (Fig. 3b). These results suggest that the ability of PLCβs to associate with Fhit and increase the interaction between Fhit and $G\alpha_q$ may be specific.

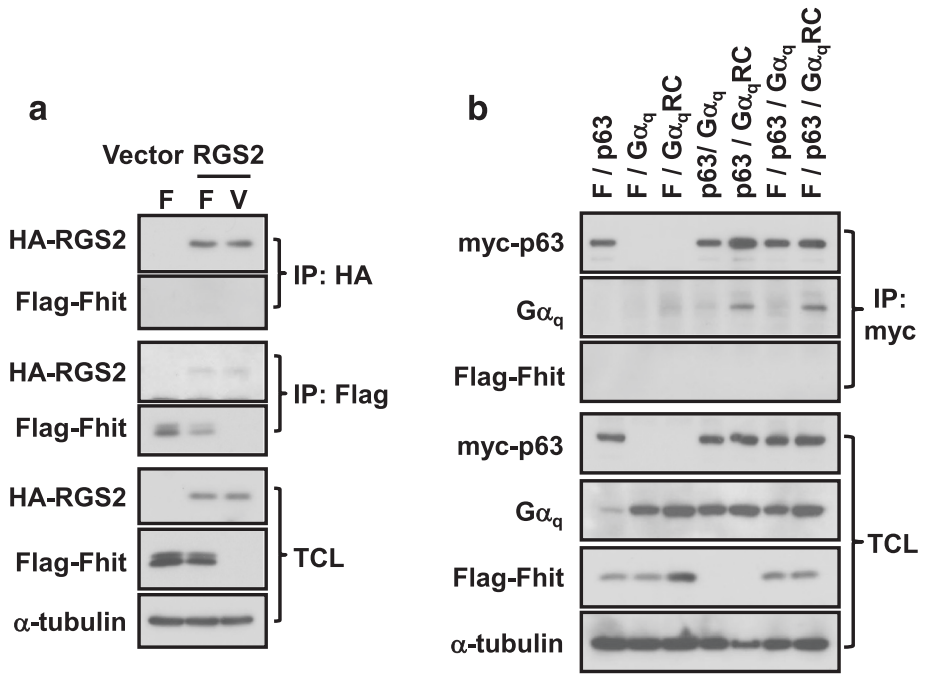

Fig. 3 Fhit does not interact with RGS2 or p63RhoGEF. **a** HEK293 cells were transfected with pcDNA3 vector or HA-tagged RGS2 in combination with pFlag-CMV2 (V) or Flag-Fhit (F). Cell lysates were subjected to co-immunoprecipitation assay with anti-Flag or anti-HA affinity gel. RGS2, $G\alpha_q$, Fhit and α-tubulin were detected by Western blotting. **b** HEK293 cells were transfected with different combinations of Flag-Fhit, myc-p63RhoGEF and $G\alpha_q$ or $G\alpha_q$RC. After 1 day, cells were subjected to the co-immunoprecipation with anti-myc affinity gel. The immunoprecipitates and total cell lysates were analyzed by Western blot

Since overexpression of PLCβs appeared to enhance the interaction between Fhit and $G\alpha_{16}$ (Fig. 1), we assessed whether Fhit can reciprocally enhance the interaction of PLCβ with $G\alpha_q$ members. We performed co-immunoprecipitation assay with anti-PLCβ3 antiserum because it has the best specificity among the different anti-PLCβ antisera. $G\alpha_{16}$QL as well as Fhit was co-immunoprecipitated with PLCβ3 (Fig. 4). Co-expression of Fhit appeared to weaken the interaction between $G\alpha_{16}$QL and PLCβ3 (Fig. 4 *cf* lanes 2 and 4 of row two). The reduction of $G\alpha_{16}$QL in the PLCβ3-immunoprecipitates was not due to variations in the expression levels or pull down efficiency, as these parameters were essentially similar in the different samples (Fig. 4 rows one and five).

By using a series of $G\alpha_{16/z}$ chimeras with discrete regions of $G\alpha_{16}$ replaced by those of $G\alpha_z$ (a $G\alpha_i$ subfamily member which does not interact with Fhit), we have previously identified the α2-β4 region of $G\alpha_{16}$ as critical for Fhit interaction [15]. Interestingly, the α2 region of $G\alpha_q$ is seemingly involved in binding to PLCβ [25]. To further investigate the associations among $G\alpha_q$, PLCβ and Fhit, we constructed two new chimeras named zα2β4 ($G\alpha_{16}$ backbone with α2β4 region from $G\alpha_z$) and 16α2β4 ($G\alpha_z$ backbone with α2β4 region from $G\alpha_{16}$), wherein the α2-β4 region of $G\alpha_{16}$ or $G\alpha_z$ was swapped with each other (Fig. 5a). As shown in Fig. 5b, both wild-type and constitutively active mutants of the zα2β4 and 16α2β4 chimeras can be expressed in HEK293 cells to levels that were comparable to those of $G\alpha_{16}$, $G\alpha_z$, or C128 (a previously characterized chimera with the C-terminal 128 residues of $G\alpha_{16}$ swapped with the corresponding sequences of $G\alpha_z$). Because the Gα-specific antibodies are N-terminal targeting, the zα2β4 and 16α2β4 chimeras were recognized by anti-$G\alpha_{16}$ and anti-$G\alpha_z$ antisera, respectively. When examined for their ability to stimulate PLCβ, activated zα2β4QL efficiently stimulated the formation of inositol phosphates to an extent similar to that of $G\alpha_{16}$QL (Fig. 5b). The constitutively active $G\alpha_z$QL did not activate PLCβ because $G\alpha_z$ belongs to the $G\alpha_i$ subfamily (Fig. 5b). Since neither 16α2β4QL nor C128QL was able to stimulate PLCβ (Fig. 5b), it indicated that the α2-β4 region of $G\alpha_{16}$ alone was not sufficient to stimulate PLCβ.

A hallmark of Fhit/$G\alpha_q$ interaction is the enhanced association with the activated $G\alpha_q$ subunits over their wild-type counterparts [15]. Activation state-dependent interaction with Fhit was reproducibly observed in the present study (Fig. 1 and Additional file 1: Figure S1) and this feature was therefore used as an indicator of Fhit association with the chimeras. In co-immunoprecipitation assays, both the wild-type and constitutively active mutant of zα2β4 were pulled down by Flag-Fhit to similar extents

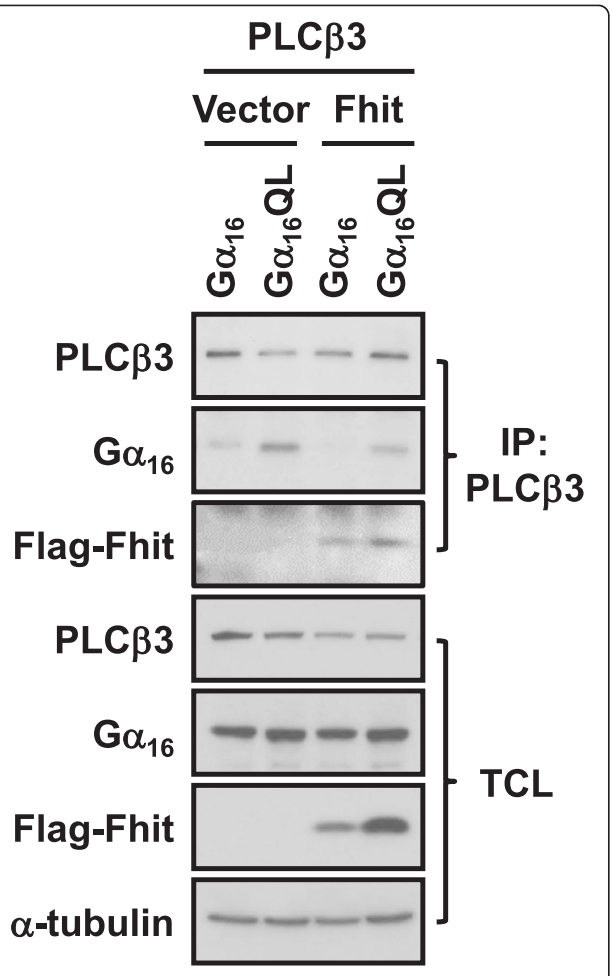

Fig. 4 Fhit does not enhance the association of $G\alpha_{16}QL$ with PLCβ3. $G\alpha_{16}$ or $G\alpha_{16}QL$ was co-transfected into HEK293 cells with pFlag-CMV2 (Vector) or Flag-Fhit in combination with PLCβ3. One day after transfection, cell lysates were immunoprecipitated with anti-PLCβ3 antibody and Protein G agrose, and subjected to Western blot analysis

(Fig. 5c), despite the fact that zα2β4QL was fully capable of stimulating PLCβ (Fig. 5b). Thus the α2β4 region of activated $G\alpha_{16}$ is required by Fhit association but not by PLCβ activation. In the control groups, more $G\alpha_{16}QL$ was detected in the Fhit-immunoprecipitates than wild-type $G\alpha_{16}$ whereas both $G\alpha_z$ and $G\alpha_zQL$ were hardly detected (Fig. 5c). These control groups produced the same results as in our previous report [15]. The presence of 16α2β4 or 16α2β4QL in the Fhit-immunoprecipitates was even weaker than those $G\alpha_z$ (Fig. 5c), suggesting that this chimera could not be recognized by Fhit. It should be noted that activation state-dependent association with Fhit has previously been demonstrated for the C128 chimera [15], while C128QL was not able to stimulate PLCβ (Fig. 5b). According to the above results of zα2β4, 16α2β4 and C128, the capability of activated $G\alpha_{16}QL$ to associate with Fhit did not affect its ability to stimulate PLCβ and it may explain why PLCβ enhanced the association of Fhit and $G\alpha_q$ instead of competing with Fhit for $G\alpha_q$,

Discussion

The diversity of pathways downstream of G_q has endowed mammalian cells with a complex signaling network for the delicate regulation of a multitude of biological effects, with some responses being cell type-specific. One example is the fact that activation of G_q leads to proliferation in some cells while it induces apoptosis in other cell types [22]. The tumor suppressor Fhit taps into the G_q signaling network through its ability to associate with activated $G\alpha_q$ [15, 23]. Interestingly, Fhit suppresses G_q-mediated cell growth in H1299 lung cancer cells via an unknown mechanism [15, 23]. Here, we showed that the canonical $G\alpha_q$ effector PLCβ can form a complex with activated $G\alpha_q$ and Fhit, and can increase the overall association of the latter two proteins. Many tripartite or even higher order complexes involving $G\alpha_q$ subunits are known to exist [28]. For instance, $G\alpha_q$ can simultaneously bind to p63RhoGEF and RhoA [27], to G protein-coupled receptor kinase 2 (GRK2) and Gβγ [29], as well as to ADP-ribosylation factor 6 (ARF6) and ARNO (a GEF for ARF6) [30]. Hence, the existence of a $G\alpha_q$/PLCβ/Fhit complex seems plausible.

Given the prior demonstration that direct binding of Fhit to activated $G\alpha_q$ does not affect PLCβ activity [15], the ability of PLCβ to form a complex with Fhit/$G\alpha_q$ is rather surprising. All three PLCβ isoforms (PLCβ1, 2 and 3) tested as well as the endogenously expressed PLCβ1 could be detected in the immunoprecipitates of Fhit in the absence of $G\alpha_q$ overexpression (Fig. 2). Therefore, it is possible that PLCβ can directly interact with Fhit. Although the PLCβ association with Fhit may also occur via binding to endogenous $G\alpha_q$ subunits, the interaction between PLCβ and Fhit appears to be specific because other $G\alpha_q$ effectors such as RGS2 and p63RhoGEF did not interact with Fhit even in the presence of activated $G\alpha_q$ (Fig. 3). PLCβs have weak affinities for inactive $G\alpha_q$ subunits (EC_{50} at ~10 μM range) [31] but this basal interaction between PLCβ3 and inactive $G\alpha_q$ could be detected (Fig. 4). The increased affinity of Fhit with inactive $G\alpha_q$ upon overexpression of PLCβs (Figs. 1 and 2b) may result from the basal interactions between PLCβs and inactive $G\alpha_q$ and the interaction between PLCβ and Fhit. It remains to be demonstrated if PLCβ can directly interact with Fhit.

The α2-β4 region of $G\alpha_q$ is essential for the binding of Fhit [15] and other regions may also be required (Fig. 5b). According to the structure of $G\alpha_q$-PLCβ3 complex, PLCβ3 interacts with the α2 and α3 region of $G\alpha_q$ by a helix-turn-helix domain [25] and a similar interaction domain is also present in the $G\alpha_q$/p63RhoGEF complex [27]. Different from PLCβ, p63RhoGEF could

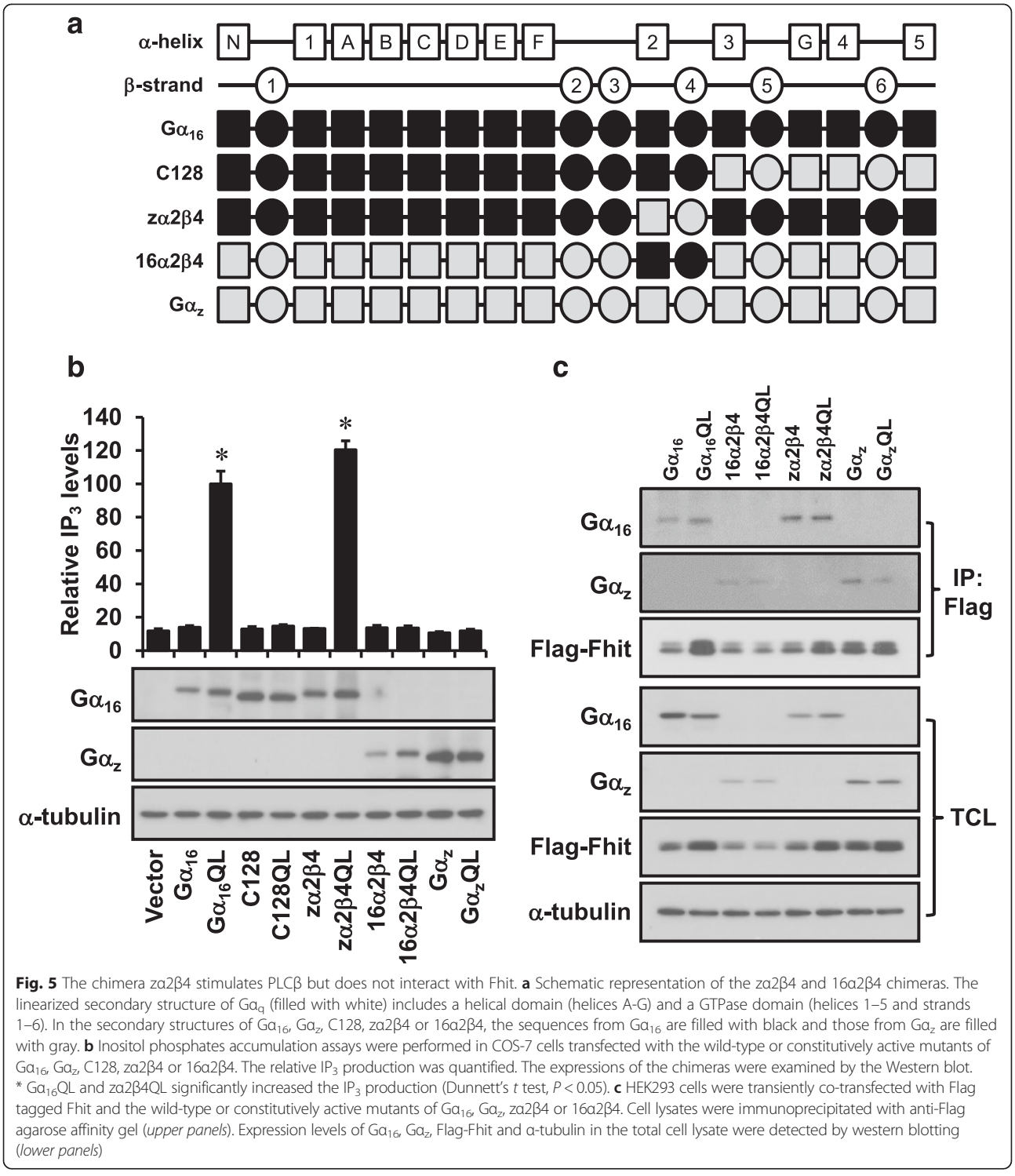

Fig. 5 The chimera zα2β4 stimulates PLCβ but does not interact with Fhit. **a** Schematic representation of the zα2β4 and 16α2β4 chimeras. The linearized secondary structure of $G\alpha_q$ (filled with white) includes a helical domain (helices A-G) and a GTPase domain (helices 1–5 and strands 1–6). In the secondary structures of $G\alpha_{16}$, $G\alpha_z$, C128, zα2β4 or 16α2β4, the sequences from $G\alpha_{16}$ are filled with black and those from $G\alpha_z$ are filled with gray. **b** Inositol phosphates accumulation assays were performed in COS-7 cells transfected with the wild-type or constitutively active mutants of $G\alpha_{16}$, $G\alpha_z$, C128, zα2β4 or 16α2β4. The relative IP_3 production was quantified. The expressions of the chimeras were examined by the Western blot. * $G\alpha_{16}$QL and zα2β4QL significantly increased the IP_3 production (Dunnett's *t* test, $P < 0.05$). **c** HEK293 cells were transiently co-transfected with Flag tagged Fhit and the wild-type or constitutively active mutants of $G\alpha_{16}$, $G\alpha_z$, zα2β4 or 16α2β4. Cell lysates were immunoprecipitated with anti-Flag agarose affinity gel (*upper panels*). Expression levels of $G\alpha_{16}$, $G\alpha_z$, Flag-Fhit and α-tubulin in the total cell lysate were detected by western blotting (*lower panels*)

not interact with Fhit irrespective of whether activated $G\alpha_q$ was present (Fig. 3b). This tends to suggest that when activated $G\alpha_q$ is bound to an effector other than PLCβ, Fhit is precluded from interacting with $G\alpha_q$. The complete lack of evidence on Fhit and PLCβ competing for activated $G\alpha_q$ further supports the existence of a tripartite complex of Fhit/$G\alpha_q$/PLCβ. A prerequisite for the simultaneous binding of Fhit and PLCβ to activated $G\alpha_q$ is that the two molecules should not use identical regions on $G\alpha_q$ for interaction. This notion is indirectly supported by the results pertaining to the zα2β4QL chimera, which stimulated PLCβ activity as robustly as

$G\alpha_{16}$QL (Fig. 5b) but failed to interact with Fhit beyond that of wild-type zα2β4 (Fig. 5c). It would appear that the conformation of activated zα2β4QL can be recognized by PLCβ but not by Fhit. Although PLCβ has additional contact points (e.g., the α3-β5 region) on $G\alpha_q$ [25], the lack of detrimental effect upon the replacement of the α2-β4 region in the zα2β4QL chimera is rather intriguing and warrant some discussion. In the α2-β4 region of $G\alpha_q$, nine of the ten residues (Q209-K215, H218, C219 and E221) for PLCβ3 interaction are the same with members of $G\alpha_i$ subfamily while the exceptional residue on $G\alpha_i$ subunits corresponding to R214 of $G\alpha_q$ is identical to that of $G\alpha_{16}$ [25]. Hence, it is possible that substitution of α2-β4 region of $G\alpha_{16}$ (a $G\alpha_q$ member) with the corresponding region of $G\alpha_z$ (a $G\alpha_i$ subfamily member) would still allow the zα2β4QL chimera to interact productively with PLCβ (Fig. 5b). In contrast, the mere presence of the α2-β4 region of $G\alpha_{16}$ in the 16α2β4QL chimera was insufficient to support efficient interaction with PLCβ or Fhit. Collectively, these results indicate that the substitution-induced conformational changes on the binding interface of $G\alpha_q$ are tolerated by PLCβ but not by Fhit.

PLCβ is a key molecule in transducing activated G_q protein signal to its downstream signal pathways, and similar to G_q protein, it also plays complicated roles in regulating cell growth. PLCβ2 expression level is positively correlated with breast cancer [32] and it promotes mitosis and migration of breast tumor cells [33]. On the other hand, in human erythroleukemia cells, PLCβ1 suppresses proliferation probably through regulating cyclin D3 [34]. PLCβ3-deficiency in mice leads to lymphoma and other tumors [35]. The interaction between PLCβs and Fhit as well as the complex formation among Fhit, $G\alpha_q$ and PLCβ reveals new pathway(s) of cell growth inhibition by $G\alpha_q$ and PLCβ.

Activated $G\alpha_q$ binds to Fhit through the α2-β4 region of $G\alpha_q$ (Fig. 6a and [15]), and it binds to PLCβ through multiple regions of $G\alpha_q$ including the α2 region (Fig. 6b and [25]). The overlapping PLCβ and Fhit binding domain on the activated $G\alpha_q$ is the α2 region (Fig. 6c). As substitution of α2-β4 region of $G\alpha_{16}$ with the corresponding region of $G\alpha_z$ did not affect the activated $G\alpha_{16}$-induced PLCβ activation (Fig. 5b), Fhit may bind to this overlapped interacting region on $G\alpha_q$ and change

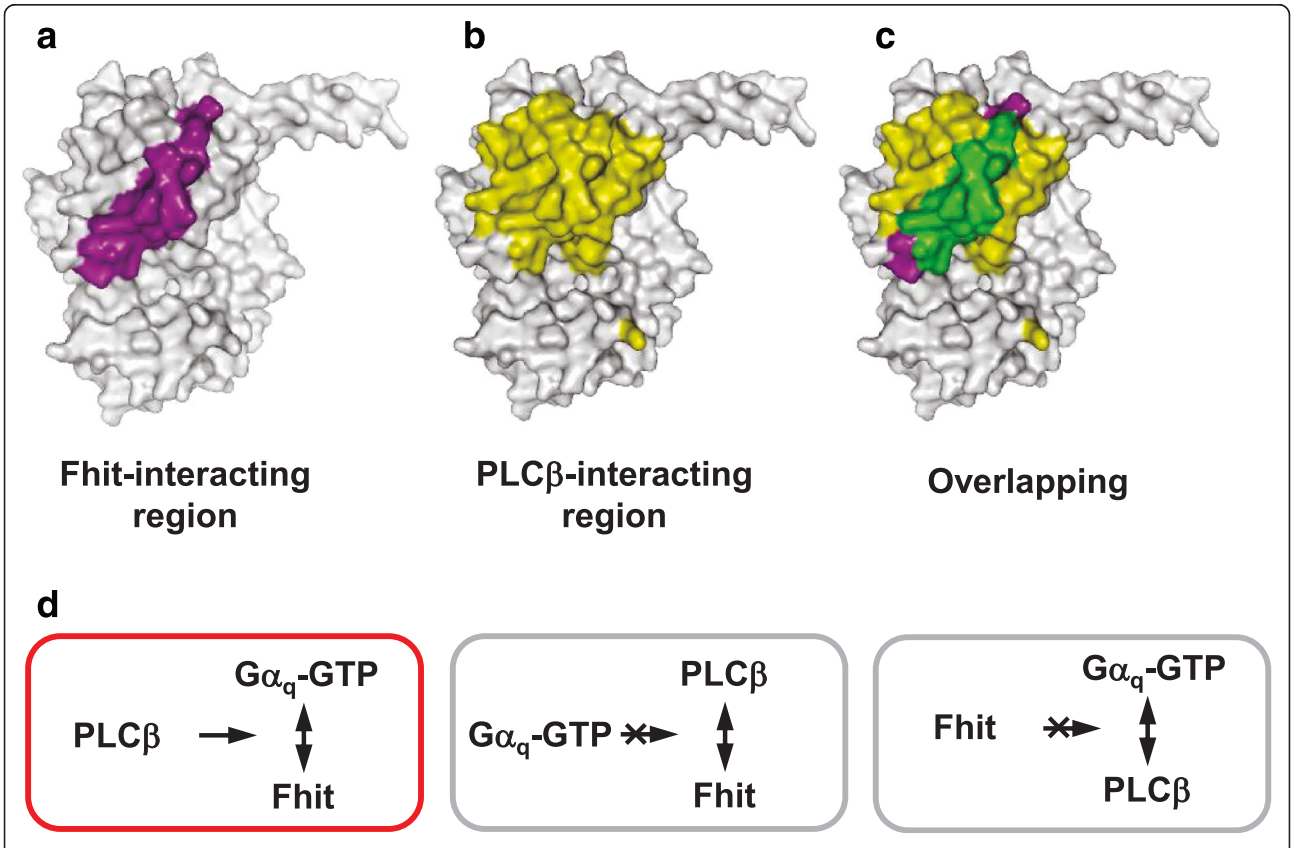

Fig. 6 The binding regions of Fhit and PLCβ on activated $G\alpha_q$ surface. Molecular surface of activated $G\alpha_q$ was modeled based on the crystal structures of activated $G\alpha_q$ and PLCβ3 (PDB: 4GNK). **a** The location of the Fhit-interacting α2-β4 regions (Gly208-Asp243, purple) relative to the other domains (white) on $G\alpha_q$ is illustrated. **b** The contact interface of activated $G\alpha_q$ with PLCβ3 is highlighted in yellow. **c** Overlapped binding regions of PLCβ and Fhit on activated $G\alpha_q$ is shown in green. **d** PLCβ increases the interaction between $G\alpha_q$ and Fhit. $G\alpha_q$ does not enhance the interaction between PLCβ and Fhit. And Fhit is unable to strengthen the association of $G\alpha_q$ and PLCβ

the binding interface between $G\alpha_q$ and PLCβ without affecting the PLCβ activity. The altered binding interfaces of activated $G\alpha_q$ and PLCβ may trigger $G\alpha_q$ and PLCβ to form a 'clamp' around Fhit. Beside protein interactions that were found between two protein pairs among $G\alpha_q$, PLCβ and Fhit, PLCβs increased the interaction between $G\alpha_q$ and Fhit. But Fhit or $G\alpha_q$ did not enhance the interaction between PLCβ and $G\alpha_q$ or Fhit, respectively (Fig. 6d). One possibility for increased affinities of Fhit to $G\alpha_q$ by PLCβ is that PLCβ may interact with and stabilize the complex of Fhit and $G\alpha_q$. Another possibility is that when binding to $G\alpha_q$, PLCβ may provide direct binding sites on itself for Fhit which also leads to the formation of a heterotrimeric protein complex. In both possibilities, the activated $G\alpha_q$ recruits PLCβ which acts as a positive regulator for the association of Fhit with activated $G\alpha_q$. In the future, the involvement of PLCβ in the regulation of Fhit by $G\alpha_q$ and their possible roles on cancer therapy should be demonstrated.

Conclusions

We showed that PLCβ could interact with Fhit, and the expression of PLCβ increased the interaction between $G\alpha_q$ and Fhit. This regulatory effect appears to be unique to PLCβ because other $G\alpha_q$ effectors such as RGS2 and p63RhoGEF could not interact with Fhit. Substitution of the α2-β4 region of $G\alpha_q$ with $G\alpha_i$ did not affect $G\alpha_q$-induced PLCβ activation but eliminated the interaction between $G\alpha_q$ with Fhit. This new $G\alpha_q$/PLCβ/Fhit signaling complex represents a novel pathway of $G\alpha_q$ regulation on tumor suppression.

Additional file

Additional file 1: Figure S1. Fhit preferentially associates with activated $G\alpha_{16}$ in HEK293 cells co-expressing PLCβ. HEK293 cells were co-transfected with different combinations of pFlag-CMV2 (V), Flag-Fhit (F), $G\alpha_{16}$, $G\alpha_{16}$QL, PLCβ1, PLCβ2 or PLCβ3. After co-immunoprecipitation assay with anti-Flag affinity gel, PLCβ1, 2, 3, $G\alpha_{16}$, Fhit and α-tubulin were determined by Western blotting. Flag-Fhit pulled down detectably more $G\alpha_{16}$QL than $G\alpha_{16}$ (*cf* lanes 1 and 3 of row two), but wild-type $G\alpha_{16}$ became able to interact with Fhit upon overexpression of PLCβs (*cf* lanes 1 and 2 of row two). All three forms of PLCβ were co-immunoprecipitated with Flag-Fhit but the co-expression of $G\alpha_{16}$QL did not enhance the interaction between PLCβs and Fhit, rather such interactions were attenuated ($G\alpha_{16}$ versus $G\alpha_{16}$QL lanes). (PDF 185 kb)

Abbreviations

PLCβ: Phospholipase Cβ; IP_3: Inositol 1,4,5-trisphosphate; Ap_3A: Diadenosine 5′,5′-P1,P3-triphosphate; RGS: Regulator of G protein signaling; IP: Immunoprecipitation; TCL: Total cell lysate.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HZ performed the experiments, participated in the design of the study and wrote the manuscript. YHW participated in the design of the study and the writing of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work was supported in part by grants from the National Key Basic Research Program of China (2013CB530900), the Research Grants Council of Hong Kong (HKUST 661808), and the Hong Kong Jockey Club.

Author details

[1]Division of Life Sciences, and the Biotechnology Research Institute, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China. [2]State Key Laboratory of Molecular Neuroscience, and the Molecular Neuroscience Center, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong. [3]Present address: Department of Pharmacology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390, USA.

Received: 28 July 2015 Accepted: 16 October 2015
Published online: 24 October 2015

References

1. Barnes LD, Garrison PN, Siprashvili Z, Guranowski A, Robinson AK, Ingram SW, et al. Fhit, a putative tumor suppressor in humans, is a dinucleoside 5′,5‴-P1, P3-triphosphate hydrolase. Biochemistry. 1996;35(36):11529–35.
2. Pekarsky Y, Zanesi N, Palamarchuk A, Huebner K, Croce CM. FHIT: from gene discovery to cancer treatment and prevention. Lancet Oncol. 2002;3(12):748–54.
3. Ji L, Fang B, Yen N, Fong K, Minna JD, Roth JA. Induction of apoptosis and inhibition of tumorigenicity and tumor growth by adenovirus vector-mediated fragile histidine triad (FHIT) gene overexpression. Cancer Res. 1999;59(14):3333–9.
4. Roz L, Gramegna M, Ishii H, Croce CM, Sozzi G. Restoration of fragile histidine triad (FHIT) expression induces apoptosis and suppresses tumorigenicity in lung and cervical cancer cell lines. Proc Natl Acad Sci U S A. 2002;99(6):3615–20.
5. Siprashvili Z, Sozzi G, Barnes LD, McCue P, Robinson AK, Eryomin V, et al. Replacement of Fhit in cancer cells suppresses tumorigenicity. Proc Natl Acad Sci U S A. 1997;94(25):13771–6.
6. Waters CE, Saldivar JC, Hosseini SA, Huebner K. The FHIT gene product: tumor suppressor and genome "caretaker". Cell Mol Life Sci. 2014;71(23):4577–87.
7. Karras JR, Paisie CA, Huebner K. Replicative stress and the FHIT gene: roles in tumor suppression, genome stability and prevention of carcinogenesis. Cancers (Basel). 2014;6(2):1208–19.
8. Saldivar JC, Miuma S, Bene J, Hosseini SA, Shibata H, Sun J, et al. Initiation of genome instability and preneoplastic processes through loss of Fhit expression. PLoS Genet. 2012;8(11):e1003077.
9. Joannes A, Bonnomet A, Bindels S, Polette M, Gilles C, Burlet H, et al. Fhit regulates invasion of lung tumor cells. Oncogene. 2010;29(8):1203–13.
10. Pace HC, Garrison PN, Robinson AK, Barnes LD, Draganescu A, Rosler A, et al. Genetic, biochemical, and crystallographic characterization of Fhit-substrate complexes as the active signaling form of Fhit. Proc Natl Acad Sci U S A. 1998;95(10):5484–9.
11. Weiske J, Albring KF, Huber O. The tumor suppressor Fhit acts as a repressor of β-catenin transcriptional activity. Proc Natl Acad Sci U S A. 2007;104(51):20344–9.
12. Trapasso F, Pichiorri F, Gaspari M, Palumbo T, Aqeilan RI, Gaudio E, et al. Fhit interaction with ferredoxin reductase triggers generation of reactive oxygen species and apoptosis of cancer cells. J Biol Chem. 2008;283(20):13736–44.
13. Pekarsky Y, Garrison PN, Palamarchuk A, Zanesi N, Aqeilan RI, Huebner K, et al. Fhit is a physiological target of the protein kinase Src. Proc Natl Acad Sci U S A. 2004;101(11):3775–9.
14. Shi Y, Zou M, Farid NR, Paterson MC. Association of FHIT (fragile histidine triad), a candidate tumour suppressor gene, with the ubiquitin-conjugating enzyme hUBC9. Biochem J. 2000;352(Pt 2):443–8.
15. Zuo H, Chan GP, Zhu J, Yeung WW, Chan AS, Ammer H, et al. Activation state-dependent interaction between $G\alpha_q$ subunits and the Fhit tumor suppressor. Cell Commun Signal. 2013;11:59.
16. Joannes A, Grelet S, Duca L, Gilles C, Kileztky C, Dalstein V, et al. Fhit regulates EMT targets through an EGFR/Src/ERK/Slug signaling axis in human bronchial cells. Mol Cancer Res. 2014;12(5):775–83.
17. Hubbard KB, Hepler JR. Cell signalling diversity of the $G_q\alpha$ family of heterotrimeric G proteins. Cell Signal. 2006;18(2):135–50.

18. Sanchez-Fernandez G, Cabezudo S, Garcia-Hoz C, Beninca C, Aragay AM, Mayor Jr F, et al. $G\alpha_q$ signalling: the new and the old. Cell Signal. 2014;26(5):833–48.
19. Nakagawa Y, Akao Y. Fhit protein inhibits cell growth by attenuating the signaling mediated by nuclear factor-kappaB in colon cancer cell lines. Exp Cell Res. 2006;312(13):2433–42.
20. Liu AM, Wong YH. G_{16}-mediated activation of nuclear factor κB by the adenosine A1 receptor involves c-Src, protein kinase C, and ERK signaling. J Biol Chem. 2004;279(51):53196–204.
21. Rozengurt E. Mitogenic signaling pathways induced by G protein-coupled receptors. J Cell Physiol. 2007;213(3):589–602.
22. New DC, Wong YH. Molecular mechanisms mediating the G protein-coupled receptor regulation of cell cycle progression. J Mol Signal. 2007;2:2.
23. Zuo H, Chan AS, Ammer H, Wong YH. Activation of $G\alpha_q$ subunits up-regulates the expression of the tumor suppressor Fhit. Cell Signal. 2013;25(12):2440–52.
24. Tsu RC, Chan JS, Wong YH. Regulation of multiple effectors by the cloned δ-opioid receptor: stimulation of phospholipase C and type II adenylyl cyclase. J Neurochem. 1995;64(6):2700–7.
25. Waldo GL, Ricks TK, Hicks SN, Cheever ML, Kawano T, Tsuboi K, et al. Kinetic scaffolding mediated by a phospholipase C-β and G_q signaling complex. Science. 2010;330(6006):974–80.
26. Heximer SP, Watson N, Linder ME, Blumer KJ, Hepler JR. RGS2/G0S8 is a selective inhibitor of $G\alpha_q$ function. Proc Natl Acad Sci U S A. 1997;94(26):14389–93.
27. Lutz S, Shankaranarayanan A, Coco C, Ridilla M, Nance MR, Vettel C, et al. Structure of $G\alpha_q$-p63RhoGEF-RhoA complex reveals a pathway for the activation of RhoA by GPCRs. Science. 2007;318(5858):1923–7.
28. Lyon AM, Taylor VG, Tesmer JJ. Strike a pose: $G\alpha_q$ complexes at the membrane. Trends Pharmacol Sci. 2014;35(1):23–30.
29. Tesmer VM, Kawano T, Shankaranarayanan A, Kozasa T, Tesmer JJ. Snapshot of activated G proteins at the membrane: the $G\alpha_q$-GRK2-Gβγ complex. Science. 2005;310(5754):1686–90.
30. Giguere P, Rochdi MD, Laroche G, Dupre E, Whorton MR, Sunahara RK, et al. ARF6 activation by $G\alpha_q$ signaling: $G\alpha_q$ forms molecular complexes with ARNO and ARF6. Cell Signal. 2006;18(11):1988–94.
31. Runnels LW, Scarlata SF. Determination of the affinities between heterotrimeric G protein subunits and their phospholipase C-β effectors. Biochemistry. 1999;38(5):1488–96.
32. Bertagnolo V, Benedusi M, Querzoli P, Pedriali M, Magri E, Brugnoli F, et al. PLC-β2 is highly expressed in breast cancer and is associated with a poor outcome: a study on tissue microarrays. Int J Oncol. 2006;28(4):863–72.
33. Bertagnolo V, Benedusi M, Brugnoli F, Lanuti P, Marchisio M, Querzoli P, et al. Phospholipase C-β2 promotes mitosis and migration of human breast cancer-derived cells. Carcinogenesis. 2007;28(8):1638–45.
34. Poli A, Faenza I, Chiarini F, Matteucci A, McCubrey JA, Cocco L. K562 cell proliferation is modulated by PLCβ1 through a PKCα-mediated pathway. Cell Cycle. 2013;12(11):1713–21.
35. Xiao W, Hong H, Kawakami Y, Kato Y, Wu D, Yasudo H, et al. Tumor suppression by phospholipase C-β3 via SHP-1-mediated dephosphorylation of Stat5. Cancer Cell. 2009;16(2):161–71.