

# G-protein–coupled receptor GPR161 is overexpressed in breast cancer and is a promoter of cell proliferation and invasion

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Triple-negative breast cancer (TNBC) accounts for 20% of breast cancer in women and lacks an effective targeted therapy. Therefore, finding common vulnerabilities in these tumors represents an opportunity for more effective treatment. Despite the growing appreciation of G-protein–coupled receptor (GPCR)-mediated signaling in cancer pathogenesis, very little is known about the role GPCRs play in TNBC. Using genomic information of human breast cancer, we have discovered that the orphan GPCR, G-protein–coupled receptor 161 (*GPR161*) is overexpressed specifically in TNBC and correlates with poor prognosis. Knockdown of *GPR161* impairs proliferation of human basal breast cancer cell lines. Overexpression of *GPR161* in human mammary epithelial cells increases cell proliferation, migration, intracellular accumulation of E-cadherin, and formation of multiacinar structures in 3D culture. *GPR161* forms a signaling complex with the scaffold proteins  $\beta$ -arrestin 2 and Ile Gln motif containing GTPase Activating Protein 1, a regulator of mammalian target of rapamycin complex 1 and E-cadherin. Consistently, *GPR161* amplified breast tumors and cells overexpressing *GPR161* activate mammalian target of rapamycin signaling and decrease Ile Gln motif containing GTPase Activating Protein 1 phosphorylation. Thus, we identify the orphan GPCR, *GPR161*, as an important regulator and a potential drug target for TNBC.

G-protein–coupled receptors (GPCRs) are heptahelical membrane proteins responsible for transducing signals from a diverse range of ligands to affect numerous physiological processes, including vision, olfaction, behavior, and autonomic nervous system transmission (1). These properties have allowed the widespread development of GPCR-targeted drugs, which represent nearly 30% of all currently used therapeutics, for indications ranging from allergy to depression to hypertension. However, the clinical utility of targeting GPCRs in cancer therapy remains poorly defined (2, 3). GPCRs regulate many aspects of tumorigenesis, including proliferation, invasion, survival at the secondary site, and immune cell function, as well as several cancer-associated signaling pathways (4). Emerging large-scale genomic analyses have recently provided further evidence of frequent GPCR alterations in human tumors (5–10). For example, 20% of all human tumors sequenced contain mutations in GPCRs; the phenotypic outcome of these mutations remains unknown and thus provides a wealth of information for the development of hypothesis-driven experiments (5). In addition to mutations, alterations in gene expression, copy number, and promoter methylation of GPCRs have been detected. Determining the contribution of such alterations to cancer initiation and progression remains a significant challenge yet critical both for discovery of driver oncogenes and for the development of targeted therapeutics.

Triple-negative breast cancer (TNBC), characterized by lack of expression of estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor receptor 2 (*HER2*), is associated with early recurrence and poor outcome (11). Mutations in *BRCA1* and *BRCA2* account for 15% of TNBC, and several

other susceptibility loci have been identified (12). Representing nearly a quarter of all breast cancers, TNBC lacks an effective targeted therapy due to high levels of genetic heterogeneity. Therefore, finding common druggable targets is a critical endeavor. We used large-scale genomic analysis to discover GPCRs up-regulated in TNBC. This method uncovered the poorly characterized class A rhodopsin family orphan GPCR, *GPR161*.

Several studies have identified an important role for *GPR161* during normal development. An 8 bp mutation in *GPR161*, resulting in a premature stop codon and truncated C terminus, was identified as the cause of the vacuolated lens spontaneous mouse model, exhibiting neural tube defects and congenital cataracts (13). Consistent with these phenotypes, *GPR161* is expressed in the lateral neural folds and in the developing lens (13). Knockdown of *GPR161* in the zebrafish embryo disrupts left–right patterning in the lateral plate mesoderm through modulation of  $\text{Ca}^{2+}$  levels, resulting in aberrant cardiac morphogenesis (14). Recently, *GPR161* was shown to localize to primary cilia and regulate Sonic hedgehog (Shh) signaling (15). Expression of *GPR161* inhibits Shh through G $\alpha$ s-induced cyclic adenosine monophosphate (cAMP) accumulation, resulting in protein kinase A–mediated processing of Gli3 (15). In addition, *GPR161* is part of a signaling network that confers resistance to MAP kinase pathway inhibition in melanoma (16).

We describe a role for *GPR161* in the pathogenesis of human TNBC. We provide evidence that *GPR161* promotes proliferation through activation of mammalian target of rapamycin

## Significance

Recent advances in DNA sequencing and bioinformatics tools have allowed the large-scale characterization of genetic alterations in human tumors. We exploited genomics data to identify commonly occurring alterations in G-protein–coupled receptors (GPCRs) in triple-negative breast cancer (TNBC), a subset of breast cancer with poor prognosis and lacking effective targeted therapy. We have discovered that the orphan GPCR, G-protein–coupled receptor 161 (*GPR161*), is overexpressed specifically in TNBC and correlates with poor prognosis. We identify *GPR161* as a regulator of mammary epithelial cell proliferation and invasion in a mammalian target of rapamycin signaling-pathway-dependent manner. Down-regulation of *GPR161* in a TNBC-derived cell line impairs cell growth, suggesting that *GPR161* is a drug target for breast cancer.

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The authors declare no conflict of interest.

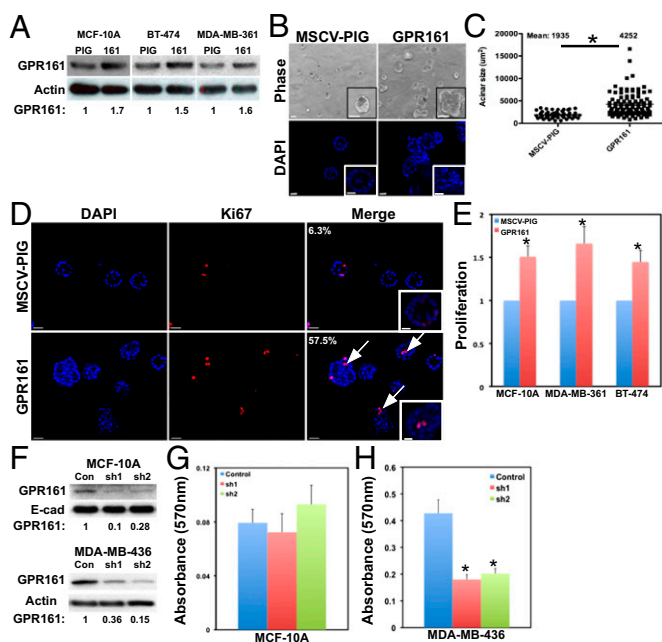
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**Fig. 2.** GPR161 overexpression induces cell proliferation. (A) Western blot analysis of GPR161 overexpression in MCF-10A, BT-474, and MDA-MB-361 cells. (B) MCF-10A cells, either MSCV-PIG or GPR161, were grown as 3D acini for 14 d. Phase contrast (upper panels) and DAPI staining (lower panels) were used to monitor acinar morphology. [Scale bar, 100  $\mu$ m (Upper), 75  $\mu$ m (Upper Inset), 50  $\mu$ m (Lower), and 20  $\mu$ m (Lower Inset).] (C) Acinar area was measured for MSCV-PIG and GPR161 MCF-10A acini. \* $P$  < 0.05 for the difference between GPR161 cells and those expressing the empty vector using an unpaired  $t$  test. (D) Proliferation was analyzed in MCF-10A acini by IF staining of Ki67. Arrows denote Ki67-positive central acinar cells. [Scale bar, 50  $\mu$ m and (Inset) 20  $\mu$ m.] (E) MTT assay was used to measure cell proliferation in the nontransformed mammary epithelial cell line MCF-10A and the luminal cancer cell lines MDA-MB-361 and BT-474, stably expressing an empty vector (MSCV-PIG) or GPR161. \* $P$  < 0.05 for the difference between GPR161 cells and those expressing the empty vector using an unpaired  $t$  test. (F) Western blot analysis of GPR161 knockdown in MCF-10A and MDA-MB-436 cells. (G) MTT assay was used to measure cell proliferation in control or GPR161-deficient MCF-10A cells. (H) MTT assay was used to measure cell proliferation in control or GPR161-deficient MDA-MB-436 cells. \* $P$  < 0.05 for the difference between cells expressing a control shRNA and those with a GPR161 shRNA using an unpaired  $t$  test.

(see arrows in Fig. 2D). Therefore, overexpression of GPR161 induced cell proliferation and disrupted morphogenesis of mammary epithelial cells grown in 3D culture.

**GPR161 Regulates Proliferation in Mammary Cell Lines.** To rule out the possibility that GPR161-induced proliferation was restricted to basal epithelial cells, we also overexpressed (less than 2.0-fold) GPR161 in two luminal-like breast cancer cell lines (BT-474 and MDA-MB-361) (Fig. 2A). Overexpression of GPR161 increased proliferation of MCF-10A cells compared with cells expressing an empty vector (MSCV-PIG) (Fig. 2E). Similarly, GPR161 overexpression increased proliferation in both MDA-MB-361 and BT-474 cells (Fig. 2E and Fig. S1F). Therefore, overexpression of GPR161 to the levels seen in TNBC can enhance proliferation in mammary epithelial cells and cancer-derived luminal tumor cell lines.

To determine if GPR161 is required for proliferation of basal breast cancer cells, we used shRNA to knock down GPR161 in MCF-10A and MDA-MB-436 TNBC cell lines (Fig. 2F). Two hairpins significantly reduced GPR161 expression compared with a control shRNA in both cell lines. GPR161 knockdown had no significant effect on proliferation of nontransformed MCF-10A cells (Fig. 2G). However, proliferation was reduced more

than twofold in MDA-MB-436 cells, likely because the cancer cells and not MCF-10A have developed a dependence on GPR161-regulated pathways (Fig. 2H). Therefore, GPR161 is both necessary and sufficient for regulating proliferation in breast cancer cells.

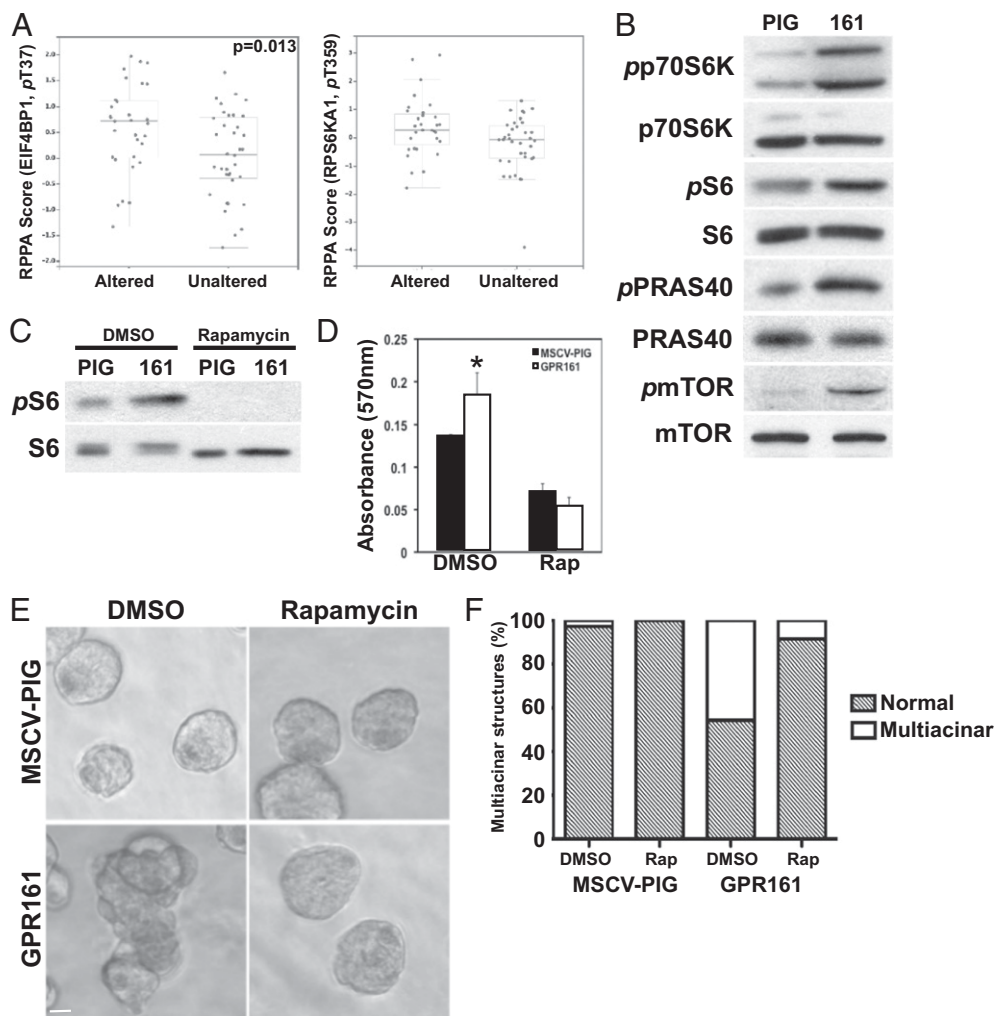
**GPR161 Induces Cell Proliferation Through mTOR.** To obtain insight into pathways activated in tumors with high levels of GPR161, we analyzed reverse phase protein array (RPPA) data from the TCGA database. Tumors with high levels of GPR161 had higher phosphorylation of mammalian target of rapamycin (mTOR) pathway components EIF4BP1, RPS6KA1, JUN, and RB1 compared with tumors expressing lower levels of GPR161 (Fig. 3A). We investigated if GPR161 overexpression activated the mTOR signaling pathway in cells in culture. Under normal growth conditions, control MDA-MD-361 cells display low levels of phosphorylation of the AKT pathway component p70S6 kinase (p70S6K) at T389 and the downstream target, ribosomal protein S6, at S235/236 (Fig. 3B). In contrast, cells expressing GPR161 display enhanced phospho-p70S6K and phospho-S6. A similar pattern of S6 phosphorylation is observed in control and GPR161-expressing BT-474 cells (Fig. S2A). mTOR activation is inhibited by PRAS40 and phosphorylation of PRAS40 at Thr246 relieves this inhibition (18). Therefore, we monitored PRAS40 phosphorylation in MDA-MD-361 cells expressing an empty vector or GPR161. Phospho-PRAS40 was markedly increased in GPR161-expressing cells (Fig. 3B). Similarly, we detected increased mTOR phosphorylation at S2448 in GPR161-expressing cells (Fig. 3B). To determine the relevance of mTOR activation in the GPR161-induced phosphorylation of S6, we treated the cells with rapamycin, an mTOR inhibitor (Fig. 3C). Although phospho-S6 levels were higher in GPR161-expressing cells, this increase was abolished upon rapamycin treatment. Therefore, GPR161-induced S6 phosphorylation was mTORC1-dependent. To determine the relevance of the mTOR pathway to GPR161-induced proliferation, MDA-MB-361 cell growth was measured in the absence and presence of rapamycin (Fig. 3D). GPR161-induced cell proliferation was inhibited in the presence of rapamycin. Furthermore, rapamycin treatment inhibited multiacinar structure formation in GPR161-expressing MCF-10A acini (Fig. 3E and F). These observations suggest that GPR161 mediates cell proliferation in an mTORC1-dependent manner.

#### GPR161 Induces Migration and Invasion of Mammary Epithelial Cells.

When grown on standard tissue culture dishes, GPR161-expressing cells displayed robust changes in cell shape. Subconfluent MCF-10A and MDA-MB-361 cells formed epithelial cell colonies with smooth, round edges (Fig. S2B and C). In contrast, cells expressing GPR161 formed colonies with poorly defined colony borders, with sharp edges and protrusions (Fig. S2B and C), suggesting migratory/invasive properties. Consistent with this possibility, overexpression of GPR161 led to a twofold increase in the migratory ability of MDA-MB-361 and BT-474 cells, as monitored by transwell migration assay over 24 h (Fig. 4A). Invasive potential was determined by culturing GPR161-expressing MCF-10A cells in 3D in a 1:1 mix of Matrigel and collagen. Whereas control cells formed round, noninvasive structures, GPR161-expressing MCF-10A acini were multiacinar and invasive (Fig. 4B). GPR161-expressing acini also had disrupted Laminin-V staining, a hallmark of invasion in MCF-10A culture (Fig. 4C).

**GPR161 Induces Intracellular Accumulation of E-cad.** The GPR161-induced change in cell shape and invasive ability led us to investigate the possibility that the cells had undergone an epithelial-to-mesenchymal transition. However, Western blot analysis of protein lysates from control and GPR161-expressing MCF-10A cells failed to show changes in the levels of the mesenchymal markers vimentin, snail, fibronectin, and N-cadherin (Fig. S2D).

Close inspection of the GPR161-expressing MCF-10A acinar structures revealed a rough surface topology, suggesting a defect in cell-cell adhesion (Fig. 4D). Therefore, we probed the localization of the adherens junction protein E-cad in GPR161-expressing



cells. Total levels of E-cad were modestly reduced upon expression of GPR161. In addition, GPR161-expressing MCF-10A cells displayed markedly higher levels of intracellular E-cad than control cells as measured by immunoblot (Fig. 4E). This pattern was similar in MDA-MB-361 cells, where E-cad was localized to the cytosol upon GPR161 expression as detected by immunofluorescence (Fig. 4F). This observation was extended to human tumors, where four TNBC samples expressing high levels of GPR161 displayed intracellular E-cad, compared with predominantly plasma-membrane-localized E-cad in six ER+/GPR161- tumors (Fig. 4G and Fig. S2E). Therefore, GPR161 expression is associated with both decrease in levels and redistribution of E-cad from the plasma membrane in epithelial cells.

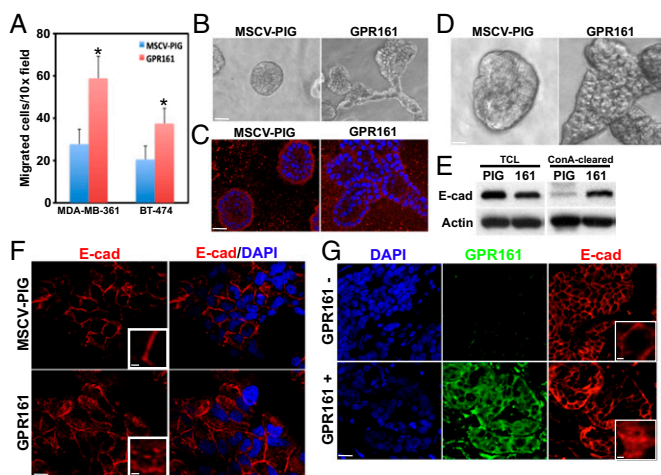
**GPR161 Forms a Complex with IQGAP1 and  $\beta$ Arr2.** To define the mechanism by which GPR161 induces proliferation, migration, and invasion, we focused on an adherens junction-localized scaffold protein that is related to GPCR signaling. IQGAP1 regulates a vast array of cellular functions through interactions with proteins such as E-cad, Neural Wiskott-Aldrich syndrome protein (N-WASP), Cdc42, and the exocyst complex (19). IQGAP1 has been reported to interact with  $\beta$ Arr2, a scaffold protein that binds to activated GPCRs and regulates signaling and receptor endocytosis (20). IQGAP1 promotes proliferation and migration, and overexpression has been associated with poor prog-nosis in advanced colorectal cancer (21–23). Interestingly, IQGAP1 can coordinate cell growth and division in a phosphorylation-dependent manner (24). Phosphorylation of IQGAP1 at Ser1339 by protein kinase C allows

binding of Cdc42 (25), whereas unphosphorylated IQGAP1 binds mTOR and leads to activation of downstream signals, including phosphorylation of S6 (26, 27). To determine if GPR161 overexpression influences the phosphorylation status of IQGAP1, we immunoprecipitated endogenous IQGAP1 from control or GPR161-expressing MCF-10A and MDA-MB-361 cells and analyzed serine phosphorylation by immunoblot analysis (Fig. 5A). In both cell lines, expression of GPR161 induced a striking decrease in IQGAP1 serine phosphorylation, consistent with GPR161-induced mTOR activation.

To test the possibility that GPR161 forms a signaling complex with IQGAP1 and  $\beta$ Arr2, we immunoprecipitated myc-tagged IQGAP1 from 293T cell lysates coexpressing FLAG-tagged GPR161 (Fig. 5B). Myc-IQGAP1 was able to pull down FLAG-GPR161. Immunoprecipitation (IP) of  $\beta$ Arr1 failed to pull down either myc-IQGAP1 or FLAG-GPR161 (Fig. 5C, center lane). In contrast, IP of  $\beta$ Arr2 pulled down both myc-IQGAP1 and FLAG-GPR161 (Fig. 5C, right lane) in cells coexpressing FLAG-GPR161, myc-IQGAP1 with either HA-tagged  $\beta$ Arr1 or  $\beta$ Arr2. Furthermore, IP of endogenous GPR161 immunoprecipitated both IQGAP1 and  $\beta$ Arr2, and IQGAP1 immunoprecipitated GPR161 and  $\beta$ Arr2 (Fig. 5D). Although these results do not prove the existence of a trimeric complex, they suggest that  $\beta$ Arr2 may serve as a scaffold connecting GPR161 and IQGAP1.

To determine the requirement for IQGAP1 in GPR161-induced proliferation and migration, we used siRNA to knock down IQGAP1 in MDA-MB-361 cells (Fig. 5E). Knockdown of IQGAP1 attenuated both GPR161-induced proliferation (Fig. 5F)





**Fig. 4.** GPR161 induces migration and invasion of mammary epithelial cells. (A) Quantification of transwell migration assay of MDA-MB-361 and BT-474 cells either stably expressing an empty vector (MSCV-PIG) or GPR161.  $*P < 0.05$  using an unpaired  $t$  test. (B) MCF-10A cells, either MSCV-PIG or GPR161, were grown as 3D acini in 1:1 Matrigel/collagen for 14 d. Phase contrast was used to monitor acinar morphology. (Scale bar, 20  $\mu$ m.) (C) Invasion was analyzed in MCF-10A acini by IF staining of Laminin V. (Scale bar, 20  $\mu$ m.) (D) MCF-10A cells, either MSCV-PIG or GPR161, were grown as 3D acini in 1:1 Matrigel/collagen for 14 d. Phase contrast was used to monitor acinar morphology. (Scale bar, 10  $\mu$ m.) (E) Western blot analysis was used to measure E-cad levels from total MCF-10A cell lysates (TCLs) or those treated with Con A. (F) IF analysis of E-cad localization in MDA-MB-361 cells, either stably expressing an empty vector (MSCV-PIG) or GPR161. [Scale bar, 10  $\mu$ m and (Inset) 20  $\mu$ m.] (G) IF analysis of E-cad localization in human tumors expressing low or high levels of GPR161. [Scale bar, 20  $\mu$ m and (Inset) 10  $\mu$ m.]

and transwell migration (Fig. 5G). Therefore, GPR161 promotes cell proliferation and cell migrations in an IQGAP1-dependent manner.

**GPR161 and IQGAP1 Are Coamplified in Human Breast Cancer.** As both *GPR161* (this study) and *IQGAP1* (28) have been implicated in human breast cancer pathogenesis, we sought to investigate the cooccurrence of alterations in these two genes. Of the 748 tumors analyzed from the TCGA, 166 (22.2%) contained amplification of *GPR161* and 39 (5.2%) contained amplification of *IQGAP1* (Fig. 5H). Thirteen tumors contained alterations in both genes, a statistically significant outcome ( $P < 0.036$ ) compared with that expected from random overlap. Despite the significant association observed in all breast cancers, TNBC represented a small subset and hence did not reach statistical significance. Therefore, there is likely a selection for coamplification of *GPR161* and *IQGAP1* during breast cancer progression.

## Discussion

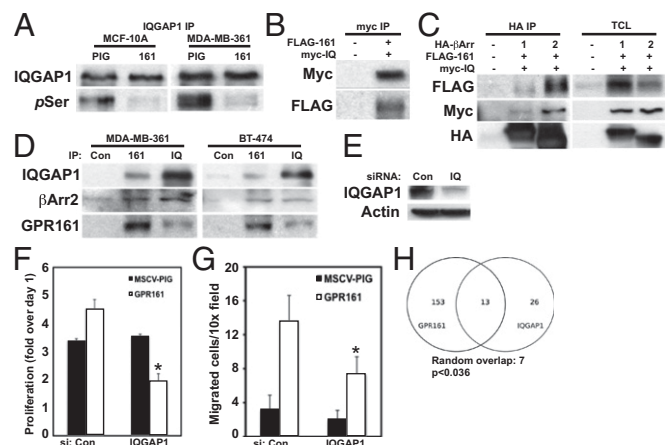
Taken together, our results identify *GPR161* as a prognostic biomarker for TNBC and demonstrate that GPR161 is an important regulator of cell proliferation and migration in breast cancer cells. We identify activation of mTORC1/S6K as an effector pathway and identify IQGAP1 and  $\beta$ Arr2 as GPR161 binding partners. In addition, we uncover genetic interactions between *GPR161* and *IQGAP1* in human breast cancer.

Phosphorylation of the scaffold protein IQGAP1 has recently been recognized as a regulator of nutrient and growth factor signaling (24). Phosphorylation of IQGAP1 at Ser1443 allows binding of CDC42, whereas unphosphorylated IQGAP1 promotes activation of mTOR. We find that overexpression of GPR161 decreases IQGAP1 serine phosphorylation, consistent with the observed activation of mTORC1. Whether GPR161 reduces the activity of PKC (the IQGAP1 Ser1443 kinase), or activates an unknown phosphatase, remains to be determined.

Genomic analyses of human tumors have uncovered widespread alterations in GPCRs in numerous cancer types (5, 29). However, the contribution of these alterations to human tumorigenesis remains unknown. Our study used genomic information to identify *GPR161* as a regulator of mammary epithelial cell proliferation and invasion, and a putative drug target, in TNBC. We also identified two breast cancer *GPR161* mutations in the Catalog of Somatic Mutations in Cancer database. One mutation (R91G) is found within the first extracellular loop and may therefore play a role in ligand binding. The other, S251G, is within the third intracellular loop, a region known to be phosphorylated in response to activation of many GPCRs and the site of interaction of  $\beta$ -arrestins. The importance of these mutations in cancer remains to be investigated. However, we demonstrate that expression of *GPR161* is prognostic, suggesting a role for *GPR161* in determining patient outcomes. Interestingly, TCGA data across multiple cancer types uncover recurrent amplifications of *GPR161* in bladder urothelial carcinoma, lung adenocarcinoma, and melanoma. It is likely that developing ways to target *GPR161* may have implications beyond breast cancer.

## Materials and Methods

**Plasmids and Antibodies.** The GPR161 cDNA was obtained from Origene and cloned into the MSCV-PIG retroviral vector. Expression vectors containing myc-IQGAP1, HA- $\beta$ Arr1, and HA- $\beta$ Arr2 were obtained from Addgene. Antibodies were obtained from the following sources: GPR161 (Abcam), E-cad (BD Biosciences), Actin (Sigma), Ki67 (Invitrogen), phospho-p70S6K (Cell Signaling), p70S6K (Cell Signaling), phospho-S6 (Cell Signaling), S6 (Cell Signaling), phospho-mTOR (Cell Signaling), mTOR (Cell Signaling), Laminin V



**Fig. 5.** GPR161 forms a protein complex with IQGAP1 and  $\beta$ Arr2. (A) Western blot analysis of IQGAP1 serine phosphorylation in MCF-10A and MDA-MB-361 cells either stably expressing an empty vector (MSCV-PIG) or GPR161. (B) Myc-IQGAP1 and GPR161-FLAG were transfected into 293T cells, followed 48 h later by Myc-IP. Western blot analysis was used to detect Myc and FLAG. (C) Myc-IQGAP1, GPR161-FLAG, HA- $\beta$ Arr1, and HA- $\beta$ Arr2 were transfected into 293T cells, followed 48 h later by HA-IP. Western blot analysis was used to detect Myc, FLAG, and HA. (D) GPR161 and IQGAP1 antibodies were conjugated to protein G-Sepharose beads, followed by incubation with MDA-MB-361 and BT-474 cell lysates. Western blot analysis was used to detect co-IP of endogenous IQGAP1,  $\beta$ Arr2, and GPR161. (E) Western blot analysis was used to detect knockdown of IQGAP1 in MDA-MB-361 cells. (F) MTT assay was used to measure cell proliferation in MDA-MB-361 cells (MSCV-PIG or GPR161) transfected with control or IQGAP1 siRNA.  $*P < 0.05$  for the difference between GPR161-expressing cells treated with control or IQGAP1 siRNA using an unpaired  $t$  test. (G) Quantification of transwell migration assay of MDA-MB-361 cells either stably expressing an empty vector (MSCV-PIG) or GPR161 and transfected with control or IQGAP1 siRNA.  $*P < 0.05$  for the difference between GPR161-expressing cells treated with control or IQGAP1 siRNA using an unpaired  $t$  test. (H) Expression of GPR161 and IQGAP1 was analyzed in 748 breast tumor samples.  $P$  values were calculated with Fisher's exact test.

(Millipore), IQGAP1 (Santa Cruz), phospho-Serine (Abcam), HA (Covance), myc (Cell Signaling), and Flag (Sigma).

**Cell Culture and Transfection.** Cells were cultured in the following medium: MCF-10A [DMEM/F12, 5% (vol/vol) horse serum, epidermal growth factor (20 ng/mL), hydrocortisone (0.5 µg/mL), cholera toxin (100 ng/mL), insulin (10 µg/mL), penicillin/streptomycin (P/S)], BT-474 [Improved Minimal Essential Medium, 10% FBS, Insulin (10 µg/mL), P/S], MDA-MB-361 [DMEM, 15% FBS, L-Glutamine (5 mL), nonessential amino acids (5 mL), P/S], BT-20 [DMEM, 15% (vol/vol) FBS, P/S], MDA-MB-157 (DMEM, 10% FBS, P/S), MDA-MB-436 (DMEM, 10% FBS, P/S), and 293T (DMEM, 10% FBS, P/S). Cells were transfected by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's suggested protocol. Retroviruses expressing MSCV-PIG and GPR161 were prepared as described (30). GPR161 and control shRNA were ordered from Thermo Scientific. The GPR161 sequences are as follows: TCTGTACAAGGTGACCAC and AGGACTTCTGTACAAGGT. MCF-10A cells were cultured in 3D as previously described (17). Control and IQGAP1 siRNA were obtained from Sigma.

**Cytosolic E-cad Assay.** Samples were treated with Con A covalently linked to Sepharose (Amersham). Confluent cells were washed with PBS and lysed in radioimmunoprecipitation assay buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) plus aprotinin, leupeptin, and sodium orthovanadate. Lysates were rotated at 4 °C for 20 min and then centrifuged for 20 min at 20,000 × g. A total of 1.25 mg of each sample was diluted in 500 µL RIPA buffer. A total of 50 µL of Con A-Sepharose was added to each tube, which then were incubated at 4 °C for 1 h with rotation. After a brief centrifugation, the supernatant was removed to a fresh microfuge tube, 25 mL of Con A-Sepharose was added, and the samples were rotated at 4 °C for 1 h. After brief centrifugation, the supernatant was removed and samples were subjected to SDS/PAGE on 10% acrylamide gels.

**Indirect Immunofluorescence.** Cells grown on glass coverslips in 12-well plates were fixed in 2% paraformaldehyde for 10 min, then washed three times in PBS:glycine. Cells were permeabilized in 0.2% Triton X-100 for 10 min at 4 °C, then washed three times with immunofluorescence wash, and blocked with 10% goat serum for 1 h. Unconjugated primary antibodies were incubated in 10% goat serum for 2 h with gentle rocking, followed by three washes. Secondary antibodies conjugated to Alexa Fluor dyes (Invitrogen) were

added for 1 h. Coverslips were then washed, incubated with DAPI for 5 min, and then mounted on glass slides with Prolong (Molecular Probes).

The 3D acinar structures grown in eight-well chamber slides were washed once in PBS, fixed in 5% formalin for 10 min, washed three times with PBS:glycine, permeabilized with 0.5% Triton X-100, washed three times with PBS:glycine, and then incubated in primary block (IF wash, 10% goat serum) for 1 h. Acini were incubated for 30 min in secondary block [IF wash, 10% goat serum, F(ab')<sub>2</sub>]. Antibodies were diluted in secondary block and incubated for 2 h, followed by three washes with IF wash. Secondary antibodies were diluted in primary block and incubated for 1 h, followed by two IF washes and incubation with DAPI. Acini were mounted with Prolong. Tissue sections were processed for immunofluorescence as previously described (31). All fluorescence images were collected using an Axiovert 200M equipped with an Apo-tome imaging system. Images were analyzed using Axiovision software.

**Proliferation Assay.** Cells were seeded in 96-well plates (5,000 cells per well) and allowed to grow for the indicated lengths of time. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) Cell Proliferation Assay (ATCC) was used to monitor cell proliferation according to the manufacturer's suggested protocol.

**Transwell Migration Assay.** Cells were seeded in 24-well plates containing 8 µm transwell migration filters, then fixed and stained with DAPI, and the migrated cells quantified.

**IP.** Cells grown in 100 mm plates were lysed in a buffer composed of 50 mM Tris-HCl, 150 mM NaCl, 10 mM NaF, 0.7% CHAPS, and protease inhibitors. We incubated a 500 µg protein with 1 µg antibody and 25 µL Protein G Sepharose beads (GE Healthcare) at 4 °C for 2 h with rotation. The beads were washed twice in lysis buffer and the bound proteins eluted with sample buffer. The samples were subjected to SDS/PAGE on 10% acrylamide gels.

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