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A genome-wide RNAi screen reveals a Trio-regulated Rho GTPase circuitry transducing GPCR-initiated mitogenic signals

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Abstract

Activating mutations in *GNAQ* and *GNA11*, encoding members of the Gαq family of G protein α subunits, are the driver uveal melanoma oncogenes, while mutations in Gq-linked G proteincoupled receptors (GPCRs) have been identified recently in numerous human malignancies. How Gαq and its coupled receptors transduce mitogenic signals is still unclear, due to the complexity of signaling events perturbed upon Gq activation. Using of a synthetic biology approach and a genome-wide RNAi screen, we found that a highly conserved guanine nucleotide exchange factor, Trio, is essential to activate Rho- and Rac-regulated signaling pathways acting on JNK and p38, thereby transducing proliferative signals from Gαq to the nucleus independently of PLC-β. Indeed, while many biological responses elicited by Gq depend on the transient activation of second messenger system, Gq utilizes a hardwired protein-protein interaction-based signaling circuitry to achieve the sustained stimulation of proliferative pathways, thereby controlling normal and aberrant cell growth.

Keywords

MAPK; JNK; p38; Rho GTPases; Signal Transduction; G Proteins; Cancer

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Supplemental Information

Supplemental information includes five figures, two movies and three tables, Supplemental Experimental Procedures and Supplemental References.

Introduction

With more than 800 members, G protein coupled receptors (GPCRs) represent the largest family of cell-surface molecules involved in signal transmission (Pierce et al., 2002). These receptors control a large variety of key biological functions ranging from development, neurotransmission, secretion from endocrine and exocrine glands, inflammatory responses, blood pressure control, hemostasis, and cardiac function to name but a few (Dorsam and Gutkind, 2007; Pierce et al., 2002). GPCRs can also control fate decisions of stem cell progenitors during development (Knox et al., 2010; Kobayashi et al., 2010), and normal and tumor cell growth (Dorsam and Gutkind, 2007; Pierce et al., 2002; Rozengurt, 2007). Indeed, many potent mitogens including polypeptides (i.e., gastrin releasing peptide (GRP), endothelin, bradykinin, and angiotensin II), proteolytic enzymes (i.e., thrombin), chemokines (i.e., SDF-1), lipid mediators (i.e., prostaglandins, lysophospatidic acid (LPA) and sphingosine 1-phosphate (S1P)), and neurotransmitters (i.e., acetylcholine and noradrenaline), can promote cell proliferation by activating their cognate G protein-linked receptors in a variety of cells types (reviewed in (Dorsam and Gutkind, 2007; Rozengurt, 2007). In addition, two of the six known human cancerassociated viruses, Epstein-Barr virus and Kaposi's sarcoma-associated human Herpesvirus express constitutively active mutant GPCRs from their viral genome (Slinger et al., 2011), while recent cancer sequencing initiatives have revealed a surprisingly high incidence of GPCR mutations (5–30%), in some of the most prevalent human malignancies (Kan et al., 2010).

Most mitogens acting on GPCRs stimulate the Gq family of heterotrimeric G proteins (Rozengurt, 2007). In line with this coupling selectivity, activated Gαq mutants harbor transforming potential (Kalinec et al., 1992), and Gq-linked receptors can act as potent oncogenes in experimental model systems (Gutkind et al., 1991; Young et al., 1986). Mutations in Gq-coupled receptors (Gq-GPCRs) have been recently demonstrated in multiple human cancers (Kan et al., 2010), and activating mutations in the genes for Gαq family members, Gαq (*GNAQ*) and Gα11 (*GNA11*), have been identified in approximately 80% of uveal melanomas, where they are now considered to represent the driver uveal melanoma oncogene (Van Raamsdonk et al., 2009; Van Raamsdonk et al., 2010). While the growth promoting activity of Gαq and Gq-GPCRs is already well established, the precise signaling events by which these G proteins and their linked receptors transduce sustained proliferative signals is not yet well defined, primarily due to the large number of second messenger generating systems and signaling events that can be perturbed upon Gq activation (Hubbard and Hepler, 2006).

In this report, we took advantage of receptors coupled to Gq that are exclusively activated by synthetic ligands (Armbruster et al., 2007; Conklin et al., 2008), referred herein as Synthetic Gq-coupled Receptors (Sy-Rq), to study mitogenic signaling by Gq-GPCRs in a variety of cell types. We combined this synthetic biology approach with an unbiased genome-wide RNAi screen in Drosophila cells for molecules linking GPCRs to the activation of growth-promoting transcription factors. We found that whereas most transient responses elicited by Gq-GPCRs involve the rapid generation of diffusible second messenger systems, Gq and the *GNAQ*/*GNA11* oncogenes utilize a novel protein-protein interaction-based signaling axis to transduce sustained growth inducing signals from the membrane to the nucleus.

Experimental Procedures

For expanded experimental details and information, please see Supplemental Experimental Procedures.

Cell lines, culture procedures, and tumor xenografts

NIH3T3 cells stably expressing Sy-Rq were obtained by transfection with pCEFL-HA-Sy-Rq. Other normal and cancer cell lines used and the establishment of shRNA-control and shRNA-Trio knock down cells are described in Supplemental Experimental Procedures. Results of animal experiments were expressed as mean ± SEM of a total of 10 tumors analyzed.

siRNA, DNA constructs, and RNAi library screen

Human and mouse siRNA sequences are described in supplemental information. Screening was performed at the Drosophila RNAi Screening Center at Harvard Medical School (Boston, MA) (http://www.flyrnai.org), using a library of ~18,000 dsRNAs (Mohr et al., 2010). Data analysis was performed as reported (Mohr et al., 2010) (http://www.flyrnai.org). Each well was expressed as the number of standard deviations from the plate average (z-score). Samples that scored at least 2 standard deviations above or below the plate average were identified as hits.

DNA-synthesis, focus formation, PI breakdown, and binding assays

Thymidine incorporation, focus formation, PI breakdown and binding assays were performed essentially as previously described (Gutkind et al., 1991). EdU incorporation was measured using the Click–It kit (Invitrogen) according to manufacturer's instructions.

Calcium analysis, Trio recruitment to the plasma membrane, and immunofluorescence

For calcium analysis, cells were incubated with Rhod-3AM according to manufacturer's instructions (Invitrogen). For Trio translocation assays, cells were incubate with CellMask™ Orange plasma membrane stain (Invitrogen). For immunofluorescence, cells were stimulated with the appropriate mitogen prior to processing as described in in the Supplemental Experimental Procedures.

Small GTPase activation assays, co-immunoprecipitation, immunoblot, and luciferase assays in mammalian cells: assays

NIH3T3-Sy-Gq were co-transfected with AP-1 luc and pRLNull and appropriate siRNAs and stimulated for 4h prior to the detection of the luciferase activity. Detailed information on the procedures and antibodies used for *in vivo* RhoA, Rac1, Cdc42 and Ras activity co-immunoprecipitation of G protein associated proteins, and Western blotting techniques are described in Supplemental Experimental Procedures.

Statistical analysis

All data analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).

Results

A synthetic biology approach to study mitogenic signaling through Gq-GPCRs in mammalian cells

We used Sy-Receptors to engineer GPCR-regulated signaling networks in a variety of cellular systems, thereby circumventing the known variability of GPCR expression levels and the presence of potential autocrine loops resulting in distinct basal GPCR activity (Rozengurt, 2007). We used Sy-Rq, a mutant M3 muscarinic GPCR that cannot be activated by its natural ligand (acetylcholine) but gained the ability to be activated by clozapine-N-oxide (CNO), a pharmacologically inert small molecule (Armbruster et al., 2007; Conklin et

al., 2008) (Fig. 1A). HA-Sy-Rq was stably expressed in NIH3T3 cells, as judged by immunofluorescence and [^{3}H]-N-methyl scopolamine (^{3}H-NMS) binding (Fig. 1B, 1C, S1A and S1C). Detailed binding analysis revealed that Sy-Rq cells express ~100,000 receptors per cell, within the physiological level of endogenous GPCRs for polypeptide mitogens (Fig. 1C). Furthermore, CNO stimulated phospholipase C (PLC) activation and a rapid raise in cytoplasmatic [Ca^{2+}] only in cells expressing Sy-Rq (Fig. 1D), confirming the activation of classical Gq downstream effectors. Prolonged activation of Sy-Rq led to oncogenic transformation of NIH3T3 cells, with foci of transformed cells expressing Sy-Rq (Fig. 1E and Fig. S1A and S1B). Sy-Rq activation elicited a potent proliferative response, comparable to that induced by PDGF when used as control (Fig. 1F), which was visualized at the individual cell level (Fig. 1F). We also used mass cultures of NIH3T3 cells expressing Sy-Rq in a tetracycline-inducible fashion to avoid clonal selection. These experiments supported that Sy-Rq transduces growth promoting signals efficiently, as expression of ~20,000 Sy-Rq per cell is sufficient to promote cell proliferation in response to CNO rather than requiring gross overexpression (Fig S1A-C). Gq-GPCRs are best known for their ability to stimulate second messenger generating systems through PLC. Thus, we first tested the contribution of PLC to mitogenic signaling by Sy-Rq by the use of a small molecule PLC-inhibitor (U73122) (Bleasdale et al., 1989) (PLCi). PLC inhibition reduced the stimulation of the ERK MAPK by Sy-Rq, albeit partially (Fig. 1G). Surprisingly, inhibition of PLC had only a limited impact on DNA-synthesis in response to CNO, despite completely abolishing its ability to increase intracellular Ca^{2+} levels, a direct downstream consequence of PLC activation (Fig. 1G). Taken together, we can conclude that an engineered Gq-coupled receptor can activate cells to undergo normal and malignant cell growth. However, mitogenic signaling downstream from Gq may involve multiple mechanisms, some of which may bypass the strict requirement of PLC activation for cell growth promotion.

AP-1 is a key downstream target in cell growth promotion by Gq-GPCRs

In addition to PLC, Gq-linked GPCRs can regulate a complex network of signaling events (reviewed in (Dorsam and Gutkind, 2007; Pierce et al., 2002; Rozengurt, 2007). Indeed, exposure of Sy-Rq cells to CNO led to the stimulation of Ras, RhoA, Rac1 and Cdc42, and promoted a prolonged activation of endogenous MAPKs, including ERK2, JNK1/2, p38 and ERK5 (Fig. 2A). Activation of the AP-1 transcription factor, which is composed by homodimers of c-Jun family members or heterodimers between c-Jun and c-Fos family members (Karin et al., 1997), is an integral component of mitogenic signaling by Gq-GPCRs (Dorsam and Gutkind, 2007). As such, CNO stimulated AP-1 potently in a concentration dependent manner in Sy-Rq cells, which correlated with the increased transcription of both *c-jun* and *c-fos* mRNAs and the expression and nuclear accumulation of their protein products, c-Jun and c-Fos (Fig. 2B-C). Knock down of these AP-1 family members revealed a key role for c-Jun in both AP-1 reporter activation and DNA-synthesis, with a significant but slightly less dramatic impact for c-Fos (Fig. 2D). Together, these results support that Gq-GPCRs are endowed with the ability to stimulate second messenger generating systems, small GTPases of the Ras and Rho family, and multiple MAPKs.

A genome-wide Drosophila RNAi screen identifies novel regulators of AP-1 activation downstream of Gq

How the multiple signals initiated upon GPCR activation are integrated to control nuclear events is still poorly defined. As this process is likely highly conserved across species, we took advantage of the ease of performing knock down strategies in Drosophila cells (Mohr et al., 2010) to explore the molecular events involved in signaling from Gq-GPCRs at the plasma membrane to AP-1-dependent nuclear gene transcription. Drosophila S2 cells were transfected with plasmids expressing M1 muscarinic receptor, a prototypical Gq-linked

GPCR similar to M3 that was used for the generation of Sy-Rq. Receptor expression was confirmed (Fig. S2A) and its functionality examined by the ability to stimulate PLC activity, AP-1, and JNK with a synthetic muscarinic agonist, carbachol (Fig. 3A, B). dsRNAs designed to inhibit the expression of each Gα protein confirmed that Gαq is specifically required to activate JNK in this cellular system (Fig. 3C). As expected, knock down of Gαq and NorpA, a PLC isoform acting downstream of Gαq (Lee et al., 1994), but not dJun, impaired the activation of PI breakdown by M1 Gq- GPCR (Fig. 3D). Surprisingly, however, AP-1 activation was dependent on Gαq and dJun, but not on NorpA or another Drosophila PLC isoform under multiple experimental conditions (Fig. 3.D). Aligned with this observation, PLCi had a limited impact on AP-1 activation in Sy-Rq expressing mammalian cells (Fig. S2B).

These findings prompted us to investigate the signaling networks linking Gq-GPCRs to AP-1 by the use of an unbiased genome-wide dsRNA screen. Data were analyzed using a "z-score" method, establishing as a "hit" a dsRNA whose value is at least double (above or below) the average standard deviation of each plate value (Fig. 3E) (Mohr et al., 2010). Hits from the primary screen spanned molecules involved in cytoskeletal organization and metabolism, proteins with kinase or phosphatase activity, and those involved in the ubiquitin cycle (Fig. 3F). A list of representative negative and positive hits identified is provided in Table S1, and a complete list in Table S2. This screen revealed multiple predicted and unexpected molecules increasing or decreasing AP-1 activity upon Gq stimulation.

Key components of the AP-1 complex and molecules known to regulate its expression and activity were positive hits, as expected. These molecules included the Drosophila orthologs of JNK kinase (MAP2K7), SRF, c-Fos, c-Jun, and p300 (Table S1A). We also found that dsRNAs targeting the Drosophila REL-A NFκB subunit (dl) decreased AP-1 activation, which was aligned with the positive impact of reducing IκB (cact) expression in our screen (Table S1B), thus suggesting a cooperating effect of NFκB on AP-1. Unexpected molecules whose targeting dsRNAs increased AP-1 activation included the orthologs of Twist (twi), an oncogenic transcriptional repressor (Yang et al., 2004), and two members of the hippo pathway, NF2 and LATS1 (mer and wts, respectively) (Zhao et al., 2008). The latter were also identified in a Drosophila screen for molecules controlling JNK (Bakal et al., 2008), together suggesting an interplay between the hippo and JNK/AP-1 regulated networks.

The most remarkable finding from our screen was the identification of multiple Rho GTPases (Rho1, RhoL, Rac1, and Rac2, which are Drosophila orthologs of mammalian RhoA, RhoG, Rac3 and Rac1, respectively) and their direct downstream targets, mbt, pkn, and slpr, which are the Drosophila orthologs of PAK, PKN, and MLK (Davis, 2000), as some of the most significant hits (Table S1A). These kinases can act as part of a repertoire of JNKKKs activating MAP2K7, a JNKK, in mammalian cells (Davis, 2000), the ortholog of hep in Drosophila, which was also a strong hit in our screen (Table S1A). Overall, this RNAi screen revealed a central role of Rho GTPase and their regulated biochemical networks in signaling from Gq-GPCRs to AP-1. Secondary screening with alternate amplicons targeting a subset of genes of interest confirmed the importance of Rho GTPases, hep (JNKK), Jun, and Fos, among others (Table S1).

These observations prompted us to focus on candidate molecules linking Gq to Rho GTPases. Only one Rho-specific guanine nucleotide exchange factor (GEF), Drosophila Trio, depicted as dTrio herein, was identified as a positive hit in our RNAi screen (Table S1A and Table S2A). Trio is a highly conserved GEF containing a series of N-terminal spectrin domains, an SH3 domain and two tandem Dbl homology-Plekstrin homology (DH-PH) domains that act as GEFs for Rho GTPases (Debant et al., 1996). Effective knock down of dTrio (Fig. 3G) did not affect M1-receptor expression (Fig. S2C), but reduced the

activation of Jnk as judged by the reduced phosphorylation of dJnk (Fig. 3H) and Jra (dJun) (Fig. S2D). Furthermore, dTrio knockdown inhibited AP-1 activation potently (Fig. 3I) without affecting GPCR-stimulated PLC activity (Fig. 3J), whereas knockdown of dGq inhibited both. Defective AP-1 activation due to dTrio knockdown was rescued by re-expressing a truncated form of dTrio comprising the Cterminal region including the two DH-PH GEF domains and a C-terminal area of predicted interaction with Gαq (see below), but not by its N-terminal spectrin region (Fig. 3K). While the Trio N-terminal DH-PH (GEF1) domain activates Rac proteins, the C-terminal DH-PH (GEF2) domain stimulates RhoA *in vivo* (Chhatriwala et al., 2007; Debant et al., 1996). AP-1 activation required both of these GEFs domains, as activation of AP-1 was rescued only partially when expressing mutant forms of dTrio in which either of the two GEF domains were inactivated (Fig. 3K). Similar results were obtained in mammalian cells rescued by the expression of human Trio and its GEF mutants (see below). We can conclude that while dTrio does not contribute to PLC activation, this GEF provides a link between Gq-GPCRs and AP-1-dependent gene transcription through its GEF1 and GEF2 domains, thereby supporting the central role of Rho GTPases in AP- 1 activation by Gq-GPCRs in Drosophila cells.

Trio-dependent signaling circuitries elicited by mitogenic Gq activation

Murine fibroblasts express Trio, and knock down of Trio (Fig. 4A, upper panel) impaired the ability to stimulate AP-1 by Sy-Rq, which was rescued by the co-transfection of expression vectors for the dTrio Cterminal region that includes its two GEF domains (Fig. 4B). Cells in which Trio was knocked down retained their ability to activate PLC (Fig. 4C and 4D). We then transfected Sy-Rq cells with control shRNA or Trio shRNAs. The latter cells exhibited reduced Trio levels (Fig. 4A) without affecting Sy-Rq expression (Fig. S3A), and had impaired ability to activate DNA-synthesis in response to CNO but not in response to serum as a specificity control (Fig. 4E). Reduced Trio expression did not affect the activation of Rac1 and RhoA in response to serum (Figs. 4F-4H), but nearly abolished the activation of these GTPases downstream of Gq-GPCRs (Fig. 4I-K). In turn, reduced expression of Rac1 and RhoA, but not of Cdc42, diminished the ability of cells to undergo DNA-synthesis upon CNO stimulation (Fig. 4L and 4M, S3B and S3C). Furthermore, both Rac1 and RhoA knockdown impaired the ability to activate AP-1 in response to CNO, without affecting PLC activation (Fig. 4N and 4O). Taking together, we can conclude that Trio specifically links Gq-GPCRs to the activation of Rac1 and RhoA GTPase in mammalian cells, thereby triggering AP-1 activity and DNA-synthesis.

Trio activation is required for mitogenic signaling elicited through Gq

Trio knock down did not affect the activation of JNK, p38, and ERK in response to serum (Fig. 5A), and had only a slight effect on the activation of ERK by CNO (Fig 5B). However, JNK activation in response to CNO was nearly abolished in cells in which Trio expression was reduced, while p38 activation was also inhibited, albeit to a lesser extent (Fig. 5B). This reduced JNK and p38 activation by Sy-Rq was reflected at the level of c-*jun* and c-*fos* mRNA expression (Fig. S4A). Furthermore, cells with reduced Trio were highly resistant to the transforming ability of Gq-GPCRs (Fig. S4B). These observations support that Trio acts as a key molecular intermediate linking Gq to the stimulation of the JNK and p38 MAPK cascades, which ultimately regulate the expression and activity of c-Jun and c-Fos AP-1 family of transcription factors, thereby promoting normal and aberrant cell growth.

How Gq stimulates Trio *in vivo* and whether this process requires an interplay with Gqmediated activation of PLCβ is at the present unknown. In this regard, we observed that active mutants of Gαq can form stable molecular complexes with Trio (Fig S4C), and that the activation of Sy-Rq leads to a progressive accumulation of a GFP-tagged form of mammalian Trio at the level of the plasma membrane (Fig. 5C, and Movie S1). Of interest,

Trio translocation was delayed and sustained when compared to the rapid and transient Gq-induced [Ca^{2+}] elevation (Fig. 5D). Furthermore, the accumulation of Trio at the plasma membrane was insensitive to PLC inhibition (Fig. 5E, S4D and Movie S2). Together, these findings suggest that Gq can associate with Trio directly, thereby causing its translocation to the membrane and activation independently of Gq-regulated second messenger systems.

Trio mediates normal and aberrant cell growth elicited by the activation of endogenous Gq-GPCRs and the *GNAQ* oncogene

We next tested the biological relevance of Trio in Swiss 3T3 cells, a well-defined cellular system in which activation of endogenous Gq-GPCRs, such as the GRP receptor (GRPR), can induce potent proliferative responses (Rozengurt, 2007). Treatment of Swiss 3T3 cells with GRP stimulated DNA-synthesis (Fig. S5A), and Trio knockdown diminished the ability of the cells to proliferate in response to GRP without affecting PLC (Fig. S5B-D), thus confirming our previous results using Sy-Rq. This effect was specific, as Trio knockdown did not affect the mitogenic response to PDGF or serum (Fig. S5E), supporting that Trio transduces proliferative signaling when initiated by endogenous Gq-GPCRs.

These results prompted us to explore the possibility that Trio activity may also participate in aberrant proliferation in human malignancies. The *Trio* gene locus, 5p15.2 is frequently amplified in cervical (Kloth et al., 2007) and head and neck cancer (HNSCC) (Baldwin et al., 2005), and meta-analysis of deregulated Trio expression showed that Trio mRNA is deregulated in a variety of human malignancies (Table S3; Figure 6A and Figure S5F). We established Trio knock down and shRNA control cells using two different representative cervical cancer and HNSCC cell lines, HeLa and HN12 (Fig. 6B and S5G), respectively, both of which are able to generate tumors in animal models. As a control, we also expressed Sy-Rq in HeLa cells, and confirmed that CNO increased AP-1 activity and DNA-synthesis in a Trio-dependent manner, and increased cytoplasmatic [Ca^{2+}] levels independently of Trio (Fig. 6C-E). Both Trio Rho- and Rac-GEF regions are required for nuclear signaling through Gq, as a wild type human Trio but not Trio mutants harboring mutations in their individual GEF1 and GEF2 domains were able to rescue AP-1 activation after Trio knock down in HeLa-Sy-Rq cells (Fig. 6C), paralleling our observations in drosophila cells. Endogenous Gq-GPCRs, such as those activated by GRP and endothelin, promoted DNA synthesis in cervical cancer- and HNSCC-derived cells, which was strikingly dependent on Trio expression (Fig. 6F and S5H). Furthermore, Trio deficiency resulted in the dramatic reduced growth of HeLa (Fig. 6G) and HN12 (Fig. S5I) tumors *in-vivo*. We can conclude that Trio is required to promote cell growth by engineered and endogenously expressed Gq-GPCRs, and that this GEF and its upstream activators, including Gαq, and its downstream signaling targets can play an unexplored role in multiple human malignancies.

More than 80% of human uveal melanoma cases harbor activating mutations in the gene encoding $G\alpha_q$ or its related $G\alpha_{11}$, *GNAQ* and *GNA11*, respectively (Van Raamsdonk et al., 2009; Van Raamsdonk et al., 2010). These mutated forms, collectively known as the *GNAQ* oncogene, result in the activation of ERK in the absence of mutations in the *B-RAF* or *N-RAS* oncogenes, which are frequently mutated in cutaneous melanomas (Davies et al., 2002). Indeed, knock down of $G\alpha_q$ in cell lines derived from primary or metastatic uveal melanomas resulted in decreased ERK activation and reduced DNA-synthesis (Fig. 6H and S5J), as previously reported (Van Raamsdonk et al., 2009). Of interest, $G\alpha_q$ levels reduced the activity of ERK in primary uveal melanoma cells efficiently, while this was less obvious in cells derived from a metastatic uveal melanoma lesion (Fig. 6H and S5J) both of which harbor *GNAQ* mutations (Van Raamsdonk et al., 2009). In both cases, however, knock down of active $G\alpha_q$ decreased JNK and p38 activity, and these effects were phenocopied by Trio knock down (Fig. 6H and S5J). Furthermore, Trio knock down diminished AP-1 activation and the aberrant proliferation and tumorigenicity of uveal melanoma cells,

without affecting PLC activation or p-ERK levels (Fig. 6H-J and S5K). Overall, as depicted in Figure 6K, we can conclude that whereas many of the biological responses elicited by Gq-GPCRs are mediated by the rapid and transient generation of second messengers downstream from PLC activation, Trio may provide a direct biochemical link between Gq and the sustained activation of signaling routes controlling cell proliferation by GPCRs and *GNAQ* oncogene.

Discussion

Activating mutations in *GNAQ* and *GNA11* represent the driver oncogenic event in human uveal melanoma (Van Raamsdonk et al., 2010), while mutations in a large number of GPCRs, many of which are predicted to signal through Gq, have been recently identified in some of the most prevalent human malignancies (Kan et al., 2010). However, how Gq and its linked receptors transduce growth promoting signals is still far from being understood due to the complexity of downstream targets and second messenger systems regulated by this GPCR family. Using a synthetic biology approach, we now show that the activation of PLC and the consequent generation of diffusible second messengers can explain only partially the mitogenic activity of Gq-GPCRs. Instead, by focusing on how this receptor family controls the nuclear expression of growth promoting genes, we found that a highly conserved GEF, Trio, is essential to promote the activation of Rho GTPase and their signaling circuitries transducing proliferative signals from Gq to the nucleus. Overall, while many of the rapid biological effects elicited upon Gq activation can be explained by the transient stimulation of conventional second messenger systems, Trio may provide a direct link between Gq and the sustained stimulation of growth promoting pathways controlling normal and aberrant cell proliferation.

GPCRs coupled to Gq stimulate the PLC-β family, which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce two second messengers: inositol 1,4,5- trisphosphate (IP_3) and diacylglycerol (DAG) (Hubbard and Hepler, 2006). IP_3 raises cytoplasmic Ca^{2+} levels, which stimulates multiple calcium-regulated pathways and together with DAG, activates classic PKC isoforms (Griner and Kazanietz, 2007). Hence, the stimulation of PLCs and PKCs has been traditionally associated with the stimulation of cell growth by Gq-GPCRs (Dorsam and Gutkind, 2007; Rozengurt, 2007). However, individual PKCs can have both positive and negative effects on cell proliferation and cancer in distinct cells and tissues, leading to the concept that the effects of PKCs are isoform-specific and dependent on cell context (Griner and Kazanietz, 2007). Gq-GPCRs can stimulate ERK and cell proliferation by both PKC-dependent and PKC-independent mechanisms (reviewed in (Dorsam and Gutkind, 2007; Miller and Lefkowitz, 2001; Rozengurt, 2007). Indeed, a small molecule inhibitor that abolished the activation of PLC exerted only a limited impact on the mitogenic responses elicited by the Sy-Rq, causing only a partial decrease in the activation of ERK and AP-1. Activation of PLC by tyrosine kinase receptors that stimulate PLC-γ potently (Meisenhelder et al., 1989), plays similarly only a partial role in cell growth promotion, while mutant tryrosine kinase receptors that specifically stimulate PLC but no other downstream targets are not effective in promoting cell growth (Coughlin et al., 1989). Thus, activation of PLC by both tyrosine kinases and G protein-linked receptors may not alone be sufficient or strictly necessary to elicit a mitogenic response.

The activation of PLC-β by Gαq is often rapid and yet transient, as multiple structural features in PLC-β enable its fast binding to activated Gq while acting as a potent guanosine triphosphatase (GTPase)–activating protein (GAP) for Gαq, thereby promoting its rapid deactivation (Ross, 2011). While the interaction between Gq and PLC-β is the most thoroughly investigated, Gαq can interact directly with a large number of downstream molecules, including serine-threonine and tyrosine kinases, Rho GEFs, scaffolding

molecules, and tetratricopeptide repeat containing proteins (Hubbard and Hepler, 2006). Many of these Gq-targets have well known functions in cell signaling independently of PLC, and hence could play a role in AP-1 activation. Indeed, the stimulation of nuclear events independently of PLC activation represents a highly conserved mechanism, as judged by the stimulation of AP-1 by Gq-GPCRs in Drosophila cells after knock-down of NorpA, the Drosophila Gq-linked PLC-β ortholog, which hence prevented Gq-induced PI-turnover. This observation provided an opportunity to identify the critical components linking Gq to AP-1 by the use of a genome-wide RNAi approach in Drosophila cells.

This siRNA screen revealed an unexpected large number of molecules regulating the activation of AP-1 upon stimulation of Gq-GPCRs. While their contribution to signaling from Gq to the nuclear activity of AP-1 warrants further investigation, we focused our attention on dTrio, a large protein exhibiting multiple structural features, which include two DH-PH domains, named GEF1 and GEF2, which activate Rac and Rho GTPases, respectively (Bellanger et al., 2003). dTrio knockdown did not affect PLC activation, but it abolished the stimulation of AP-1 by Gq-GPCRs, which was fully rescued when re-expressing the C-terminal region of dTrio as long as both GEF1 and GEF2 domains of dTrio remained intact, each acting in an additive manner. Knockdown of C. elegans Trio ortholog, UNC-73, suppresses the egg-laying phenotypes caused by an active Gαq (Williams et al., 2007). In mammals, Trio is essential for embryo development, receiving inputs from numerous signaling systems (O'Brien et al., 2000). Whether Gq activates Rho in mammalian cells was initially debated (reviewed in (Sah et al., 2000), but it is now known that Gq-linked GPCRs stimulate RhoA potently (Chikumi et al., 2002). Gαq can interact with multiple GEFs, including LARG, Lbc, p63-RhoGEF, and Trio in overexpression systems, resulting in specific RhoA activation (Booden et al., 2002; Lutz et al., 2005; Rojas et al., 2007; Sagi et al., 2001). However, Lbc and p63-RhoGEF exhibit restricted tissue distribution (Souchet et al., 2002; Wuertz et al., 2010), and cells derived from LARG knockout mice exhibit limited defective RhoA activation upon Gq stimulation (Wirth et al., 2008) and unpublished observations). In this regard, Trio is expressed in most cells and normal and cancer human tissues (O'Brien et al., 2000), and see Table S3). Indeed, knock down of Trio abolished the ability of GPCRs acting on Gαq to stimulate RhoA in fibroblasts and multiple cancer cells. We now show that Gq-coupled GPCRs can also stimulate Rac1 potently, which was prevented by reducing Trio. Overall, while multiple RhoGEF may contribute to Rho activation in specialized cell types and tissues, Trio is a widely expressed GEF mediating the activation of Rho and Rac GTPases by Gq and its coupled receptors as part of a signaling pathway conserved through evolution, ranging from nematode, to fruit flies and humans. Furthermore, AP-1 activation by GPCRs linked to Gq may require the activation of both RhoA and Rac1, which likely act in nuclear signaling by GPCRs in a cooperating fashion. Thus, Trio, which is unique as it exhibits two distinct GEF regions, may endow Gq-linked GPCRs with the ability to stimulate Rho and Rac GTPases concomitantly, thereby initiating the activation of a MAPK signaling network, including JNK and p38, which controls nuclear AP-1 activity and ultimately cell growth promotion.

Upregulation of *Trio* gene expression was observed in multiple human cancer types, including small cell lung carcinoma (Coe et al., 2005), which has been traditionally associated with the concomitant secretion of neuropeptides and the overexpression of their cognate Gq- GPCR, hence persistently stimulating Gq in an autocrine fashion (Rozengurt, 2007). Using cervical and oral squamous carcinoma cells as an example of Trio overexpressing cancers, we observed that Trio knock down prevents the proliferative response to mitogens acting on endogenous GPCRs. In fact, Trio knock down converted the pro-proliferative effect of GRP and endothelin, both stimulating Gq-GPCRs, into growth inhibitory. Thus, de-regulated Trio may mediate the growth promoting function of GPCRs acting on Gq in cancer cells. This requirement was even more evident in uveal melanoma, in

which activating mutations in *GNAQ* and *GNA11* drive the growth of this aggressive human malignancy (Van Raamsdonk et al., 2009; Van Raamsdonk et al.). Reducing Gαq expression in primary and metastatic uveal melanoma cells was sufficient to diminish the activation of PLC, JNK, p38, AP-1, and cell proliferation, which was phenocopied by Trio knock down, albeit the latter did not reduce PLC activation. Furthermore, decreased Trio expression did not affect the activation of ERK in uveal melanoma and in any other cell tested, although it had a dramatic impact diminishing the tumorigenic potential of uveal melanoma cells *in vivo*. Thus, as depicted in Figure 6K, we can conclude that stimulation of PLC and the subsequent increased in ERK activity may represent a general event caused by Gq activation. However, this effect is likely fast and transient due to structural features in PLC-β limiting the duration of signal transmission through this PLC family. Instead, GPCRs transducing signals through Gq control nuclear events resulting in cell proliferation by activating a network of Rho GTPases and MAPK cascades that are dependent on highly specific protein-protein interactions, rather than on diffusible second messenger systems.

Overall, our present findings suggest that Gαq can initiate the activation of a hardwired mechanism resulting in the sustained activation of RhoA and Rac1. This process involves the direct interaction of Gαq with Trio, which lacks GAP activity and hence does not act as part of a negative feedback loop inactivating GTP-bound Gαq, resulting in the prolonged recruitment of Trio to the membrane, where it stimulates Rho GTPases independently on PLC-β activation. Thus, Trio may represent a central node in the transmission of proliferative signals from the membrane to the nucleus upon activation of Gq-linked GPCRs. These observations also raise the possibility that targeting the Gαq-Trio interaction or the GEF activities of Trio may represent a suitable therapeutic strategy to halt cancer growth in patients harboring activating mutations in Gq-GPCRs, *GNAQ* or *GNA11*, or Trio gene amplification, and in human malignancies involving the persistent activation of Gq-GPCRs in an autocrine and paracrine fashion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Human cancers harbor frequent mutations in Gq-linked GPCRs and Gαq subunits
- A genome-wide RNAi screen revealed that Trio is essential to activate AP-1 by Gαq
- A network of Trio-regulated Rho GTPases and MAPKs links Gq to the nucleus
- A hardwired Gq-Trio signaling axis promotes the growth of many human malignancies

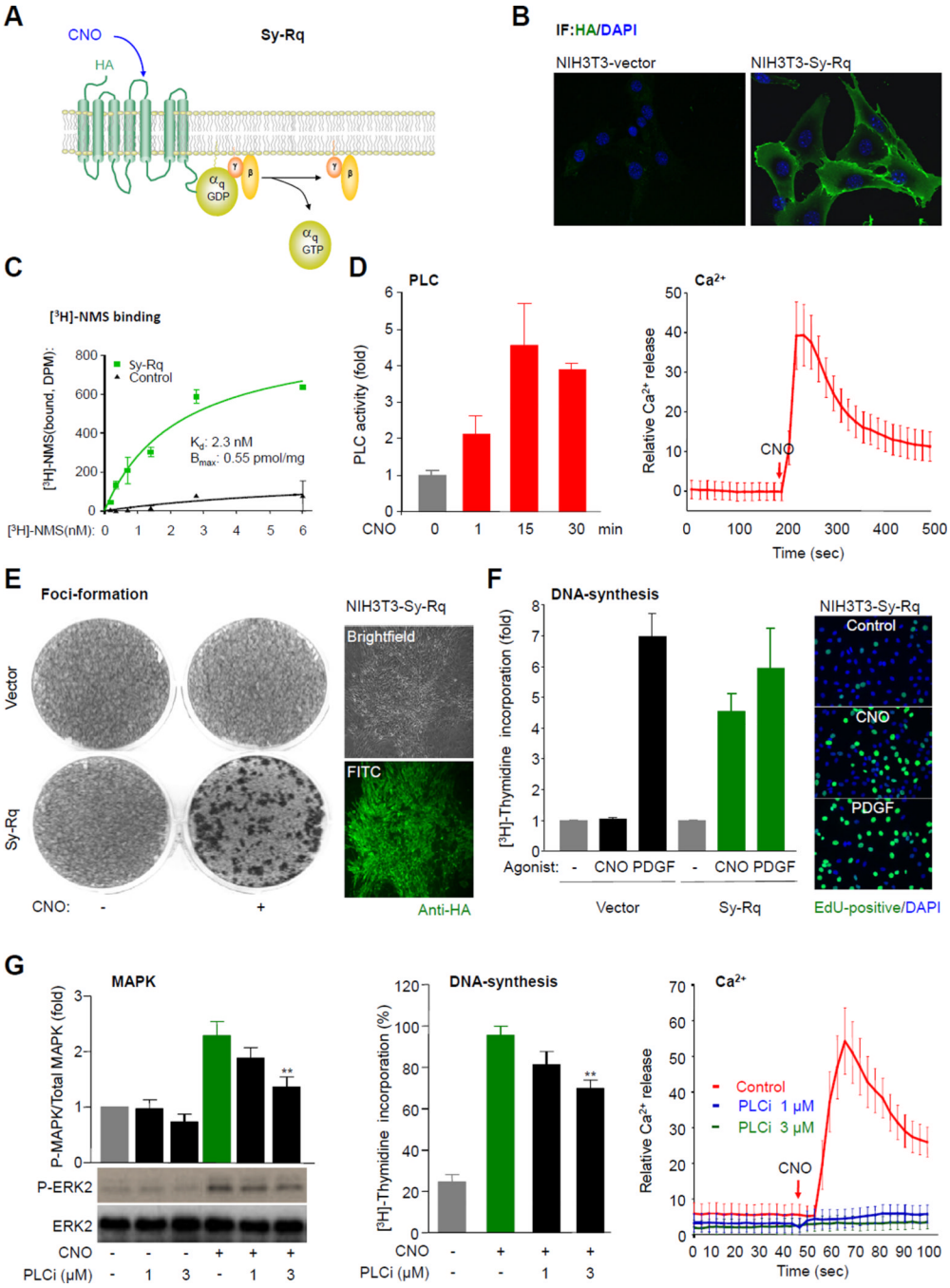

Figure 1. A synthetic biology approach to study the activity of Gα_q-coupled receptors activity in mammalian cells

(A) HA-tagged Sy-Rq receptor and its activation by the synthetic ligand CNO. (B) Expression of HA-Sy-Rq in transfected cells (NIH3T3-Sy-Rq) but not in vector transfected controls (NIH3T3-vector). Immunofluorescence anti-HA (green) and DAPI (blue). (C) Binding saturation curves of stable NIH3T3 Sy-Rq cells (Sy-Rq) and vector transfected cells (Control). [^{3}H]-NMS binding to cell membranes was analyzed using a nonlinear curve-fitting program to determine the ligand binding affinity (K_d) maximum ligand binding (B_{max}). (D, *left*) PLC activity (PI-breakdown, fold induction) in NIH3T3-Sy-Rq cells stimulated with CNO (mean ± SEM, N=3). (D, *right*) Cytosolic [Ca^{2+}] levels after CNO

treatment (arrow) (fold increase; mean ± SEM, N=9). (E) NIH3T3-control or Sy-Rq cells seeded on top of NIH3T3 cells, grown with or without CNO. (E, *left*) wells stained with Giemsa; (E, *right*) and a Sy-Rq focus (brightfield; top) showing positive anti-HA staining (immunofluorescence; bottom). (F) [^{3}H]- Thymidine incorporation in control vector and Sy-Rq cells treated with CNO or PDGF (fold induction; mean ± SEM, N=3) (*left*). EdU incorporation (green) and DAPI (blue) in 3T3-Sy-Rq cells stimulated as above (*right*). (G *left*) Western-blot and quantification of ERK2 activation in Sy-Rq cells, treated with vehicle (-) or PLCi prior to stimulation with CNO (5 min) (fold increase; mean ± SEM, N=3). (G, *center*) DNA-synthesis in Sy-Rq cells incubated with PLCi and stimulated with CNO (percentage of the response to CNO in the absence of PLCi; mean ± SEM, N=3). (G, *right*) Intracellular [Ca^{2+}] levels in Sy-Rq treated with vehicle (red) or PLCi (1 μM, blue; 3 μM green) for 15 minutes prior to CNO stimulation (arrow) (mean ± SEM, N=9). See also Figure S1.

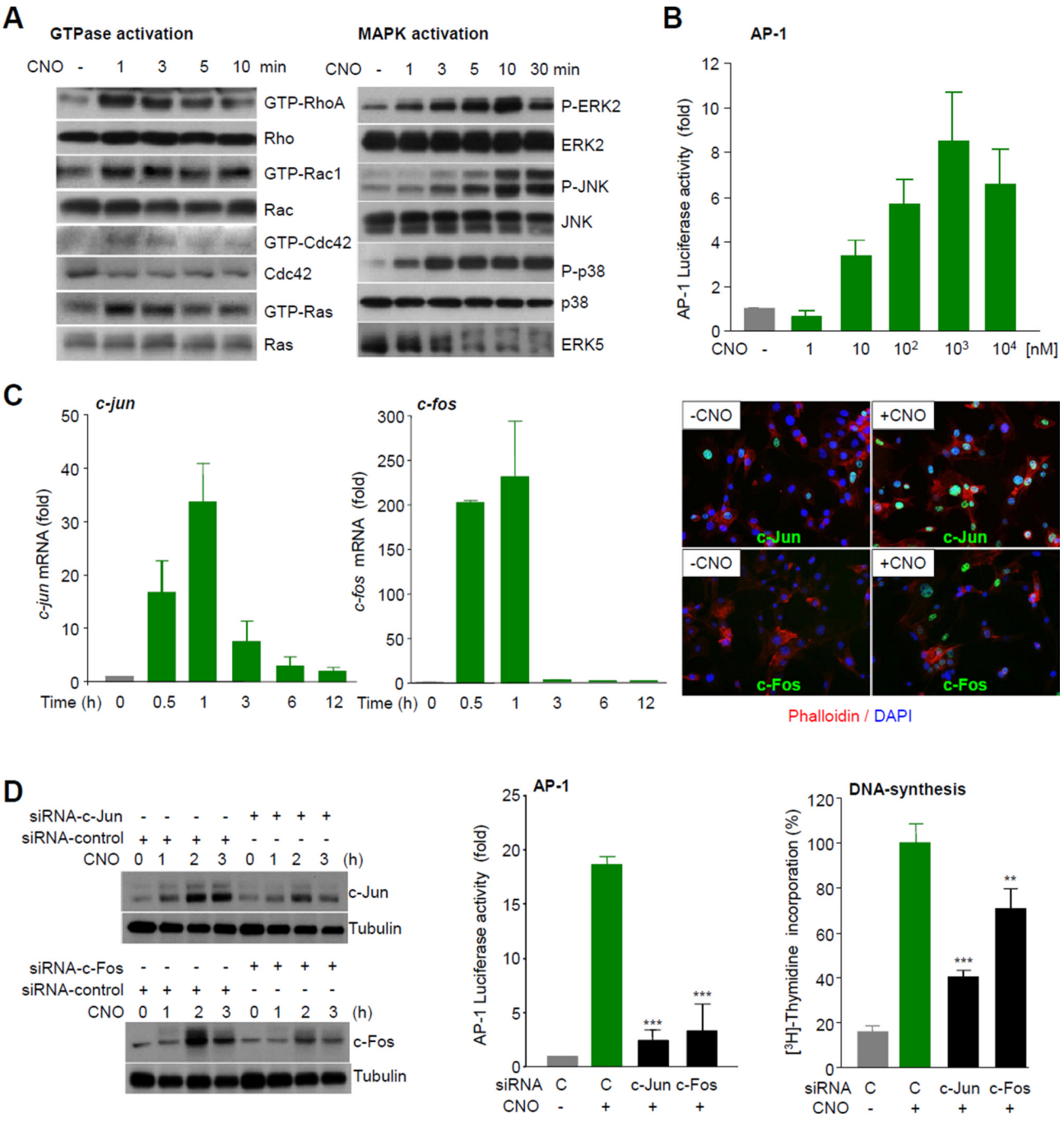

Figure 2. AP-1 is a key downstream target in cell growth promotion by Gq-GPCRs

(A) NIH3T3-Sy-Rq cells treated with CNO were analyzed for RhoA, Rac1, Cdc42 and Ras activation (*left*) and MAPKs activation (*right*). (B) AP-1 activation in Sy-Rq cells treated with CNO (fold increase *vs* control (-) (mean ± SEM, N=3). mRNA analysis of *c-jun* and *c-fos* expression in Sy-Rq cells treated with CNO (C, *left* and *center*) (fold induction *vs* untreated cells, 0; mean ± SEM, N=3), and (*Right*) immunofluorescence showing c-Jun (green) and c-Fos (green) expression, phalloidin (red), and DAPI (blue) (3h CNO treatment). (D) Western-blot analysis of c-Jun and c-Fos in NIH3T3-Sy-Rq cells transfected with siRNAs (*control*, *c-jun* or *cfos* siRNA), and stimulated with CNO (*Left*). (*Center*) AP-1

activation (fold increase vs control cells; mean ± SEM, N=3). (*Right*) DNA synthesis (% of response in siRNA control transfected cells treated with CNO; mean ± SEM, N=3).

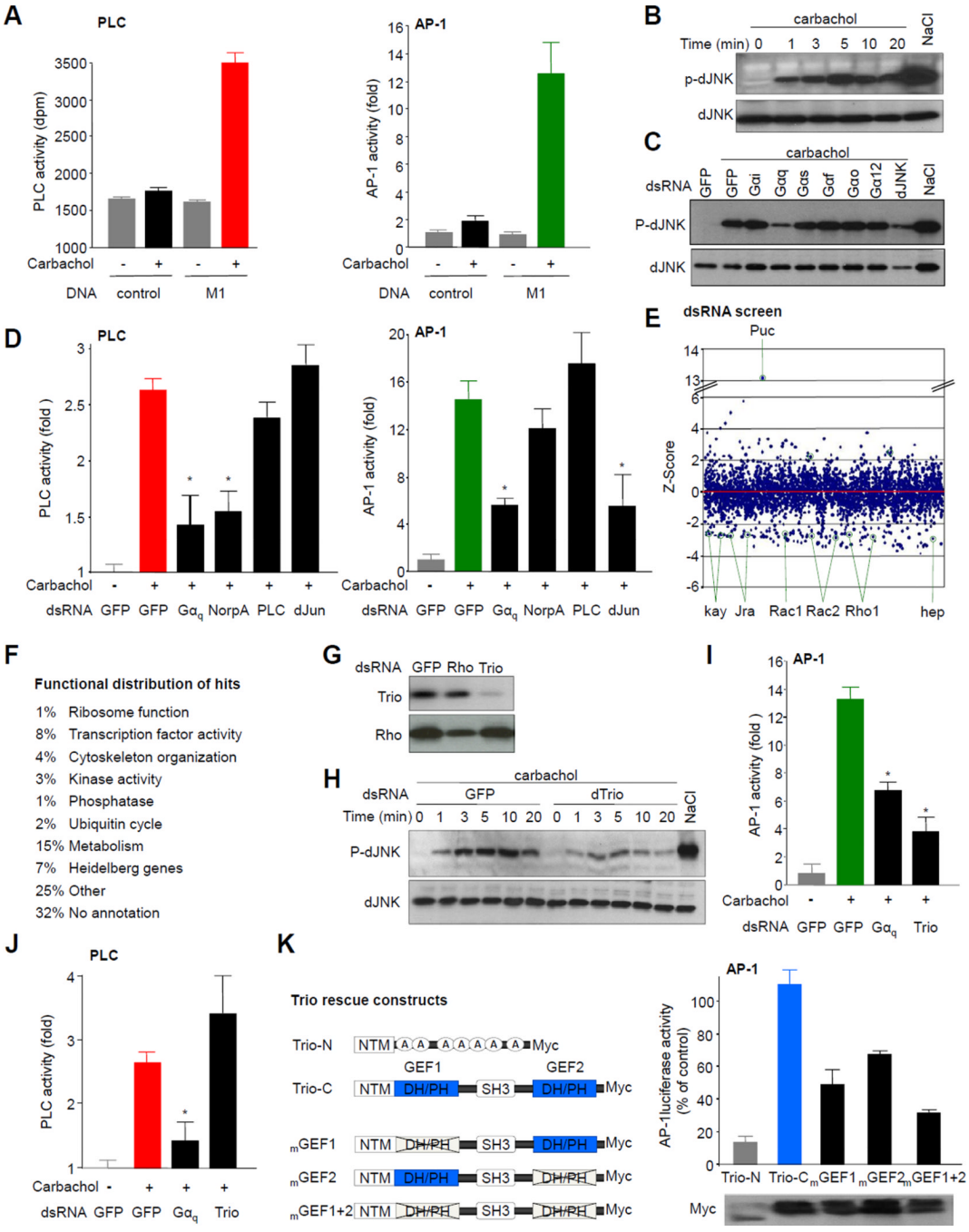

Figure 3. A genome-wide Drosophila RNAi screen identifies novel regulators of AP-1 activation downstream of Gq

(A) (*Left*) PLC activity (PI-breakdown, dpm of IP_1 accumulation) in S2 cells transiently transfected with pIZ (control) or pIZ-M1 (M1) vectors and stimulated with carbachol (mean ± SEM, N=3). (*Right*) AP-1 activation (fold induction; mean ± SEM, N=3). (B and C) JNK activation in pIZ-M1 transfected cells (B) or in cells transfected with pIZ-M1 and dsRNAs (C) after carbachol stimulation for 5 min (NaCl, 300 nM, 5 min, positive control). (D) PLC and AP1 activity in S2 cells transfected with pIZ-M1 and dsRNAs, stimulated with carbachol (*left*, fold increase IP_1 accumulation and *right*, AP-1 activity *vs* unstimulated (-) cells; mean ± SEM, N=3). (E) Scatterplot showing the distribution of hits based on their Z-

score. Examples of positive and negative hits are highlighted. (F) Functional characterization of RNAi screen hits with z-scores values <−2.0 or >2.0, expressed as % of total hits. (G) dTrio and dRho protein expression in S2 cells transfected with dsRNA for dTrio or dRho. (H, I, J) dJNK, AP1 and PLC activation in pIZ-M1 cells transfected with dsRNA control (GFP), dTrio and $G\alpha_q$ dsRNA, where indicated, and stimulated with carbachol (mean ± SEM, N=3). (K) (*Left*) Schematic representation of dTrio DNA expression constructs. The two Rho-GEF domains in Trio-C were mutated as indicated (mGEF1, mGEF2 or mGEF1+2). (*Right*) AP-1 activation in pIZ-M1 cells transfected with dTrio dsRNAs together with the indicated myc-Trio constructs, and treated with carbachol (% of response to carbachol; control dsRNA considered as 100%; mean ± SEM, N=3). Western blot below shows protein expression of myc-tagged Trio constructs. See also Figure S2.

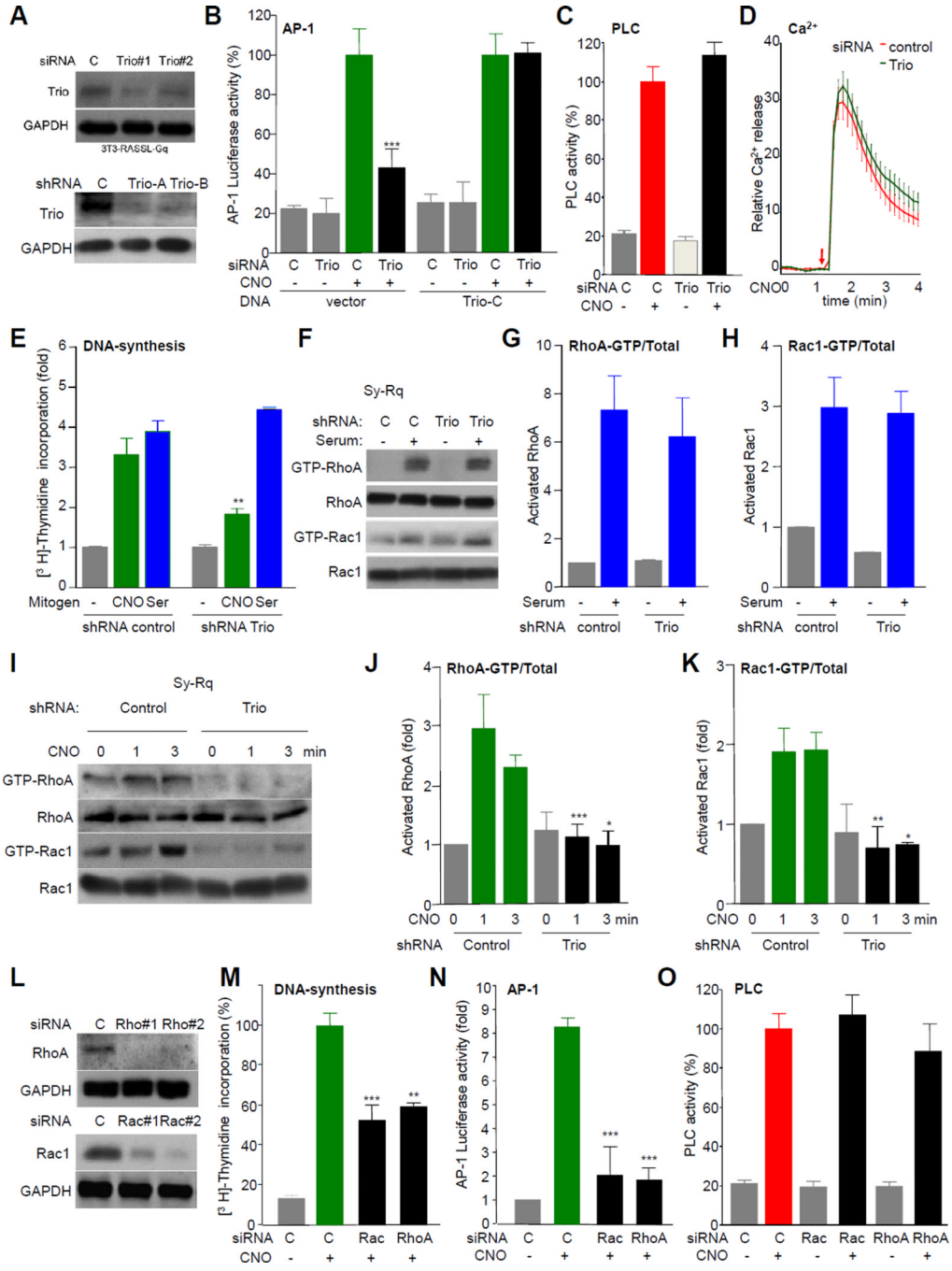

Figure 4. Trio-dependent signaling circuitries elicited by mitogenic Gq activation in mammalian cells

(A) Trio protein expression in NIH3T3-Sy-Rq cells transfected with control (C) or Trio siRNAs (top), and cells stably expressing shRNA-control or Trio sh-RNA (bottom). (B) AP-1 activation in NSy-Rq cells transfected with siRNA control (C) or targeting Trio, and vector control or Trio-C expression vector (Trio-C), expressed as percentage of AP1 response to CNO in siRNA control cells transfected with vector DNA, considered as 100% (mean ± SEM, N=3). (C) PLC activity in cells transfected with siRNA as in B, expressed as the percentage of IP_1 accumulation *vs* siRNA control transfected cells (mean ± SEM, N=3). (D) Intracellular [Ca^{2+}] in cells transfected with control or Trio siRNA stimulated with CNO

(fold increase, mean ± SEM of 9 independent measurements). (E) DNA-synthesis in Sy-Rq-shRNA control or – shRNA Trio-A cells stimulated with CNO or serum (fold increase *vs* unstimulated (-) shRNAcontrol cells; mean ± SEM, N=3). (F). RhoA and Rac1 activation in Sy-Rq shRNA-control or – Trio-A cells stimulated with serum (3 min). (G and H) Quantification of RhoA (G) and Rac1 (H) GTP-bound accumulation (fold increase *vs* unstimulated control shRNA cells; mean ± SEM, N=3). (I, J, and K) A similar RhoA and Rac1 activation analysis was performed in response to CNO at the indicated times. (L) RhoA and Rac1 expression in Sy-Rq cells transfected with control, or RhoA or Rac1 siRNAs. (M, N, and O) DNA synthesis (M), AP-1 (N) and PLC activity (O) in cells transfected with control, RhoA#1 or Rac#1 siRNAs stimulated with CNO (data are expressed as percentage of response to CNO in control siRNA, considered 100%; mean ± SEM, N=3). See also Figure S3.

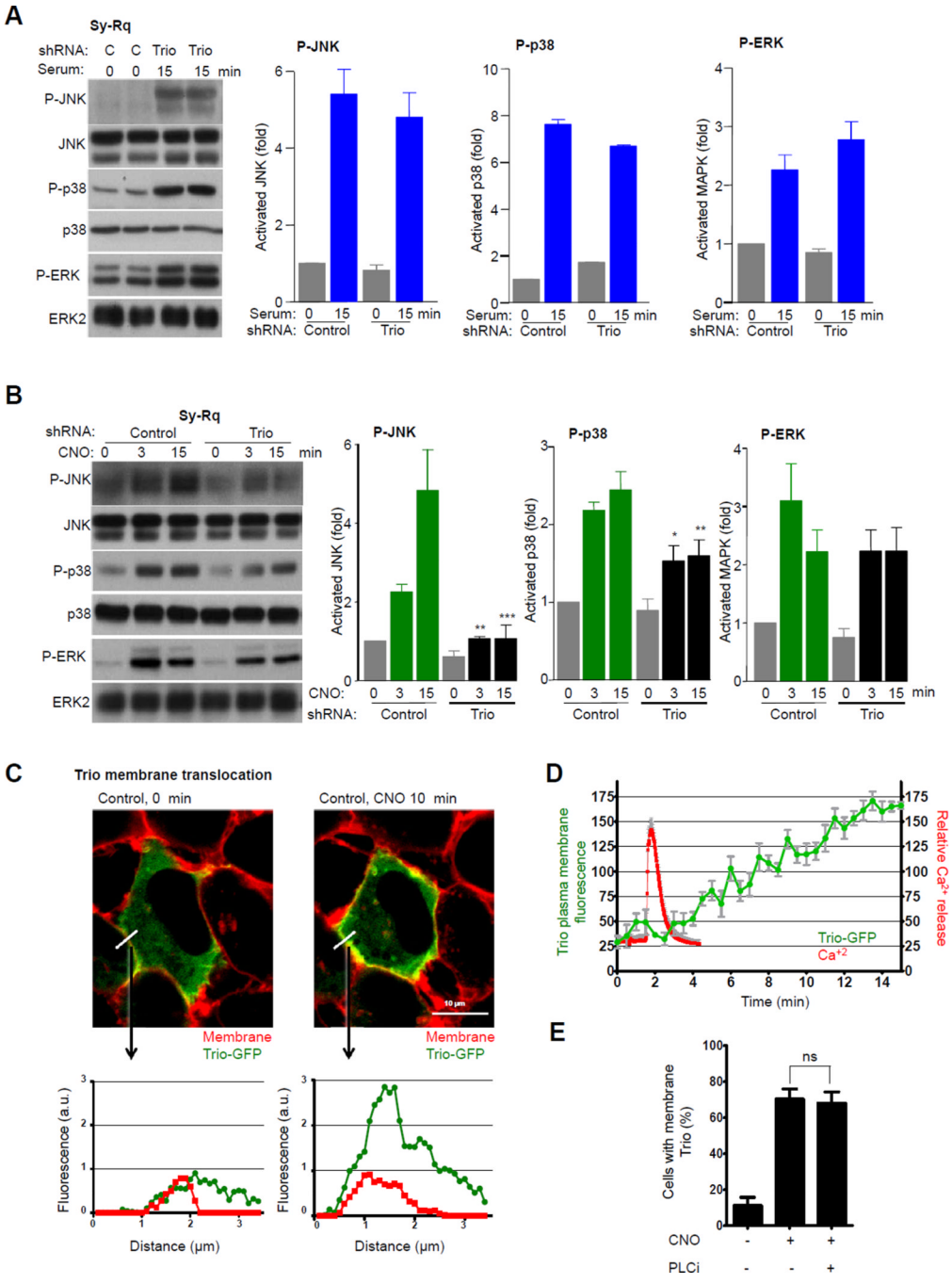

Figure 5. Trio activation is required for proliferative signaling through Gq

(A) Activation of ERK, JNK and p38 in NIH3T3-Sy-Rq shRNA-control or -Trio cells stimulated with serum. Representative Western-blots and quantification (graphics; fold increase *vs* unstimulated control shRNA cells; mean ± SEM, N=3). (B) Activation of ERK, JNK and p38 under the same conditions as in A but stimulated with CNO for the indicated time. (C) Trio recruitment to the plasma membrane. Confocal images of cells transfected with Sy-Rq and Trio-GFP (green) and stained with a plasma membrane maker (red), prior to (0 min) or 10 min after the addition of CNO. Histograms depict fluorescence intensity in arbitrary units (a.u.) along the white line to show the recruitment of Trio to the plasma membrane. No changes in localization were observed when the empty GFP expression

vector was used as a control (not shown). (D) Temporal analysis of Trio accumulation at the plasma membrane in cells transfected with Sy-Rq and Trio-GFP after stimulation with CNO (green, left axis) (see Movie S1). Calcium mobilization was measured in parallel (red, right axis). (E) Trio recruitment to the plasma membrane is independent of PLC activity. Percentage of cells per field (40×) showing membrane localization of Trio was quantified in confocal images of cells transfected with Sy-Rq and Trio-GFP before (-) and after (+) CNO stimulation (10 min) in the absence or presence of 3μM PLCi (See Fig. S4D and Movie S2). Bars, average ± S.E.M of 5 fields of view with 8 to 10 cells each; ns, not statistically significant differences. See also Figure S4.

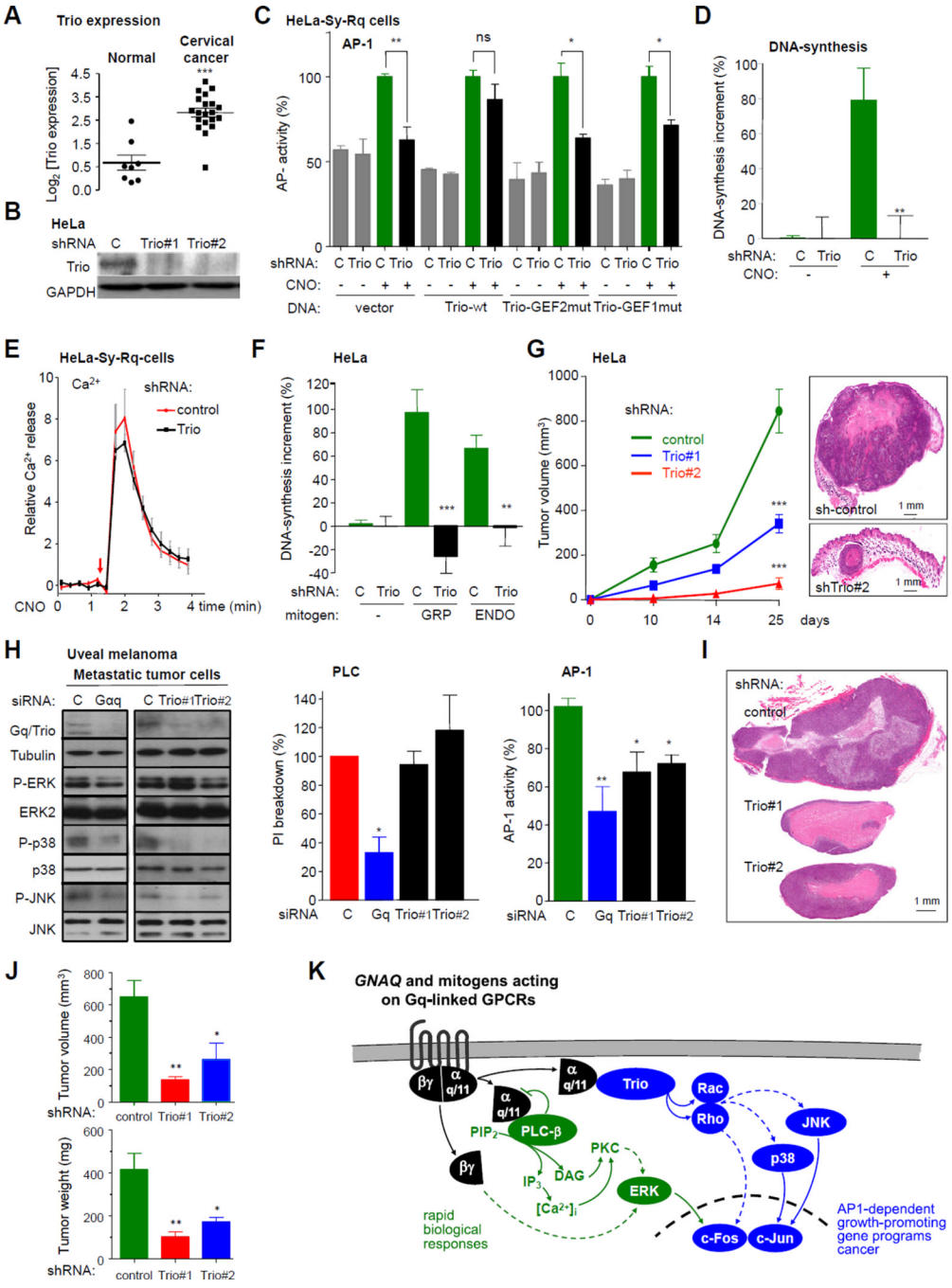

Figure 6. Trio mediates normal and aberrant cell growth elicited by the activation of endogenous Gq-GPCRs and the *GNAQ* oncogene

(A) Trio mRNA over-expression (ONCOMINE™), in human cervical cancer compared to normal, each data point represents an individual patient tissue sample (data extracted from (Pyeon et al., 2007)). (B) Trio protein expression analysis in human HeLa (cervical) cancer cells stably transfected with the indicated pGIPZ-shRNA expression vectors. (C) HeLa shRNA-control (C) or shRNA-Trio#2 (Trio) cells transfected with pCEFL-HA-Sy-Rq were transfected with vector control (vector), and expression vectors for Trio wild type (wt), and its mutants Trio AE (Trio Q1368A/L1376E, GEF1mut) and Trio L2051E (GEF2mut), and stimulated with CNO. AP-1 reporter activity was determined and expressed as percentage of

AP1 response elicited by CNO in Hela-Sy-Rq shControl (mean ± SEM, N=3). (D and E) HeLa shRNA-control or shRNA-Trio#2 cells transfected with pCEFL-HA-Sy-Rq. (D) Effect of CNO on DNA-Synthesis (percentage of increase vs unstimulated control shRNA cells; mean ± SEM, N=3) and (E) cytoplasmic Ca^{2+} levels (fold increase vs unstimulated control shRNA cells; mean ± SEM, N=3). (F) DNA synthesis in HeLa shRNA-control or shRNA-Trio#2 cells stimulated using GRP or endothelin. Data are expressed as above (mean ± SEM, N=3). (G) Xenografted tumor growth in nude mice injected with HeLa shRNA-control and shRNA-Trio#1 and –Trio#2 cells (mean ± SEM, N=10). Representative H&E-stained tissue section of tumors derived from HeLa shRNA-control and HeLa shRNA-Trio#2 cells. Tumors grow as poorly differentiated squamous cell carcinomas. HeLa cells in which Trio has been knocked down grow much slower. (H) Western-blot analysis of lysates form metastatic human uveal melanoma tumor cells (OMM 1.3) transfected with siRNA targeting Gq or 2 siRNAs targeting Trio, using antibodies against Gq (left panels) and Trio (right panels), tubulin, and total and phosphorylated forms of ERK, p38, and JNK. OMM 1.3 cells were transfected with the indicated siRNA and analyzed for PLC-mediated PI-breakdown and AP-1 activity as indicated. Data are represented as the percentage of the results obtained in cells transfected with the corresponding siRNA control (C) (mean ± SEM, N=3). (I). The picture shows representative H&E sections of metastatic (OMM 1.3) human uveal melanoma shRNA-control and shRNA-Trio#1 and #2 derived tumors. These uveal melanoma cells grow as large tumor xenografts, invading the surrounding muscle (redish stained cell remnants around the tumor mass). Tumor xenografts in which Trio has been knocked down are much smaller, less infiltrative, with large necrotic areas (pinkish area). (J) Average ± S.E.M. of the tumor volume and weight at the end the observation period (n=10 per group). (K) Scheme summarizing the signaling pathways elicited downstream form the activation of Gq-GPCRs. The transient stimulation of PLC and the subsequent increased in ERK activity may represent a general event caused by Gq activation. The duration of signal transmission through PLC-β is limited by its GAP activity of on Gαq. In parallel, Gαq promotes the membrane recruitment of Trio independently on PLC-β activation, thus initiating the prolonged activation of a hardwired mechanism resulting in the sustained activation of Rho and Rac and their downstream targets, thereby promoting the stimulation AP-1 and normal and aberrant cell proliferation. See also Figure S5.