WDR36 acts as a scaffold protein tethering a G-protein-coupled receptor, Gαq and phospholipase Cβ in a signalling complex

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Summary

We identified the WD-repeat-containing protein, WDR36, as an interacting partner of the β isoform of thromboxane A_2 receptor (TPβ) by yeast two-hybrid screening. We demonstrated that WDR36 directly interacts with the C-terminus and the first intracellular loop of TPβ by in vitro GST-pulldown assays. The interaction in a cellular context was observed by co-immunoprecipitation, which was positively affected by TPβ stimulation. TPβ–WDR36 colocalization was detected by confocal microscopy at the plasma membrane in non-stimulated HEK293 cells but the complex translocated to intracellular vesicles following receptor stimulation. Coexpression of WDR36 and its siRNA-mediated knockdown, respectively, increased and inhibited TPβ-induced Gαq signalling. Interestingly, WDR36 co-immunoprecipitated with Gαq, and promoted TPβ–Gαq interaction. WDR36 also associated with phospholipase Cβ (PLCβ) and increased the interaction between Gαq and PLCβ, but prevented sequestration of activated Gαq by GRK2. In addition, the presence of TPβ in PLCβ immunoprecipitates was augmented by expression of WDR36. Finally, disease-associated variants of WDR36 affected its ability to modulate Gαq-mediated signalling by TPβ. We report that WDR36 acts as a new scaffold protein tethering a G-protein-coupled receptor, Gαq and PLCβ in a signalling complex.

Key words: WDR36, Thromboxane A_2, GPCR, G protein, Phospholipase C

Introduction

G-protein-coupled receptors (GPCRs) are the largest family of membrane receptors responsible for mediating extracellular to intracellular signalling. They respond to a variety of stimuli, including light, hormones, neurotransmitters and odorants, resulting in various physiological responses (Bridges and Lindsley, 2008). In the case of the thromboxane A_2 receptor (TP; also known as TXA2R), a rhodopsin-like receptor, the agonist-dependent activation of the Gαq subunit of heterotrimeric G proteins leads to accumulation of inositol 1,4,5-triphosphate and diacylglycerol through the stimulation of phospholipase Cβ (PLCβ). This in turn activates Ca^{2+} release from the endoplasmic reticulum and protein kinase C (PKC) (Nakahata, 2008). Coupling of TP to $G\alpha_s$, $G\alpha_i$, $G\alpha_h$ and $G\alpha_{12/13}$ have also been reported (Hata and Breyer, 2004; Huang et al., 2004; Kinsella, 2001). In the cardiovascular system, where TP is mainly expressed, its stimulation leads to morphological changes and aggregation of platelets, and to vascular smooth muscle constriction (Narumiya et al., 1999). A variety of cardiovascular, pulmonary and kidney diseases are associated with defects in TP function (Dorn et al., 1990; Fitzgerald et al., 1990; Kinsella, 2001; Oates et al., 1988; Ogletree, 1987). TP mRNA is alternatively spliced, resulting in two isoforms (TPα, comprising 343 amino acids, and TPβ, comprising 407 amino acids), which share the first 328 amino acids (Hirata et al., 1991; Raychowdhury et al., 1994). We previously demonstrated that only TPβ, but not TPα, undergoes agonist-induced and constitutive internalization, both of which are dictated by distinct motifs in the C-terminus of the TPβ receptor (Parent et al., 1999). Looking for putative proteins implicated in the regulation of TPβ, we used the yeast two-hybrid system to screen for proteins that interact with the C-terminus of the receptor. We successfully identified WD40 repeat 36 (WDR36) as a novel interacting protein for TPβ.

Monemi et al. first associated alterations in the gene encoding WDR36 to primary open angle glaucoma (POAG), the most common subtype of glaucoma, which is the second most important cause of blindness worldwide (Monemi et al., 2005). The progression of POAG is characterized by elevated intraocular pressure and optic nerve atrophy (Kwon et al., 2009). Two other proteins are known to be associated with the formation of POAG, optineurin (OPTN) and myocilin (MYOC) and their functions are more established (Chalasani et al., 2009; Ray and Mookherjee, 2009; Tamm, 2002). Little is yet understood about the function of WDR36. High amounts of its mRNA have been detected in the heart, skeletal muscle, pancreas, liver and placenta (Monemi et al., 2005). According to a predicted protein structure, WDR36 comprises fourteen WD40 repeats, which fold into two connected seven-bladed β-propellers (Footz et al., 2009). The WD40 repeat (also known as the WD or β-transducin repeat) is a short structural motif of approximately 40 amino acids, often terminating in a tryptophan-aspartic acid (WD) dipeptide (Neer et al., 1994). The β-propeller structures found in WD40 repeat-containing proteins act as platforms for the stable or reversible association of binding

partners. This large family of proteins exhibit a wide range of cellular functions ranging from signal transduction and transcription regulation to cell cycle control and apoptosis (Li and Roberts, 2001; Paoli, 2001). By holding the receptor, Gα protein and an effector in close proximity in the plasma membrane, a scaffold protein could be the determinant for specificity and rapid onset of Gα-protein-mediated signalling. Here, we provide evidence that WDR36 acts as the first described scaffold protein that tethers Gαq in a multi-protein complex with a GPCR and PLCβ to facilitate receptor signalling.

Results

Identification of WDR36 as a TPβ-interacting protein

A yeast two-hybrid screen was performed using the TPβ C-terminus as bait on a human HeLa MATCHMAKER cDNA library. A total of 1.5×10^6 independent clones were screened, yielding over 300 positives. One hundred clones demonstrating a strong growth on selective yeast medium (Trp^-, Leu^-, His^- and Ade^-) were isolated, characterized by dideoxy sequencing and then aligned using the NCBI blast alignment search tool. Six independent clones covering the full-length WDR36 complementary DNA (cDNA) were identified in this screen. As shown in Fig. 1A, only yeast transformed with the pAS2.1TPβCT and pGAD-5C1 (a clone coding for the last 343 amino acids of WDR36) grew strongly on Trp^-, Leu^-, His^- and Ade^- medium. To confirm the interaction between TPβ and WDR36 and to determine if this interaction was direct, we performed an in vitro binding assay using the purified recombinant TPβ C-terminus (or C-tail; CT) fused to glutathione *S*-transferase (GST; GST–TPβCT) along with the purified recombinant Myc–WDR36 fused to a His tag (His_6–Myc–WDR36). We also wanted to ascertain whether WDR36 interacted with any of the three intracellular loops (ICLs) of TPβ. They were individually expressed in fusion with GST (GST–ICL) and used in the pulldown assays. The results presented in Fig. 1B illustrate that WDR36 interacted with glutathione–Sepharose-bound GST–TPβCT and to a much lesser extent with GST–ICL1, but not with glutathione–Sepharose-bound GST, GST–ICL2 or GST–ICL3. These data indicate that the interaction between TPβ and WDR36 is direct and mainly occurs through the TPβ C-terminus.

The interaction between WDR36 and TPβ in a cellular context was then assessed by co-immunoprecipitation experiments in HEK293 cells transfected with pcDNA3-myc-WDR36 and pcDNA3-FLAG-TPβ. Cell lysates were incubated with a FLAG-specific monoclonal antibody and co-immunoprecipitated WDR36 was detected by western blot analysis with an anti-Myc antibody. Our results confirm the interaction between WDR36 and TPβ in a cellular context, because WDR36 was co-immunoprecipitated with TPβ (Fig. 1C). To investigate the effect of receptor stimulation on the WDR36–TPβ interaction, we performed immunoprecipitation experiments in the presence or absence of stimulation with 500 nM U46619, a stable TP receptor agonist. The WDR36–TPβ interaction was significantly increased after 10 minutes of receptor stimulation and remained relatively stable afterwards (Fig. 1D). Compilation of densitometry analyses performed on three independent assays is shown in Fig. 1E. Incubation of TPβ-expressing cells with the TP antagonist SQ29548 did not modulate the TPβ–WDR36 co-immunoprecipitation (supplementary material Fig. S1A). Taken together, our results suggest that the direct interaction between TPβ and WDR36 occurs with the receptor in a 'naïve state' and is increased by agonist stimulation.

Cellular localization of WDR36

We performed confocal microscopy using HEK293 cells to visualize endogenous expression of WDR36, as detailed in the Materials and Methods. A peptide corresponding to the epitope that was used to generate the WDR36 antibody was fused to GST and purified on glutathione–Sepharose beads. The WDR36 antibody was pre-incubated for 3 hour with purified GST or GST–epitope peptide fusion before incubation with the cells. Fig. 2Aa shows that immunofluorescence of the WDR36 antibody was detected at the plasma membrane and on intracellular puncta when the antibody was incubated with GST. As seen in Fig. 2Ac, the plasma membrane staining was lost when the GST–epitope peptide fusion was pre-incubated with the WDR36 antibody, suggesting that the intracellular labelling was non-specific. This indicates that endogenous WDR36 is located at the plasma membrane in HEK293 cells. We then investigated whether this localization of WDR36 could be influenced by the expression and stimulation of TPβ. We used HEK293 cells transiently expressing TPβ–GFP treated or not with 500 nM U46619. The cells were incubated with the WDR36 antibody and then subjected to confocal microscopy (Fig. 2B). Without stimulation (Fig. 2Ba–d), TPβ and WDR36 colocalized at the plasma membrane. Receptor stimulation for 10, 30 and 120 minutes resulted in translocation of WDR36 from the plasma membrane to the intracellular compartments where it colocalized with TPβ. Although the analysis of WDR36 translocation to intracellular compartments is complicated by the presence of non-specific labelling, disappearance of WDR36 from the plasma membrane as well as its concentration and colocalization with TPβ could be clearly seen in a perinuclear compartment after 120 minutes of receptor stimulation.

WDR36 promotes TPβ signalling

To identify a function for the interaction between WDR36 and TPβ, we first investigated the effect of WDR36 on TPβ signalling. Total inositol phosphate production was measured in HEK293 cells transiently expressing HA–TPβ co-transfected with empty pcDNA3 vector (100% control) or pcDNA3-WDR36 that were stimulated with 500 nM U46619 for 30 minutes. Fig. 3A shows that WDR36 expression resulted in a dramatic increase in TPβ agonist-induced inositol phosphate production. The role of WDR36 was then confirmed by inhibiting endogenous WDR36 expression with small interfering RNAs (siRNAs). Efficacy of the siRNAs was verified on *WDR36* mRNA by semi-quantitative RT-PCR using GAPDH as a control (Fig. 3B), and on endogenous WDR36 protein by western blotting using actin as a control (Fig. 3C). Inhibition at the protein level was also assessed by confocal microscopy using the WDR36 antibody, and Hoechst staining of the nucleus (Fig. 3D). Both *WDR36* siRNAs used caused a significant inhibition of WDR36 mRNA and protein expression compared with the control siRNA. However, only the plasma-membrane-associated WDR36 immunofluorescence was decreased by both siRNAs, supporting our finding in Fig. 2A that WDR36-specific staining is found at the plasma membrane and not intracellularly. Fig. 3E shows the agonist-induced inositol phosphate production in HEK293 cells stably expressing TPβ and transfected with *WDR36* or control siRNAs. Inhibition of WDR36 expression resulted in a 40% decrease in TPβ-mediated inositol phosphate production following agonist treatment compared with the control siRNA. These data corroborate the results shown in

Fig. 3A, in demonstrating that WDR36 is positively involved in TPβ signalling. Radioligand binding assays confirmed that the increase of inositol phosphate production in the presence of WDR36 was not due to an augmentation of TPβ at the plasma membrane (data not shown). We next tested whether WDR36 also regulated agonist-induced endocytosis of TPβ. HEK293 cells stably expressing HA-tagged TPβ were transfected with negative control or *WDR36* siRNAs and subjected to a time course of 500

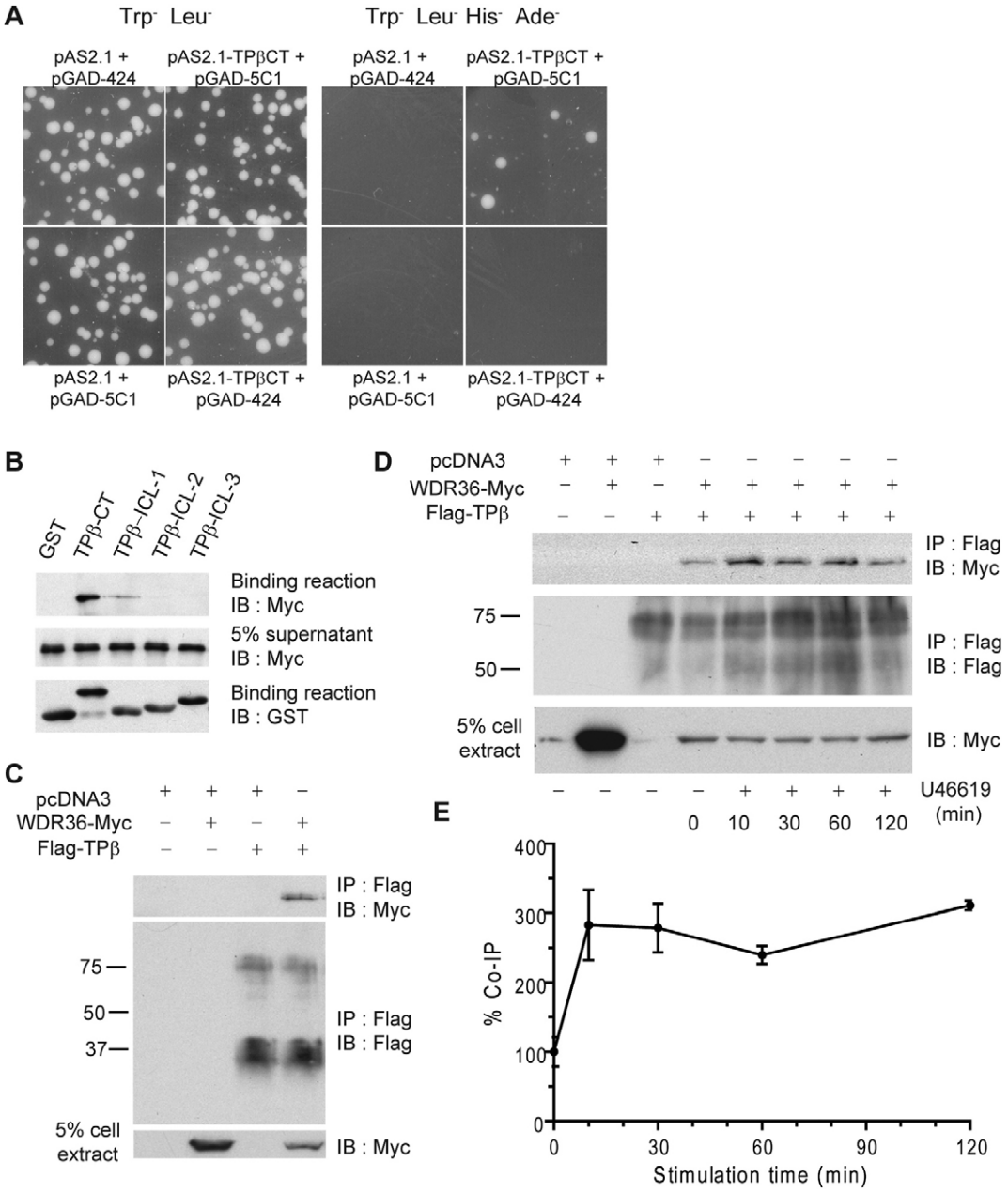


Fig. 1. WDR36 interacts with TPβ. (**A**) A yeast two-hybrid screen was performed using the TPβCT as bait on a human HeLa MATCHMAKER cDNA library. The interaction between a clone (5C1) coding for the last 343 amino acids of WDR36 and the TPβCT was confirmed by the use of the selective yeast medium Trp^-, Leu^-, His^- and Ade^-. (**B**) WDR36 interacts with the C-terminus and ICL1 (intracellular loop 1) of TPβ. The binding assays were carried out using purified glutathione–Sepharose-bound GST–TPβCT and GST–ICLs, which were incubated with purified recombinant His_6–Myc–WDR36. The binding of WDR36 was detected by immunoblotting (IB) using a HRP-conjugated Myc-specific antibody, and the GST fusion proteins present in the binding reaction were detected using a GST-specific polyclonal antibody. (**C**) WDR36 co-immunoprecipitates with TPβ in HEK293 cells. Lysates of cells that were transiently expressing WDR36–Myc and FLAG–TPβ were immunoprecipitated with a FLAG-specific monoclonal antibody, and immunoblotting was performed with FLAG-specific polyclonal or Myc-specific HRP antibodies. A blot representative of three independent experiments is shown. (**D**) The interaction of WDR36 with TPβ is modulated by receptor agonist stimulation. HEK293 cells were transfected with the indicated constructs and stimulated with 500 nM U46619, or left unstimulated, for 0–120 minutes. Immunoprecipitations and western blotting was performed as described for C. A blot representative of three independent experiments is shown. (**E**) Densitometry analysis of three independent experiments such as that shown in D was performed using ImageJ software. Values were expressed as a percentage of co-immunoprecipitation compared with the non-stimulated condition, which was set at 100%.

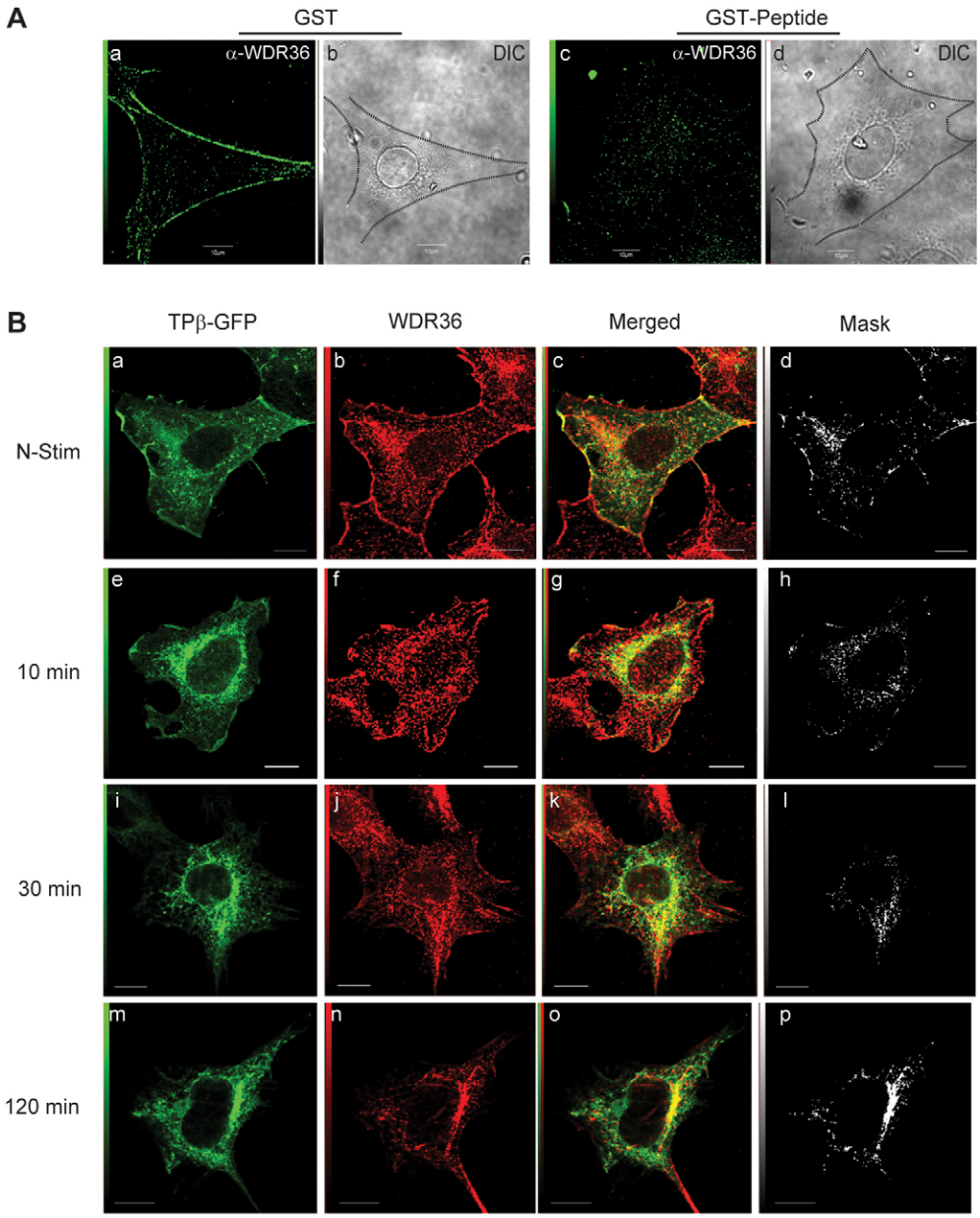


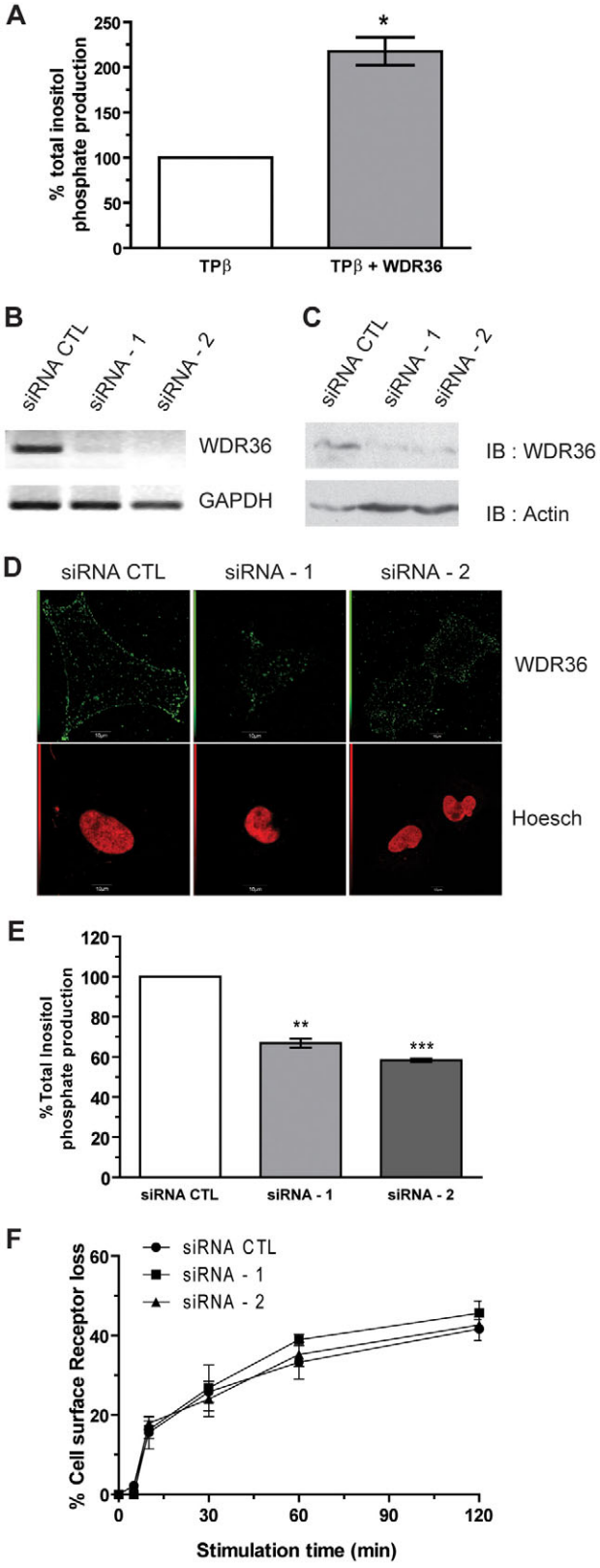
Fig. 2. Cellular localization of WDR36 is affected by TPβ stimulation. (**A**) Endogenous WDR36 is localized at the plasma in HEK293 cells. Confocal microscopy was performed on HEK293 cells fixed, permeabilized and labelled with mouse IgG2a anti-WDR36 and Alexa-Fluor 488-conjugated anti-mouse-IgG antibodies (a,c). A peptide corresponding to the epitope that was used to generate the WDR36 antibody was fused to GST and purified on glutathione–Sepharose beads. The WDR36 antibody was pre-incubated for 3 hours with purified GST (a,b) or the GST–epitope peptide fusion (c,d) before incubation with the cells. Cells are shown in differential interference contrast (DIC) with the plasma membrane outlined by dashes (b,d). (**B**) WDR36 translocates from the plasma membrane to intracellular compartments upon TPβ stimulation. HEK293 cells transiently expressing TPβ–GFP were treated with 500 nM U46619, or left untreated, for 10–120 minutes. The cells were then processed for confocal microscopy as above for visualization of endogenous WDR36. Colocalization is in yellow in the 'Merged' panels; the yellow pixels only were extracted and are shown in the 'Mask' panels. Scale bars: 10 μm.

nM U46619 stimulation. The percentage of cell-surface receptor loss was assessed by ELISA, as described previously (Parent et al., 1999). Inhibition of *WDR36* expression by siRNA did not change the rate or the amount of TPβ internalized by agonist induction (Fig. 3F). These results show that WDR36 enhances the production of inositol phosphates by TPβ, but does not affect its surface expression or its agonist-induced endocytosis.

A direct interaction with the receptor is involved in the regulation of TPβ signalling by WDR36

We then wanted to further characterize the site of WDR36 interaction on TPβ, and assess whether this interaction was necessary to promote TPβ-mediated Gαq signalling. We focused on the TPβ C-terminus because it appeared to be the major WDR36 binding domain (Fig. 1A). Truncated mutants of the TPβ C-terminus were produced as recombinant GST fusion proteins (Fig. 4A). The interaction between these mutants and WDR36 was then tested by GST pulldown assays on purified His_6–Myc–WDR36 and analyzed by western blot as shown in Fig. 4B. WDR36 bound to the full-length TPβ C-terminus, and negligibly to GST–TPβ-CT312–328. The binding of GST–TPβ-CT329–407 and GST–TPβ-CT334–407 constructs to WDR36 was similar to that of the full-length TPβ C-terminus. Significant loss in WDR36 binding was observed with the GST–TPβ-CT345–407 construct suggesting that the major WDR36 interaction site on the TPβ C-terminus is located between amino acids 334 and 345. We thus determined if WDR36 was able to modulate Gαq signalling of the TPβ-R328Stop mutant. This mutant was generated in a previous study and shown to have normal ligand binding properties (Parent et al., 1999). Wild-type TPβ and TPβ-R328Stop were individually transfected with WDR36–Myc or empty pcDNA3 vector into HEK293 cells and total inositol phosphate production was

measured after stimulating the cells for 30 minutes with 500 nM U46619. As expected, WDR36 significantly promoted Gαq signalling of wild-type TPβ but did not modulate TPβ-R328Stop signalling (Fig. 4C). These results indicate that the interaction between TPβ and WDR36 is involved in regulating Gαq signalling by the receptor.



WDR36 associates with Gαq

WDR36 has been proposed to contain 14 WD40 domains assembled in two seven-bladed propellers (Footz et al., 2009) and, as for the majority of the proteins in this family, is expected to form multi-protein complexes. Because production of inositol phosphates by TPβ occurs by the activation of Gαq, we conjectured that WDR36 could also interact with Gαq to promote TPβ signalling. Co-immunoprecipitation experiments were performed in HEK293 cells co-transfected with the indicated combinations of WDR36–Myc, Gαq and empty pcDNA3 vector (Fig. 5A). Cell lysates were incubated with a Myc-specific polyclonal antibody. Western blot analysis of the immunoprecipitation reactions with a Gαq-specific monoclonal antibody revealed that Gαq co-immunoprecipitated with WDR36, which confirmed an interaction between the two proteins in a cellular context. Colocalization of endogenous WDR36 and EE hexa-peptide (EYMPME)-tagged Gαq was detected at the plasma membrane by confocal microscopy using WDR36 monoclonal and EE polyclonal antibodies in HEK293 cells (Fig. 5B). We then hypothesized that the WDR36-Gαq interaction could promote the association between TPβ and Gαq and facilitate TPβ signalling. Consequently, we performed co-immunoprecipitations in HEK293 cells co-transfected with the indicated combinations of FLAG–TPβ, Gαq, WDR36–Myc or empty pcDNA3 vector (Fig. 5C). Cell lysates were incubated with a FLAG-specific monoclonal antibody and co-immunoprecipitated Gαq was detected by a Gαq-specific monoclonal antibody. As seen in Fig. 5C, the amount of Gαq that immunoprecipitated with TPβ was greater when WDR36 was coexpressed. These results indicate that WDR36 could positively influence TPβ signalling by interacting with Gαq and promoting its interaction with the receptor.

We then assessed the effect of receptor stimulation in the formation of the TPβ, WDR36 and Gαq complex. Lysates of HEK293 cells expressing FLAG–TPβ, WDR36–Myc and Gαq that were stimulated with 500 nM U46619 for 0–30 minutes were immunoprecipitated with a Myc-specific polyclonal antibody.

Fig. 3. WDR36 regulates TPβ signalling. (**A**) HEK293 cells were transiently transfected with pcDNA3-HA-TPβ receptor and either pcDNA3 or pcDNA3-WDR36-myc. Cells were metabolically labelled with *myo*-[^{3}H]inositol and then stimulated with 500 nM U46619 for 30 minutes. Total [^{3}H]inositol phosphates were isolated as described in the Materials and Methods, measured by liquid scintillation counting, and expressed as a percentage of the TPβ only response. Values are means ± s.e.m. of three independent experiments. (**B**–**D**) HEK293 cells transfected with negative control (CTL) or *WDR36* siRNAs were examined for *WDR36* mRNA, using semi-quantitative RT-PCR (B), for protein expression using western blotting with the anti-WDR36 antibody (C), and by confocal microscopy (D) as described in the Materials and Methods. (**E**) HEK293 cells stably expressing the HA–TPβ were transfected with negative control or WDR36 siRNAs. Inositol phosphate production was measured after 5 minutes of stimulation with 500 nM U46619. (**F**) HEK293 cells stably expressing HA–TPβ were transfected with negative control and *WDR36* siRNAs and subject to an ELISA time-course analysis of the percentage loss of cell surface receptor following receptor stimulation with 500 nM U46619. All values are the means ± s.e.m. from three separate experiments; *P<0.05, **P<0.01, ***P<0.001. IB, immunoblotting.

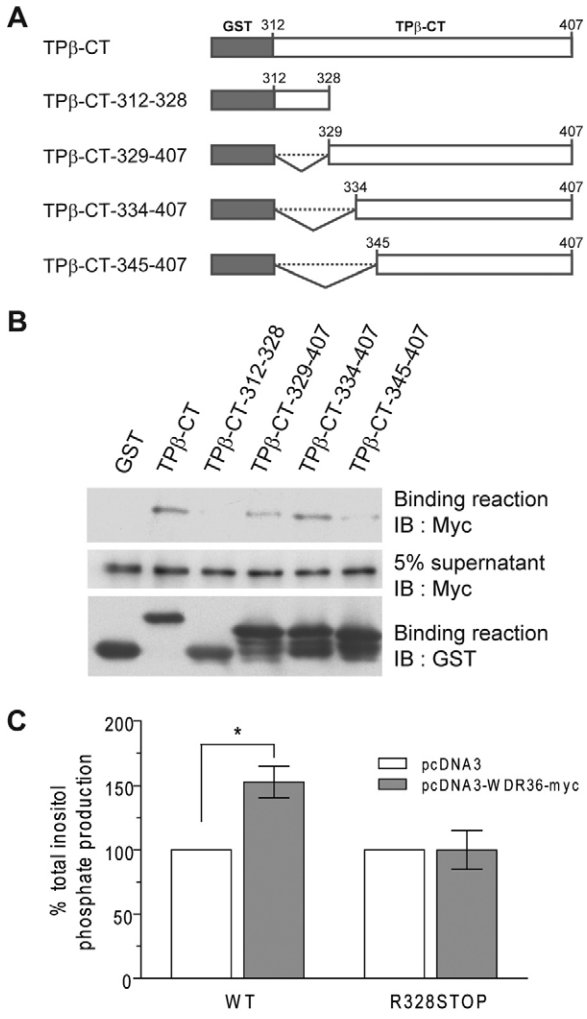


Fig. 4. WDR36 needs to directly interact with TPβ to regulate its signalling. (**A**) Schematic representation of the TPβ C-terminus deletion mutants fused to GST used in the His_6–WDR36–Myc pulldown assays to characterize the site of WDR36 binding on the TPβ C-terminus. (**B**) Pulldown assays were carried out using purified glutathione–Sepharose-bound GST fusion constructs of the full-length TPβCT or deletion mutants on purified recombinant His_6–Myc–WDR36 protein. The binding of WDR36 was detected by immunoblotting (IB) using a Myc-specific polyclonal antibody, and the GST fusion proteins present in the binding reactions were detected using a GST-specific polyclonal antibody. (**C**) HEK293 cells transiently co-transfected with the indicated combinations of empty pcDNA3 vector, pcDNA3-HA-TPβ, pcDNA3-HA-TPβR328Stop and pcDNA3-WDR36-myc were stimulated with 500 nM U46619 for 30 minutes. Total inositol phosphate production was measured as described in the Materials and Methods. Values are the means ± s.e.m. of three independent experiments; $*P<0.05$.

Western blot analysis showed that co-immunoprecipitation of Gαq with WDR36 was increased by approximately twofold after receptor stimulation with U46619, and remained relatively constant over time (Fig. 5D). The Gαq–WDR36 co-immunoprecipitation was not regulated by treating TPβ-expressing cells with the antagonist SQ29548 (supplementary material Fig. S1B). The prolonged WDR36–TPβ interaction after receptor stimulation concurs with the co-immunoprecipitation results from Fig. 1C and with the immunofluorescence data from Fig. 2B that respectively showed interaction and colocalization between the two proteins even after stimulating the receptor for 120 minutes. Together, our data indicate that WDR36 forms a complex with TPβ and Gαq at the plasma membrane in basal conditions and that the formation of this complex can be promoted by receptor stimulation, positively contributing to TPβ-mediated Gαq signalling.

WDR36 modulates the interaction of Gαq with GRK2 and PLCβ2

G-protein coupled receptor kinase 2 (GRK2; also known as β-adrenergic receptor kinase 1) has been shown to interact with the transition and active states of Gαq, which can effectively inhibit Gαq-mediated activation of PLCβ by sequestration of Gαq (Carman et al., 1999). We coexpressed a constitutively active mutant of Gαq (Gαq-Q209L) and GRK2, with or without WDR36–Myc in HEK293 cells and performed immunoprecipitations with a GRK2 polyclonal antibody. It can be seen in Fig. 6A that activated Gαq interacted with GRK2, as reported before (Carman et al., 1999). Interestingly, the amount of activated Gαq that co-immunoprecipitated with GRK2 was reduced when WDR36 was present. This suggests that WDR36 may compete against GRK2 for the interaction with activated Gαq.

We then investigated whether WDR36 was in a complex with PLCβ. HEK293 cells were transfected with empty pcDNA3 vector or pcDNA3-WDR36-myc. Cell lysates were incubated with a PLCβ2 polyclonal antibody and the immunoprecipitates analyzed by western blotting using a PLCβ2 and Myc antibodies. Interestingly, WDR36 co-immunoprecipitated with PLCβ2 (Fig. 6B). The possibility that TPβ was associated with PLCβ and that WDR36 increased this interaction was then explored. Fig. 6C shows that TPβ was indeed co-immunoprecipitated with PLCβ2 from HEK293 cells expressing FLAG–TPβ and that this interaction was promoted by the expression of WDR36. The amount of Gαq that co-immunoprecipitated with PLCβ2 was increased after 10 minutes TPβ stimulation and remained stable for the duration of the experiment in the absence of WDR36 (Fig. 6D). The Gαq–PLCβ2 co-immunoprecipitation was enhanced by the expression of WDR36 in basal conditions but, similar to the WDR36–PLCβ2 interaction, was not regulated by agonist stimulation of the receptor (Fig. 6D).

Together, these results indicate that WDR36 can promote TPβ-mediated Gαq signalling in a number of ways. It can prevent the sequestration of activated Gαq by GRK2. It can also support the interactions between TPβ and Gαq, Gαq and PLCβ, as well as TPβ and PLCβ.

Specificity of WDR36 function

We wanted to know whether the effect of WDR36 on TPβ-mediated Gαq signalling could be extended to two other Gαq-coupled receptor, TPα and the angiotensin type 1 receptor (AT1R). Total inositol phosphates were measured in HEK293 cells expressing HA–TPα or FLAG–AT1R alone or in combination with WDR36–Myc that were stimulated with agonists for 30 minutes. In contrast to TPβ, agonist-induced TPα- and AT1R-mediated Gαq signalling was not regulated by the expression of WDR36 (supplementary material Fig. S2). TPβ also signals through Gαs to induce cAMP production in our cell model. WDR36 failed to modulate cAMP production following TPβ stimulation. In agreement with this result, Gαs was not co-immunoprecipitated with WDR36 (data not shown) and there was no competition between Gαs and WDR36 for TPβ binding

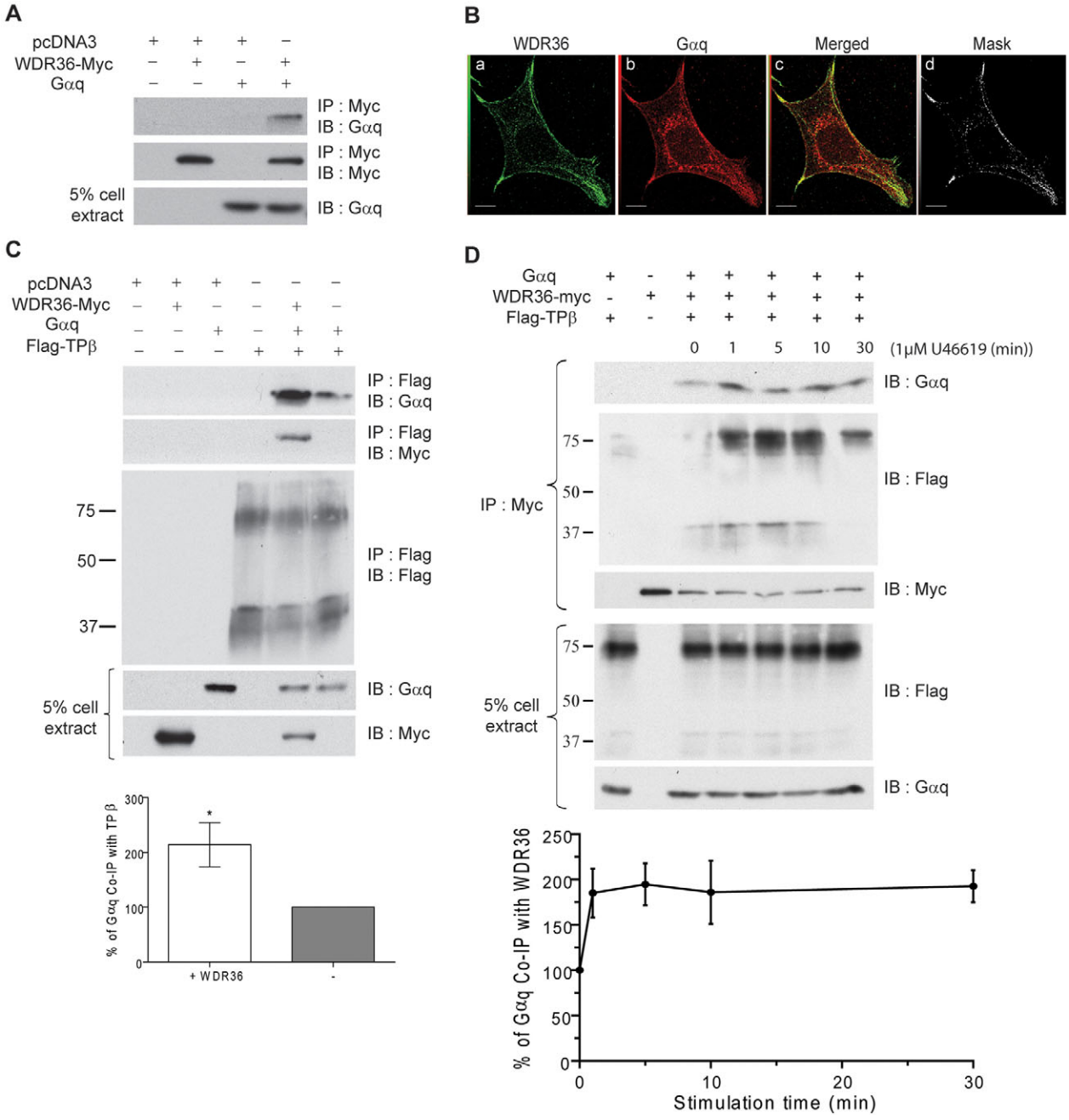


Fig. 5. WDR36 associates with Gαq and promotes the TPβ–Gαq interaction. (**A**) HEK293 cells were transiently transfected with the indicated combinations of empty pcDNA3 vector, pcDNA3-WDR36-myc and pcDNA3–Gαq. Immunoprecipitations (IP) of WDR36 were performed using a Myc-specific polyclonal antibody and immunoblotting (IB) was performed with HRP-conjugated Myc-specific or Gαq-specific monoclonal antibodies. (**B**) HEK293 cells transfected with pcDNA3-EE-Gαq were processed for confocal microscopy with mouse IgG2a, anti-WDR36 and Alexa-Fluor-488-conjugated anti-mouse IgG, and rabbit anti-EE and Alexa Fluor 546-conjugated anti-rabbit IgA antibodies as described in the Materials and Methods. The 'Merged' and 'Mask' images reveal predominant colocalization of Gαq and WDR36 at the plasma membrane. (**C**) HEK293 cells were transiently transfected with the indicated combinations of empty pcDNA3 vector, pcDNA3–FLAG–TPβ, pcDNA3-WDR36-myc and pcDNA3–Gαq. Immunoprecipitations of the receptor were performed with a FLAG-specific monoclonal antibody and immunoblotting was performed using FLAG-, Gαq- and HRP-conjugated Myc-specific antibodies. Densitometry analyses of three independent experiments were performed with the ImageJ software and values were expressed as a percentage of Gαq co-immunoprecipitation with TPβ in presence of WDR36 compared with that in absence of WDR36, which was set at 100%. (**D**) HEK293 cells were transiently transfected with the indicated combinations of pcDNA3–FLAG–TPβ, pcDNA3–WDR36–Myc and pcDNA3-Gαq. Cells were stimulated for 0–30 minutes with 500 nM U46619. Densitometry analysis of three independent experiments were performed with the ImageJ software and values were expressed as a percentage of Gαq co-immunoprecipitation with WDR36–Myc after receptor stimulation compared with the non-stimulated condition, which was set at 100%. Immunoprecipitations of the WDR36–Myc were performed with a Myc-specific antibody and immunoblotting was performed using FLAG-, Gαq- and HRP-conjugated Myc-specific antibodies. Blots shown are representative of three independent experiments.

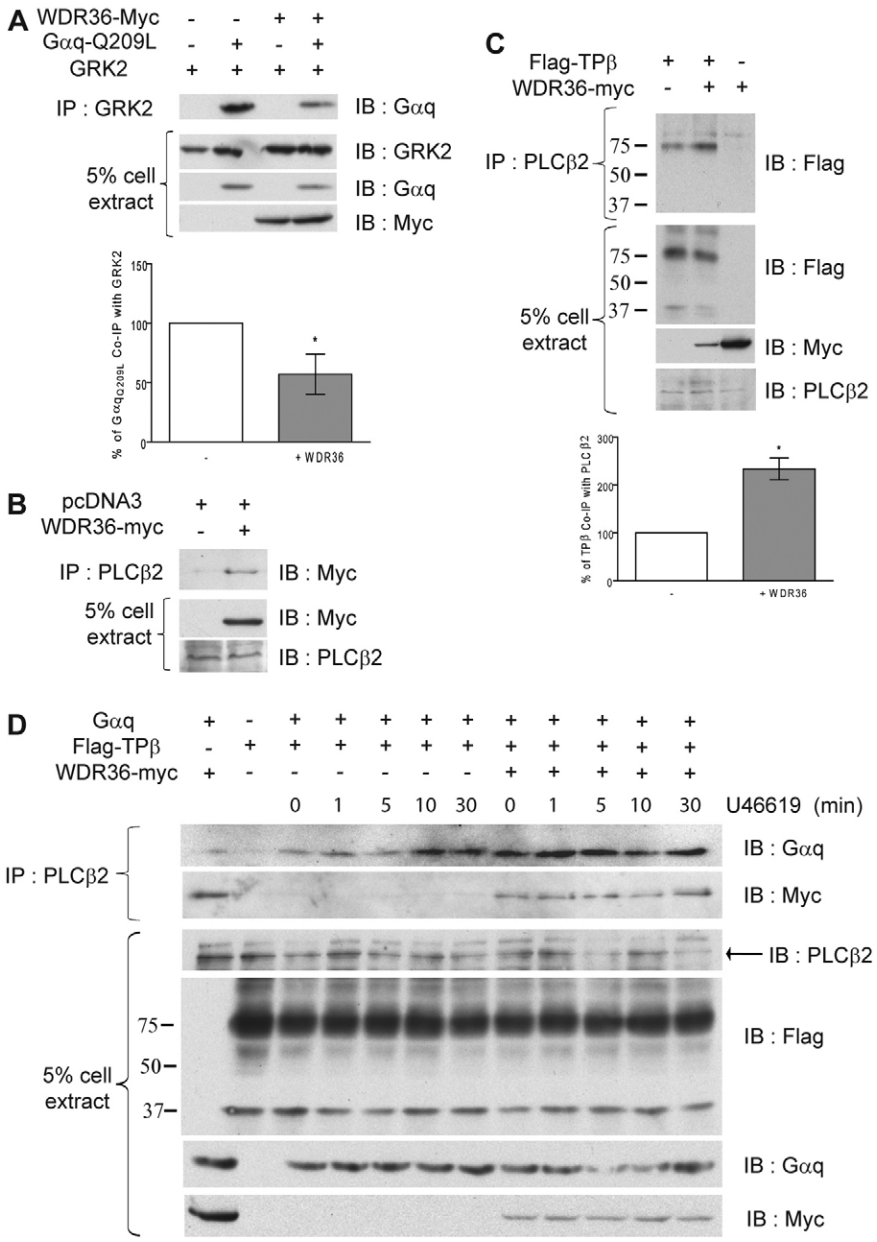


Fig. 6. WDR36 competes with GRK2 for binding to Gαq and promotes the interaction between Gαq and PLCβ2. (**A**) HEK293 cells were transiently co-transfected with the indicated combinations of pcDNA3-WDR36-Myc, -GRK2 and -Gαq-Q209L constructs. Immunoprecipitations (IP) of GRK2 were performed using a GRK2-specific polyclonal antibody and immunoblotting (IB) was performed with HRP-conjugated Myc-, Gαq- and GRK2-specific antibodies. Densitometry analyses of three independent experiments were performed with the ImageJ software and values were expressed as a percentage of Gαq-Q209L co-immunoprecipitation with GRK2 in presence of WDR36 compared with that in absence of WDR36, which was set at 100%. (**B**) HEK293 cells were transiently transfected with empty pcDNA3 vector or pcDNA3-WDR36-myc. Immunoprecipitations were performed with a PLCβ2-specific polyclonal antibody and immunoblotting was performed with HRP-conjugated Myc- and PLCβ2-specific antibodies. (**C**) HEK293 cells were transiently transfected with the indicated combinations of pcDNA3-FLAG-TPβ and pcDNA3-WDR36-myc. Immunoprecipitations (IP) were performed with a PLCβ2-specific polyclonal antibody and immunoblotting (IB) was performed with HRP-conjugated Myc-, FLAG- and PLCβ2-specific antibodies. Densitometry analyses of three independent experiments were performed with ImageJ software and values were expressed as a percentage of TPβ co-immunoprecipitation with PLCβ2 in presence of WDR36 compared with that in absence of WDR36, which was set at 100%. (**D**) HEK293 cells were transiently co-transfected with the indicated combinations of pcDNA3–FLAG–TPβ, –Gαq and pcDNA3–WDR36–Myc. The cells were subjected to stimulation for 0–30 minutes with 500 nM U46619. Immunoprecipitations were performed with a PLCβ2-specific polyclonal antibody and immunoblotting was performed with FLAG- or HRP-conjugated Myc-, Gαq- and PLCβ2-specific antibodies.

(supplementary material Fig. S3). Altogether, these data suggest that among the receptors and Gα proteins tested in the present study, WDR36 displays specificity towards TPβ and its associated Gαq signalling.

We sought to further characterize the role of WDR36 in the regulation of TPβ-mediated signalling. We compared ERK1/2 activation at various times during stimulation with TPβ in the absence or presence of WDR36. Expression of WDR36 strongly promoted TPβ-mediated ERK1/2 activation (Fig. 7A). The regulator of G-protein signalling (RGS) domain of GRK2 was then expressed with TPβ to sequester activated Gαq (Carman et al., 1999) and to assess whether TPβ-mediated ERK1/2 activation was dependent on Gαq signalling in our system. Coexpression of the RGS domain of GRK2 significantly inhibited ERK1/2 activation following TPβ stimulation (Fig. 7B). Mitogen-activated protein kinase (MAPK) activation by a number of GPCRs has been shown to be dependent on the internalization of the receptor (Sorkin and von Zastrow, 2009). We previously reported that the agonist-induced internalization of TPβ was inhibited by ~70% when a dominant-negative mutant of dynamin, Dyn-K44A, was coexpressed (Parent et al., 1999). As seen in Fig. 7C, co-transfection Dyn-K44A inhibited ERK1/2 activation following TPβ stimulation by ~50%. These data indicate that WDR36 also promotes a TPβ signalling pathway that is dependent on receptor internalization.

WDR36 variants differentially regulate TPβ signalling

Several WDR36 variants have been proposed to be associated with a polygenic form of primary open-angle glaucoma (Hauser et al., 2006; Monemi et al., 2005). We chose to test three possible disease-causing variants (N355S, R529Q and I604V) and three potential disease-susceptibility variants (L25P, D658G and M671V) (Footz et al., 2009). These variants were also selected because the corresponding residues appear accessible for protein

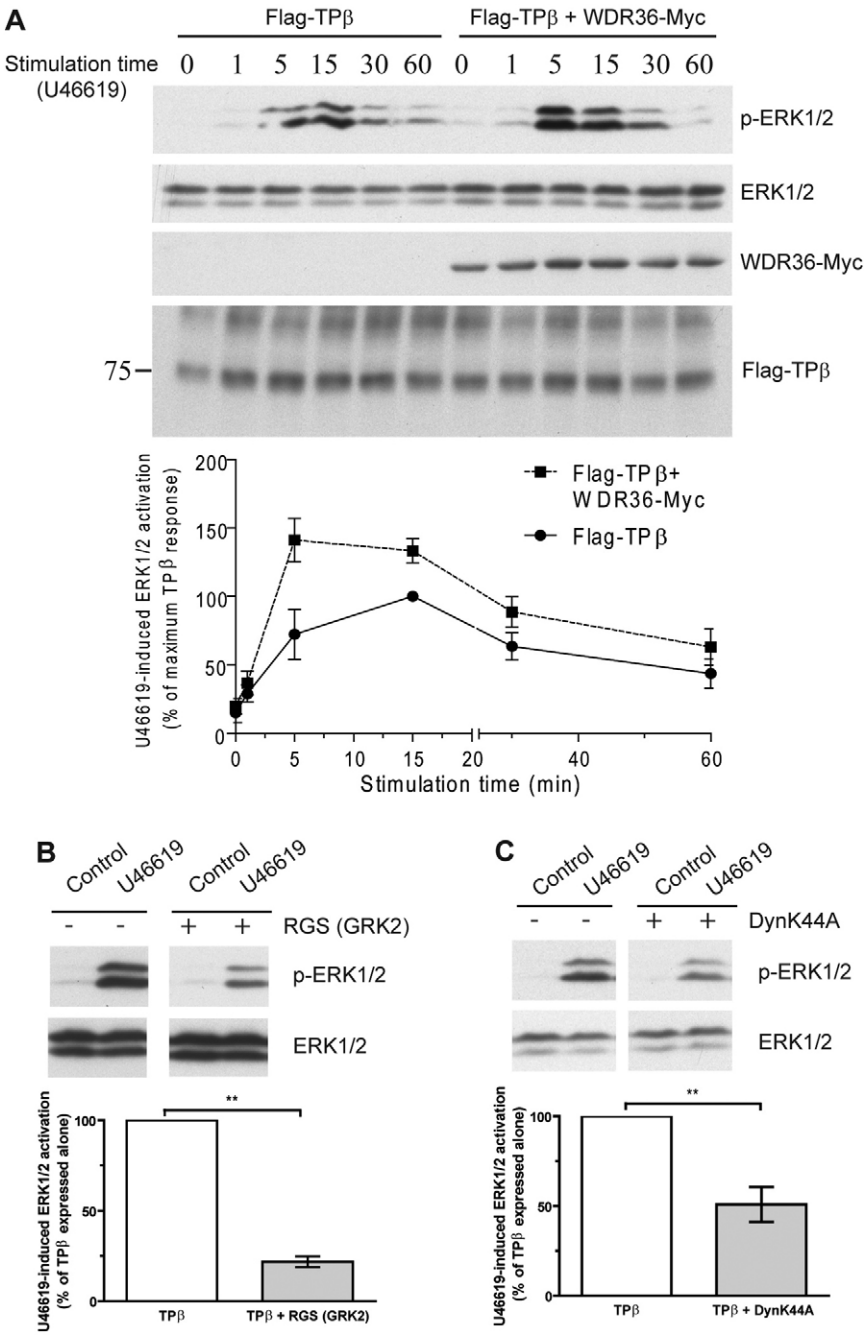


Fig. 7. WDR36 promotes TPβ-mediated ERK1/2 activation. (**A**) HEK293 cells transiently expressing TPβ in the absence or presence of WDR36 were stimulated with 1 μM U46619 for the indicated times. Western blots of a representative experiment are shown. Densitometry analysis of three independent experiments was performed with ImageJ software, and values were expressed as a percentage of the maximal response measured for TPβ expressed alone, which was set at 100%. (**B**,**C**) HEK293 cells transiently expressing TPβ alone or in combination with the RGS domain of GRK2 (B) or the dominant negative mutant of dynamin DynK44A (C) were stimulated with 1 μM U46619 for 5 minutes and samples analyzed as described in the Materials and Methods. Western blots of a representative experiment are shown. Densitometry analysis of three independent experiments was performed with ImageJ software and values were expressed as the percentage of the response measured for TPβ expressed alone, which was set at 100%. p-ERK1/2, phosphorylated ERK1/2.

interactions according to the predicted model of WDR36 (Footz et al., 2009). We were interested in comparing the ability of these WDR36 variants to modulate TPβ-mediated Gαq signalling to that of wild-type WDR36. HEK293 cells were transiently transfected with HA–TPβ in combination with pcDNA3, wild-type WDR36, or individual WDR36 variants. Total inositol phosphates were measured after stimulating the cells with 500 nM U46619 for 30 minutes. Results are presented in Fig. 8 as the ability of the WDR36 variants to promote TPβ-mediated signalling compared with that of wild-type WDR36 (100%). The L25P, N355S, D658G, M671V, and particularly the I604V variants, were all significantly more potent in promoting TPβ-mediated Gαq signalling than wild-type WDR36. The WDR36 R529Q variant, however, significantly decreased the promotion of total inositol phosphate production by TPβ compared with the wild-type protein.

Discussion

Fine-tuning of GPCR function can be attributed in many cases to receptor-interacting proteins that are differentially expressed in distinct cell types (Ritter and Hall, 2009). Some GPCR-interacting proteins augment the rapidity and efficiency of receptor signalling by acting as scaffolds to assemble downstream effectors in close proximity to the receptor (Ritter and Hall, 2009). Accumulating evidence shows that GPCRs exist as preformed complexes with downstream effectors (Dupre et al.,

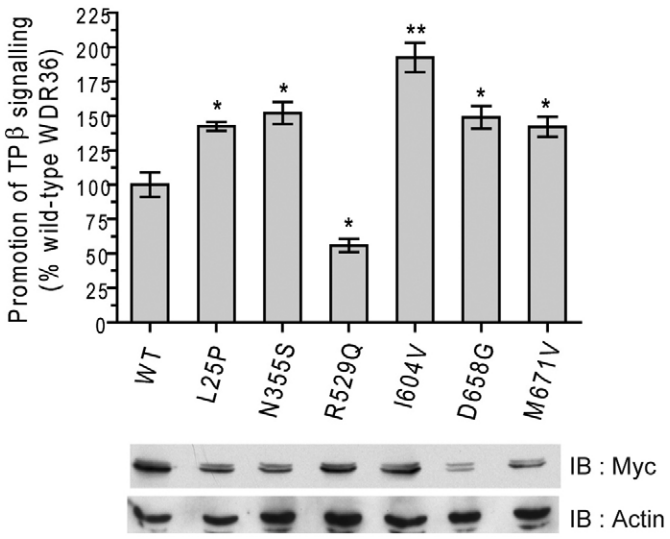


Fig. 8. WDR36 variants differentially regulate TPβ signalling. HEK293 cells were transiently co-transfected with HA–TPβ and pcDNA3, pcDNA3-WDR36–Myc or the indicated Myc-tagged WDR36 variants cloned into pcDNA3. Cells were metabolically labelled with *myo*-[^{3}H]inositol and stimulated with 500 nM U46619 for 30 minutes. Total [^{3}H]inositol phosphates were measured as described in the Materials and Methods. Results are presented as the ability of the WDR36 variants to promote TPβ-mediated signalling compared with that of wild-type WDR36 (100%). A representative western blot showing the expression levels of wild-type WDR36 and its variants in lysates from cells used for the signalling assays can be seen (IB: Myc) with actin as a loading control (IB: actin). Values are the means ± s.e.m. of three independent experiments.*$P<0.05$; **$P<0.01$.

2006; Marrari et al., 2007; Takida and Wedegaertner, 2004). However, what determines the proximity or the precoupling of GPCRs to their interacting proteins and the specificity of this process is still poorly understood. In the present study, we demonstrated that the WD40 repeat-containing protein WDR36 acts as a scaffold protein tethering a GPCR, Gαq and PLCβ in a signalling complex.

The large family of WD40 repeat-containing proteins can be engaged in a broad range of cellular events, such as signal transduction (Parent et al., 2008; Smith et al., 1999), RNA processing complexes (Mitsuzawa et al., 2001), transcriptional regulation (Pickles et al., 2002), cytoskeleton assembly and mitotic spindle formation (Welch et al., 1997), vesicle formation and trafficking (Fath et al., 2007), cell division control (Yu, 2007) and programmed cell death (Benedict et al., 2000). The basic common function of WD40 repeat-containing proteins is to coordinate multi-protein complex assembly, where the repeating units serve as a scaffold for protein interactions. The proposed β-propeller structure of these proteins enables them to create a stable platform for the simultaneous tethering of different sets of proteins (Li and Roberts, 2001).

The direct interaction between TPβ and WDR36 was shown here by two independent methods: yeast two-hybrid screening and pulldown assays with purified recombinant proteins. The TPβ C-terminus was characterized as the major WDR36 binding domain, whereas the TPβ ICL1 seemed to be involved to a lesser extent. This is similar to what we reported for RACK1 (Parent et al., 2008), another WD40 repeat-containing protein. Involvement of these two receptor domains is plausible when taking into account the three-dimensional structure of a GPCR (Cherezov et al., 2007; Rasmussen et al., 2007; Rosenbaum et al., 2007). We also demonstrated by co-immunoprecipitation experiments that WDR36 and TPβ constitutively interacted in a cellular context. This interaction was positively modulated by agonist stimulation of the receptor for 1 minute, the earliest time tested, to then remain relatively stable over time. Receptor occupancy by the antagonist SQ29548 did not increase the TPβ–WDR36 interaction. This suggests that the agonist properties of U46619 are required to recruit WDR36 to the receptor or to stabilize pre-existing TPβ–WDR36 complexes. We detected immunofluorescence specific to endogenous WDR36 at the plasma membrane in HEK293 cells where it colocalized with TPβ in basal conditions. WDR36 translocated with TPβ in a perinuclear intracellular compartment after prolonged agonist stimulation, most probably in the Rab11-positive perinuclear recycling endosome where we have previously showed TPβ to accumulate (Hamelin et al., 2005; Theriault et al., 2004).

The molecular function and the interaction partners of WDR36 have not yet been described. We showed that TPβ-mediated Gαq signalling was increased when WDR36 was overexpressed and inhibited by siRNA knockown of endogenous *WDR36* expression. Further characterization of the WDR36 binding site on the TPβ C-terminus allowed us to demonstrate that Gαq signalling of the TPβ-R328Stop mutant could no longer be regulated by WDR36. This indicated that a direct TPβ–WDR36 interaction was involved in the WDR36 functional effect on receptor signalling. The role of WDR36 in TPβ-mediated Gαq signalling can be explained by the ability of WDR36 to interact with TPβ, Gαq and PLCβ and to act as a scaffold for a preformed molecular signalling complex before agonist treatment, as evidenced by co-immunoprecipitation experiments in the absence of U46619. This would presumably improve the speed and efficiency of PLCβ activation upon TPβ activation. Our results also indicated that WDR36 can positively contribute to Gαq-mediated activation of PLCβ by competing with GRK2 for binding to activated Gαq. Indeed, GRK2 was previously reported to preferentially bind to the transition and active states of Gαq and to sequester it from other effectors (Carman et al., 1999; Day et al., 2004; Sherrill and Miller, 2006; Sterne-Marr et al., 2004).

Other scaffold proteins that enhance GPCR-PLCβ interaction and PLCβ signalling have been reported (reviewed by Ritter and Hall, 2009). However, a role of these scaffold proteins in facilitating GPCR-Gαq and Gαq-PLCβ interactions was not addressed. Signalling in the visual system of *D. melanogaster* is mediated by light stimulation of the GPCR rhodopsin, which couples to Gαq to activated PLCβ. Nearly all the components of this signalling pathway are kept together by a large PDZ domain-containing scaffold protein known as InaD, which associates with rhodopsin, TRPC, PLCβ and PKC (Popescu et al., 2006; Tsunoda et al., 2001). Choi et al. showed that PAR-3 mediated physical interaction between PLCβ1 and the bradykinin receptor, whereas NHERF2 did so between PLCβ3 and the LPA2 receptor (Choi et al., 2010). The association of NHERF1 and/or NHERF2 with the parathyroid hormone 1 receptor (PTH1R) (Mahon and Segre, 2004; Sneddon et al., 2003; Wang et al., 2009; Wheeler et al., 2008), purinergic receptor (P2RY1) (Fam et al., 2005) and metabotropic glutamate receptor 5 (mGluR5) (Paquet et al., 2006), can enhance their PLCβ-mediated signalling (Ritter and Hall, 2009). However, no evidence of Gαq involvement in the NHERF-promoted PLCβ signalling was found. In fact, in our

hands, NHERF1 inhibited the PLCβ signalling of TPβ by binding to activated Gαq and by preventing it from interacting with and activating PLCβ (Rochdi et al., 2002). Mahon et al. reported that NHERF2 reduced cAMP responses to PTH and promoted the activation of PLCβ through a pertussis toxin-sensitive mechanism, but the presence of Gα proteins in the signalling complexes was not mentioned (Mahon and Segre, 2004). These observations were extended to NHERF2 (Mahon and Segre, 2004; Wheeler et al., 2008). To our knowledge, we present the first description of a scaffold protein that promotes Gαq interaction with a GPCR and with PLCβ.

The possible role in signalling of WDR36 that internalizes with TPβ is intriguing. It is tempting to speculate that WDR36 internalizes with the receptor to preserve the integrity of a signalling complex. Persistent cAMP signalling triggered by internalized GPCRs was recently reported (Calebiro et al., 2009). However, WDR36 did not regulate TPβ-mediated cAMP generation. Gαs is known to internalize whereas Gαq stays at the plasma membrane following GPCR activation (Dave et al., 2009; Philip et al., 2007). By contrast, both Gαs and Gαq were reported to localize with their receptors on tubular recycling endosomes (Scarselli and Donaldson, 2009). One could thus hypothesize that WDR36 internalizes with the receptor to promote signalling through Gαq present on intracellular compartment membranes. PLCβ internalization was shown to be induced by protein kinase C activation (Aisiku et al., 2011; Constantinescu and Popescu, 1991). It is plausible that the persistent WDR36–PLCβ interaction that we observed could participate in the eventual removal of PLCβ from the plasma membrane. WDR36 that internalizes with the receptor could also favour activation of PLCβ on intracellular membranes (Aisiku et al., 2011; Diaz Anel, 2007). MAPK activation by a number of GPCRs might depend on their internalization and localization at endosomal compartments (Calebiro et al., 2010). We showed that TPβ-mediated activation of ERK1/2, which was dependent on receptor internalization, was increased by coexpression of WDR36. This might suggest that internalization of WDR36 with TPβ promotes receptor-mediated ERK1/2 activation on endosomal membranes. Further experiments will be needed to fully understand the role of internalization of WDR36 in TPβ signalling.

Few studies have addressed the role of WDR36 in experimental models. WDR36 loss of function results in reduced levels of 18S rRNA in zebrafish (Skarie and Link, 2008). Knockdown of *WDR36* expression causes a delay in processing 18S rRNA in mammalian cells, and loss of WDR36 in mice leads to preimplantation embryonic lethality (Gallenberger et al., 2011). Chi et al. reported that mutant WDR36 directly affects axon growth of retinal ganglion cells in transgenic mice (Chi et al., 2010). Single nucleotide polymorphisms at WDR36 that showed suggestive association with eosinophil counts were also associated with atopic asthma (Gudbjartsson et al., 2009). This is interesting with regards to the role of thromboxane A_2, the natural agonist for TPβ, as a potent bronchoconstrictor in asthma. One could speculate that the TPβ–WDR36 interaction could be involved in exacerbating asthma.

Five of the six WDR36 variants associated with polygenic primary open-angle glaucoma that we tested were significantly more potent than wild-type WDR36 at promoting TPβ-mediated Gαq signalling (L25P, N355S, I604V, D658G and M671V). By contrast, the R529Q variant was less efficient than wild-type WDR36 at enhancing receptor signalling. Our data showed that WDR36 variants that are potentially associated with disease changed the functional properties of the protein in regulating the signalling of TPβ. The functions of these WDR36 variants had not been reported before in mammalian cells.

Noteworthy, was the fact that WDR36 showed specificity among the few GPCRs that were tested in the present study. Indeed, although WDR36 expression strongly regulated PLCβ activation by TPβ, it had no effect on that by TPα and AT1R. Similarly, WDR36 did not modulate TPβ-mediated Gαs signalling. More GPCRs and Gα proteins will need to be studied to fully assess the specificity of WDR36 in regulating signalling pathways. Developing TPα- and TPβ-specific ligands has proved to be difficult because both receptors share the first 328 amino acids. The formation of specific pre-assembled signalling complexes by scaffold proteins such as WDR36 may well have implications in biased agonism. The TPβ–WDR36 interaction could constitute a specific target for pharmacological intervention in TPβ-mediated Gαq signalling pathways.

In summary, we showed, for the first time, the role of a scaffold protein, WDR36, in tethering a Gαq-coupled receptor, Gαq, and PLCβ to create an efficient signalling complex.

Materials and Methods

Reagents

Polyclonal anti-GST (glutathione transferase) antibody was from Bethyl Laboratories. Monoclonal anti-HA and polyclonal anti-EE antibodies were from Covance, whereas the anti-WDR36 monoclonal antibody was from Abnova Corporation. The actin and FLAG-specific polyclonal, FLAG-M1- and FLAG-M2-specific monoclonal antibodies were from Sigma. Anti-Myc, anti-GRK2 and anti-PLCβ2 polyclonal antibodies and protein-G–agarose were from Santa Cruz Biotechnology. Anti-Gαq monoclonal antibody was from BD Transduction Laboratories and anti-Myc–HRP from Abcam. Alexa Fluor 488 goat anti-mouse immunoglobulin M (IgM), Alexa Fluor 546 goat anti-mouse and anti-rabbit immunoglobulin G (IgG) were from Molecular Probes. Phosphorylation state-specific and phosphorylation state-independent antibodies against ERK1/2 were purchased from New England Biolabs. The TP receptor agonist U46619 was from Cayman Chemical Company. Alkaline phosphatase-conjugated goat anti-mouse antibody and the alkaline phosphatase substrate kit were purchased from Bio-Rad. IP-One HTRF assay was from Cisbio Bioassays.

Yeast two-hybrid screen

A yeast two-hybrid screen was performed following the two-hybrid system standard protocol (Gietz and Woods, 2002) using the C-terminus of TPβ cloned into the pAS2.1 vector (pAS2.1-TPβCT) to screen a human HeLa MATCHMAKER cDNA library in the yeast strain pJ69-4α, as described previously (Parent et al., 2008).

Cell culture and transfections

The HEK293 cells were maintained in DMEM (Dulbecco's modified Eagle's medium; Invitrogen) supplemented with 10% (v/v) fetal bovine serum at 37℃ in a humidified atmosphere containing 5% CO_2. Transient transfections of HEK293 cells grown to 50–70% confluence were performed using the *Trans*IT-LT1 Reagent (Mirus, Madison, WI) according to the manufacturer's instructions.

Immunoprecipitations

Immunoprecipitation experiments were performed as described previously (Parent et al., 2008) on lysates from HEK293 cells transfected with the indicated constructs that had been left unstimulated or stimulated with 500 nM of U46619 or 1 μM SQ29548 for the indicated times at 37℃. Initial lysates and immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotting using specific antibodies.

Recombinant protein production and binding assays

The pRSETa-myc-WDR36 construct was used to produce a His_6-tagged Myc–WDR36 fusion protein in the OverExpressTM C41(DE3) *Escherichia coli* strain (Avidis) following the manufacturer's instructions. The recombinant His-tagged protein was purified using Ni-NTA (Ni^{2+}-nitrilotriacetate)–agarose resin (Qiagen) following the manufacturer's instructions. The cDNA fragments coding for the ICL and the CT of TPβ were amplified by PCR and introduced into the pGEX-4-T1 vector (Amersham Biosciences) to produce the indicated GST-tagged fusion

proteins in the OverExpress C41(DE3) *E. coli* strain as described previously (Parent et al., 2008). GST fusion proteins were purified using glutathione–Sepharose 4B (Amersham Biosciences) following the manufacturer's instructions. Purified recombinant proteins were analyzed by SDS-PAGE, followed by Coomassie Brilliant Blue R-250 staining. The pulldown experiments were performed and analyzed as previously described (Parent et al., 2008). The WDR36 epitope peptide that was used to generate the WDR36 antibody (SGIETELRSLSPDCGGSIEVMQSFLKMIGMMLDRKRDFELAQAYLALFLKLH-LKMLPSEPVLLEEITNLSSQVEENWTHLQSLFNQSMCILNYLKSALL; Abcam) was produced and purified as a GST fusion epitope peptide as described above.

RNA interference

The synthetic oligonucleotides targeting the human *WDR36* gene were purchased from Ambion. siRNA 1 corresponds to the siRNA ID s43866 and siRNA 2 to the siRNA ID s43867. The negative control siRNA was also purchased from Ambion (Silencer Negative Control 1, catalogue number 4390843). HEK293 cells stably expressing HA–TPβ receptor were transfected with 25 nM oligonucleotide using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions, except for the following modification: cells were plated directly into the transfection mix at twice the cell density as in the basic protocol. Protein expression was assessed by western blotting at 72 hours post-transfection. Messenger RNA levels were assessed by reverse transcription and semi-quantitative PCR. cDNAs were synthesized with oligo(dT) Superscript II RT (Invitrogen), and amplified with a Phusion High Fidelity PCR Kit (New England BioLabs) with the following primers: forward, 59-GTGAAGGCCGACTTTTGAGA-39, and reverse, 59-GATTTGGGTACTTTGGGTGG-39 (500 bp). Controls consisted of RT-PCR of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. PCR products were separated on a 1% agarose gel and revealed by ethidium bromide staining.

Internalization assays

For quantification of receptor internalization, ELISA assays were performed on control cells and on cells after they were stimulated with 500 nM U46619 for 0–120 minutes at 37℃, as described previously (Parent et al., 1999).

Radioligand binding

Competition binding curves were generated using data from HEK293 cells expressing FLAG–TPβ transfected with empty pcDNA3 or pcDNA3-WDR36-myc as previously reported (Parent et al., 1999).

Inositol phosphate measurements

Inositol phosphate was measured as described previously (Parent et al., 1999) in HEK293 cells stably expressing HA–TPβ transfected with siRNAs, empty pcDNA3 or pcDNA3-WDR36-myc.

Immunofluorescence staining and confocal microscopy

For colocalization experiments, HEK-293 cells were plated in six-well plates at a density of 3310^5 cells per well. The following day, the cells were transiently transfected with either pcDNA3–FLAG–TPβ or pcDNA3. The following day, 2310^5 cells were transferred to coverslips coated with 0.1 mg/ml poly-L-lysine (Sigma) and grown overnight. The cells were then either left untreated or treated with 500 nM U46619 for 0–2 hours at 37℃ in DMEM. Following agonist stimulation, cells were processed for confocal microscopy as described previously (Parent et al., 2008) using a scanning confocal microscope (FV1000; Olympus) coupled to an inverted microscope with a 363 oil immersion objective (Olympus), and images were processed using Image-Pro Plus 6.0 (Media Cybernetics).

ERK1/2 activation

HEK293 cells were plated in six-well plates, transfected as described above and stimulated with the indicated concentrations of U46619. At the indicated time points, cells were washed once with cold PBS and lysed by addition of 1% SDS sample buffer. Lysates were boiled for 5 minutes, and 20 µl of cell lysate were electrophoresed on a standard 10% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membranes. To detect ERK1/2 activation, blots were probed with antibodies that recognize ERK1/2 phosphorylated on amino acids Thr202 or Tyr204. Briefly, blots were incubated for 1 hour in blocking buffer consisting of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl in Tris-buffered saline (TBS) plus 0.05% Tween 20 (TBS-T) containing 5% (w/v) dried nonfat milk, followed by incubation overnight at 4℃ in fresh blocking buffer (5% milk in TBS-T) containing a 1:1000 dilution of the phospho-specific ERK1/2 antibody. The next day, blots were washed three times with TBS-T, followed by a 1-hour incubation at room temperature with goat anti-rabbit horseradish-peroxidase-conjugated secondary antibody. Blots were then washed three times in TBS-T and visualized by enhanced chemiluminescence. Parallel blots were run and probed with antibodies that recognize ERK1/2.

Statistical analysis

Statistical analyses were performed with PRISM v4.0 (GraphPad Software) using the Student's *t*-test. Data were considered significant when *P*-values were <0.05 (*), <0.01 (**) or <0.001 (***).

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