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Kinetics of recruitment and allosteric activation of ARHGEF25 isoforms by the heterotrimeric G-protein $G\alpha_q$

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Rho GTPases are master regulators of the eukaryotic cytoskeleton. The activation of Rho GTPases is governed by Rho guanine nucleotide exchange factors (GEFs). Three RhoGEF isoforms are produced by the gene ARHGEF25; p63RhoGEF⁵⁸⁰, GEFT and a recently discovered longer isoform of 619 amino acids (p63RhoGEF⁶¹⁹). The subcellular distribution of p63RhoGEF⁵⁸⁰ and p63RhoGEF⁶¹⁹ is strikingly different in unstimulated cells, p63RhoGEF⁵⁸⁰ is located at the plasma membrane and p63RhoGEF⁶¹⁹ is confined to the cytoplasm. Interestingly, we find that both p63RhoGEF⁵⁸⁰ and p63RhoGEF⁶¹⁹ activate RhoGTPases to a similar extent after stimulation of $G\alpha_q$ coupled GPCRs. Furthermore, we show that p63RhoGEF⁶¹⁹ relocates to the plasma membrane upon activation of $G\alpha_q$ coupled GPCRs, resembling the well-known activation mechanism of RhoGEFs activated by $G\alpha_{12/13}$. Synthetic recruitment of p63RhoGEF⁶¹⁹ to the plasma membrane increases RhoGEF activity towards RhoA, but full activation requires allosteric activation via $G\alpha_q$. Together, these findings reveal a dual role for $G\alpha_q$ in RhoGEF activation, as it both recruits and allosterically activates cytosolic ARHGEF25 isoforms.

Rho GTPases are best known for their regulation of the cytoskeleton in eukaryotic cells¹. They function as molecular switches that transition between an active GTP-bound form and an inactive GDP-bound form². Rho guanine exchange factors (RhoGEFs) accelerate the exchange of GDP for GTP on RhoGTPases³, whereas Rho GTPase activating proteins (RhoGAPs) catalyze the hydrolysis of GTP to GDP on the Rho GTPase⁴.

The link between G-protein coupled receptors (GPCRs) and Rho GTPase signaling is well established^{5–8}. It was initially shown that heterotrimeric G-proteins of the $G\alpha_{12/13}$ family are involved in the activation of RGS containing RhoGEFs for the RhoA family of Rho GTPases⁹. More recently, the role of G-proteins of the $G\alpha_q$ family in GPCR mediated activation of RhoA was discovered^{10,11}. Trio¹² and p63RhoGEF¹³ are RhoGEFs that are activated by direct interaction with $G\alpha_q$. Biochemical and structural studies have well established that activation of the heterotrimeric G-protein $G\alpha_q$ relieves the DH domain of p63RhoGEF from its auto-inhibited state by allosteric interaction with the PH domain^{12,14,15}.

p63RhoGEF (encoded by the gene ARHGEF25) mediates activation of RhoA by $G\alpha_q$ in smooth muscle cells^{16,17} and has been proposed as a key regulator of angiotensin II induced effects on vascular smooth muscle tissue^{18,19}. The ARHGEF25 gene encodes for several isoforms, which are indicated as GEFT or p63RhoGEF. It is not always clear which isoform is used in a particular study. Here we use p63RhoGEF⁵⁸⁰ for the 580 a.a. protein with a predicted mass of 63 kDa. The shorter isoform lacking 106 a.a. of the N-terminus is termed GEFT here. Both p63RhoGEF⁵⁸⁰ and GEFT specifically activate RhoGTPases of the RhoA family *in vitro*^{12,20} and in cultured cells²¹. Despite their similar functionality towards RhoA, p63RhoGEF⁵⁸⁰ and GEFT have a remarkable different localization pattern inside cells. Palmitoylation of several cysteines at its N-terminus causes p63RhoGEF⁵⁸⁰ to be constitutively targeted to the plasma membrane in unstimulated cells, which is a unique feature for RhoA activating RhoGEFs. GEFT resides in the cytoplasm, it does not contain the first 106 a.a. of p63RhoGEF⁵⁸⁰, and thus

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lacks the N-terminal palmitoylation sites. The plasma membrane localization of both these p63RhoGEF isoforms have been shown to be important for their interaction with G α q^{22,23}.

More recently a third, longer variant encoded by ARHGEF25 was described in the literature²⁴. This isoform has a different N-terminus, comprises 619 a.a. in total and is indicated here as p63RhoGEF⁶¹⁹. It was found in several mass spectrometry based proteomic analyses of human tissues^{25,26}. The p63RhoGEF⁶¹⁹ variant is transcribed from two alternative N-terminal exons and, similar to GEFT, does not contain the N-terminal palmitoylation sites. The subcellular localization and functional properties of p63RhoGEF⁶¹⁹ in relation to the other two isoforms have not yet been investigated. Furthermore, the mechanism of activation of ARHGEF25 isoforms in the cytoplasm by G α q has not yet been elucidated.

Here we use advanced microscopy techniques to characterize the mechanism with which the different ARHGEF25 isoforms (p63RhoGEF⁵⁸⁰, GEFT and p63RhoGEF⁶¹⁹) are activated by G α q coupled GPCR stimulation. We show that although the subcellular localization of the different ARHGEF25 isoforms is strikingly different, they do not show any difference on basal and stimulated GEF activity towards RhoA. p63RhoGEF⁵⁸⁰ and p63RhoGEF⁶¹⁹ can activate RhoA and Cdc42 via stimulation of endogenous G α q coupled histamine-1-receptors (H₁R). Furthermore we show that p63RhoGEF⁶¹⁹ relocates to the plasma membrane upon activation of G α q. Using a chemical dimerization system, we are able to show that recruitment of p63RhoGEF⁶¹⁹ to the plasma membrane increases RhoGEF activity towards RhoA, but full activation requires allosteric activation by G α q. This study highlights the dual function of G α q, recruitment and allosteric activation, in the activation of cytosolic ARHGEF25 isoforms.

Results

The longer isoform of ARHGEF25 is located in the cytoplasm. p63RhoGEF/GEFT was first identified as a transforming gene product in a retroviral screen for oncogenes^{27,28}. Subsequently it was found that the ARHGEF25 locus produces two isoforms, p63RhoGEF⁵⁸⁰ (580 a.a.) and its shorter variant GEFT (474 a.a.)²⁰. More recently, a third and longer variant was described²⁴, p63RhoGEF⁶¹⁹, which is transcribed from two alternative exons at the N-terminus, and contains 619 a.a. in humans (see Supplemental Fig. 1 for a full sequence alignment of the three isoforms).

To compare the subcellular localization of the different ARHGEF25 isoforms, HeLa cells were transfected with a plasma membrane (PM) marker (Lck-CFP), a cytosolic marker (RFP) and either p63RhoGEF⁵⁸⁰-YFP, p63RhoGEF⁶¹⁹-YFP or YFP-GEFT. Confocal images of cells transfected with p63RhoGEF⁵⁸⁰-YFP show that p63RhoGEF⁵⁸⁰ is localized to the plasma membrane, as has been described before^{22,23}. In contrast, cells transfected with either p63RhoGEF⁶¹⁹-YFP or YFP-GEFT show only a cytosolic localization of these isoforms (Fig. 1A,B).

Previous studies have shown that p63RhoGEF⁵⁸⁰ is localized to the plasma membrane because of the palmitoylation of one or more cysteines at its the N-terminus^{22,23}. In depth sequence analysis of the first 30 amino acids shows that the N-terminus, including cysteines, of p63RhoGEF⁵⁸⁰ is well conserved throughout the whole subphylum vertebrata (Fig. 1C, top). GEFT is essentially a splice variant of p63RhoGEF⁵⁸⁰ that does not contain the first 2 N-terminal exons of p63RhoGEF⁵⁸⁰ that contain the palmitoylated cysteines, resulting in cytoplasmic localization.

Sequence analysis of the relatively unexplored isoform p63RhoGEF⁶¹⁹ shows that the N-terminus is well conserved among the vertebrate subphylum (Fig. 1C, bottom). There are no obvious PM targeting signals in the N-terminus, which is in agreement with the cytoplasmic localization observed in Fig. 1A. Of note, there seems to be a well-conserved 'GRTD' sequence at amino acid 10–13. Evolutionary sequence analysis (see Methods for details) reveals that both p63RhoGEF⁵⁸⁰ and p63RhoGEF⁶¹⁹ are not found outside the vertebrate subphylum, and even so-called early or ancient vertebrate species like the *Lancelets*²⁹ and *Lampreys*³⁰ do not contain either isoform. Our results fit with a role for ARHGEF25 in smooth muscle (see discussion). To conclude, we find that p63RhoGEF⁵⁸⁰ is located at the plasma membrane, whereas the two other isoforms show cytoplasmic distribution.

The basal GEF activity of ARHGEF25 isoforms is low in cells. It has been well established that p63RhoGEF⁵⁸⁰ can be activated through G-protein coupled receptors (GPCRs), via direct interaction with the heterotrimeric G-protein subunit G α q^{12,14,22,31}. Activated p63RhoGEF⁵⁸⁰ catalyzes the exchange of GDP for GTP on Rho GTPases of the RhoA subfamily¹². Even though it is located at the plasma membrane, p63RhoGEF⁵⁸⁰ has been confirmed to have low GEF activity towards RhoA *in vitro*¹² and in cells²¹ due to the auto-inhibition by its PH domain. To compare the GEF activity towards RhoA of the different ARHGEF25 isoforms in unstimulated cells, we transfected HeLa cells with the previously characterized DORA RhoA biosensor²¹ and p63RhoGEF⁵⁸⁰-RFP, p63RhoGEF⁶¹⁹-RFP, RFP-GEFT or only a cytosolic RFP (control) (Fig. 2A). The ratio between YFP and CFP fluorescence of the DORA RhoA biosensor, which confers the GTP loading state of RhoA, was determined for control (median = 0.61, 95% CI = 0.58–0.66) p63RhoGEF⁵⁸⁰ (median = 0.63, 95% CI = 0.61–0.67), p63RhoGEF⁶¹⁹ (median = 0.69, 95% CI = 0.61–0.73) or GEFT (median = 0.66, 95% CI = 0.61–0.72) (Fig. 2A,B). The values for p63RhoGEF⁵⁸⁰, p63RhoGEF⁶¹⁹ and GEFT are slightly elevated compared to the control condition, but are in line with low basal activity previously reported for p63RhoGEF⁵⁸⁰²¹. These results demonstrate that all different ARHGEF25 isoforms have low basal activity towards RhoA in unstimulated cells.

Both p63RhoGEF⁵⁸⁰ and p63RhoGEF⁶¹⁹ activate RhoA and Cdc42. In order to investigate whether the difference in location translates to a functional difference between the ARHGEF25 isoforms, we used DORA FRET biosensors for RhoA²¹, Rac1³² and Cdc42³³ to measure GEF specificity and activity in stimulated cells. HeLa cells were used in this study, because these cells express the endogenous histamine-1 receptor that couple to G α q. In previous experiments, we have shown that in control conditions, i.e. cells transfected with mCherry³⁴ or inactive variants of p63RhoGEF²¹, RhoA-GTP levels are not elevated upon stimulation with histamine. When

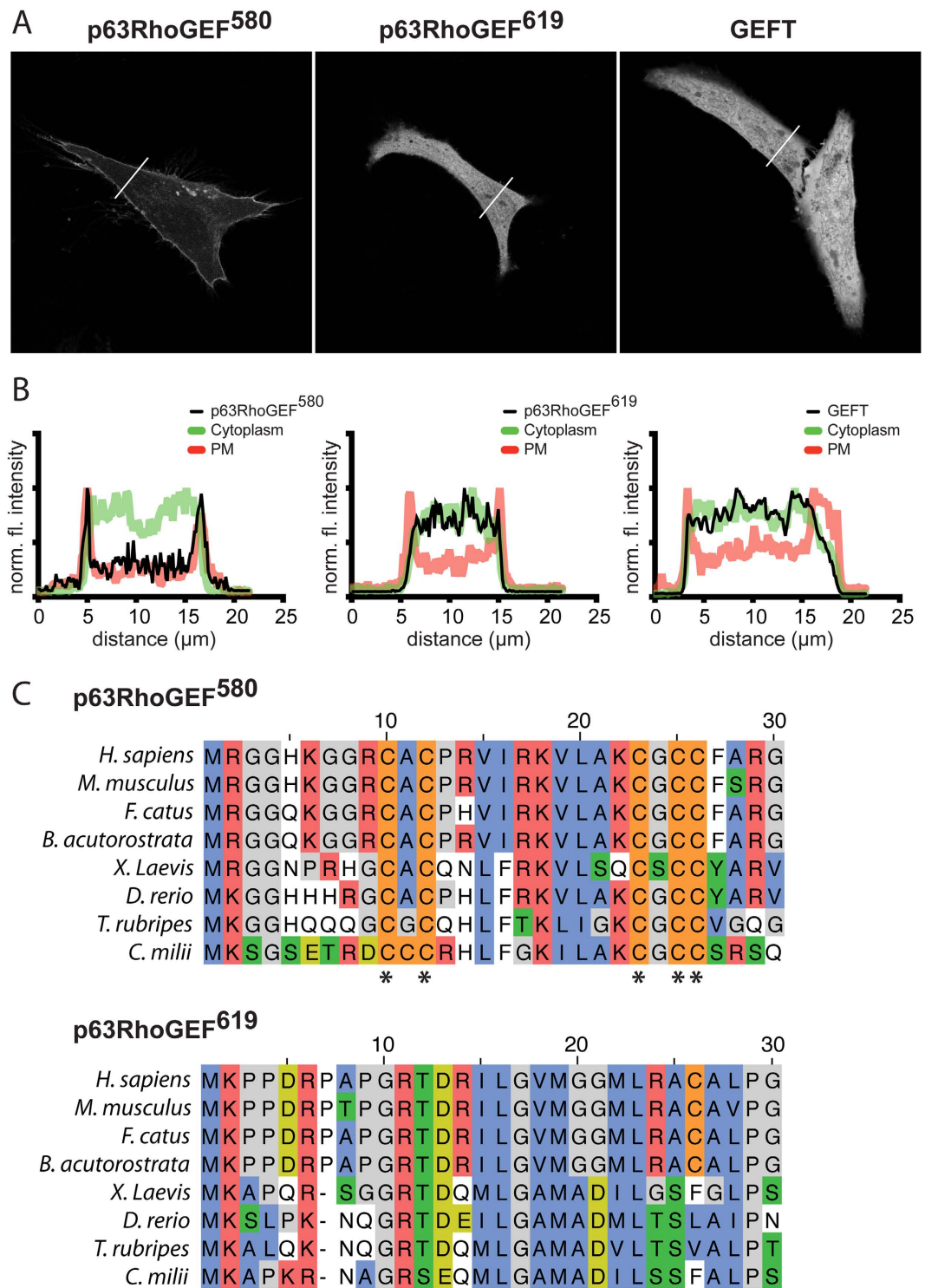


Figure 1. Localization and origin of ARHGEF25 isoforms. (a) Confocal images of HeLa cells expressing p63RhoGEF⁵⁸⁰-YFP (left), p63RhoGEF⁶¹⁹-YFP (middle) or YFP-GEFT (right). (b) Line plots showing fluorescence intensities of the different GEF constructs along the lines indicated in (a), compared with the intensities of a co-transfected plasma membrane marker (Lck-CFP) and a cytosolic marker (RFP) transfected in the same cells. The intensities of the GEF, PM marker and cytosolic marker were normalized to the maximum fluorescent intensities in their respective channels. (c) Protein sequence homology analysis of the first N-terminal 30 amino acids of p63RhoGEF⁵⁸⁰ and p63RhoGEF⁶¹⁹. Species compared: Human (*H. sapiens*), House mouse (*M. musculus*), Domestic cat (*F. Catus*), Mink whale (*B. acutorostrata*), African clawed frog (*X. Laevis*), Zebrafish (*D. rerio*), Pufferfish (*T. rubripes*) and Elephant shark/Australian ghost shark (*C. milii*). Amino acids are grouped by polar positive (red), polar negative (lightgreen), non-polar (blue), phosphorylation potential (green), palmitoylation potential (orange), or small (grey). Asterisks (*) mark the conserved potential palmitoylated cysteines of p63RhoGEF⁵⁸⁰. Width of the individual images in (a) is 121 μ m.

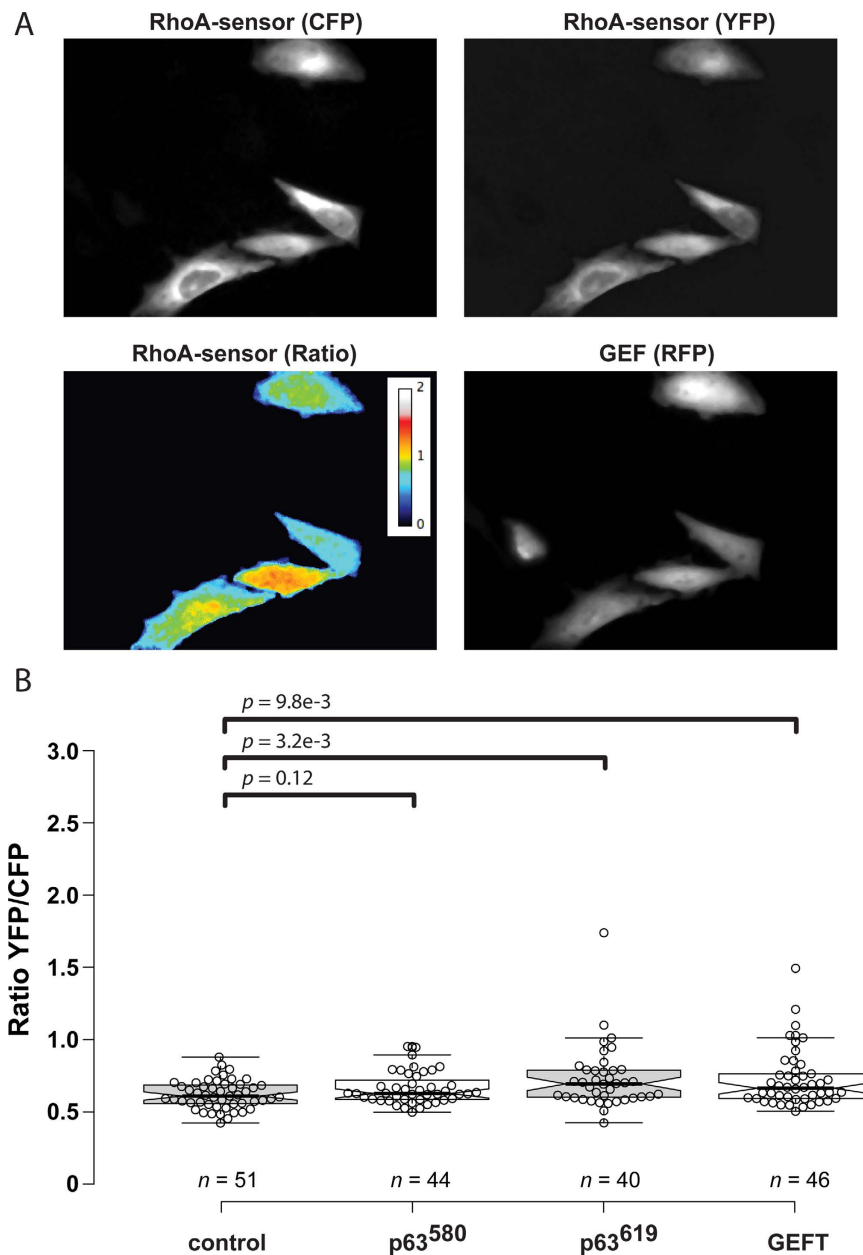


Figure 2. Basal RhoA activity of ARHGEF25 isoforms. (a) Example images of HeLa cells transfected with the DORA RhoA biosensor and p63RhoGEF⁶¹⁹, showing the background corrected CFP, YFP, Ratio between YFP/CFP of the DORA RhoA biosensor and the RFP channels. (b) Boxplot showing the median basal YFP/CFP ratio of the DORA RhoA biosensor in HeLa cells transfected with the DORA RhoA biosensor and a cytosolic RFP (n = 51, control), p63RhoGEF⁵⁸⁰-RFP (n = 44), p63RhoGEF⁶¹⁹-RFP (n = 40) or RFP-GEFT (n = 46). Individual data points are plotted as open circles. Experiments performed on 5 or more separate coverslips. Centerlines represent the median values and notches represent the 95% confidence interval for each median. The p-values were determined by a two-tailed Mann-Whitney test. Width of the individual images in (a) is 236 μm .

p63RhoGEF⁵⁸⁰-RFP is ectopically expressed in HeLa cells, a rapid activation of RhoA is observed after stimulation of the cells with histamine.

We transfected HeLa cells with p63RhoGEF⁵⁸⁰-RFP, p63RhoGEF⁶¹⁹-RFP or a cytosolic RFP (control) and one of the three aforementioned Rho GTPase FRET sensors. CFP fluorescence and YFP sensitized emission were followed over time. Cells were stimulated with histamine to activate G α q via endogenous histamine-1-receptors, and the response was antagonized with mepyramine. Stimulation of cells transfected with either p63RhoGEF⁵⁸⁰ or p63RhoGEF⁶¹⁹ showed a reversible increase in YFP/CFP ratio of the DORA RhoA biosensor, with similar kinetic profiles (Fig. 3A). Stimulation of cells in the control condition showed no change in YFP/CFP ratio of the DORA RhoA biosensor. Stimulation of cells transfected with GEFT also showed a similar response on the DORA RhoA biosensor (Supplemental Fig. 2A), which was completely inhibited if cells were incubated with the specific G α q inhibitor FR900359³⁵ (Supplemental Fig. 2B). This is in line with earlier results obtained with

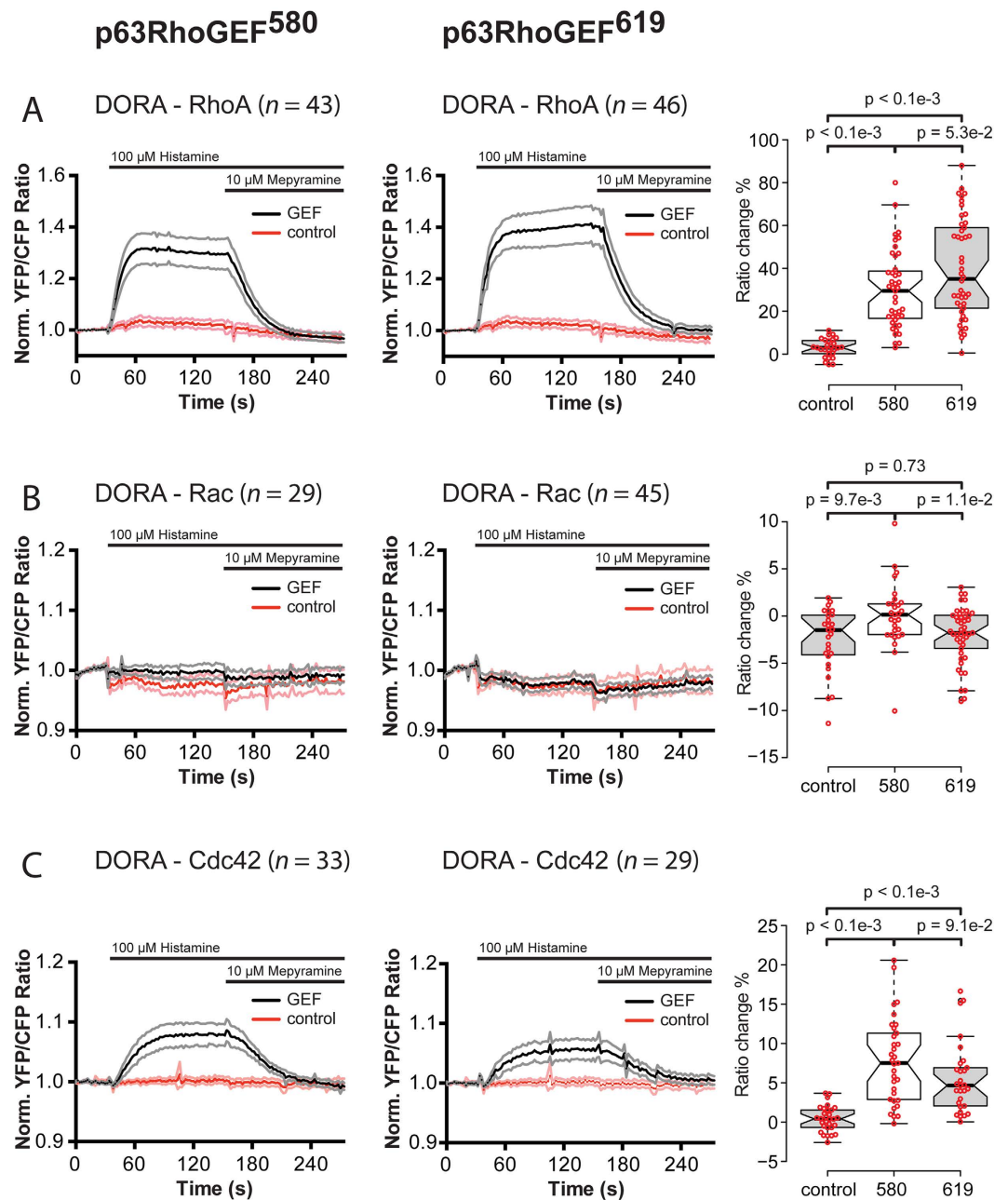


Figure 3. GPCR mediated activation of RhoA, Rac1 and Cdc42 by p63RhoGEF⁵⁸⁰ or p63RhoGEF⁶¹⁹. (a) Time-lapse FRET ratio imaging of HeLa cells transfected with the DORA-RhoA biosensor and p63RhoGEF⁵⁸⁰ (left, $n = 43$) or p63RhoGEF⁶¹⁹ (middle, $n = 46$) show a fast reversible increase in YFP/CFP ratio, indicating rapid GTP loading of RhoA upon GPCR stimulation. Control cells were transfected with the DORA-RhoA biosensor and a cytosolic RFP (red traces, $n = 27$). The median amplitude of the FRET ratio change at $t = 120$ seconds is shown on the right. (b) HeLa cells transfected with the DORA-Rac1 biosensor and p63RhoGEF⁵⁸⁰ (left, $n = 29$) or p63RhoGEF⁶¹⁹ (middle, $n = 45$) show no changes in YFP/CFP ratio. Control cells were transfected with the DORA-Rac1 biosensor and a cytosolic RFP (red traces, $n = 26$). The median amplitude of the FRET ratio change at $t = 120$ seconds is shown on the right. (c) HeLa cells transfected with the DORA-Cdc42 biosensor and p63RhoGEF⁵⁸⁰ (left, $n = 33$) or p63RhoGEF⁶¹⁹ (middle, $n = 29$) show a fast reversible increase in YFP/CFP ratio, indicating rapid GTP loading of Cdc42 upon GPCR stimulation. Control cells were transfected with the DORA-Cdc42 biosensor and a cytosolic RFP (red traces, $n = 30$). The median amplitude of the FRET ratio change at $t = 120$ seconds is shown on the right. Cells were stimulated with Histamine ($100 \mu\text{M}$) at $t = 32$ s and the response was antagonized by the addition of Mepyramine ($10 \mu\text{M}$) at $t = 152$ s. Time traces show the average ratio change of YFP/CFP fluorescence, normalized to baseline values (faded grey and pink traces depict $\pm 95\%$ CI). Boxplots show individual data points and median values. Centerlines represent the median values and notches represent the 95% confidence interval for each median. The p-values were determined by a two-tailed Mann-Whitney test.

p63RhoGEF^{580/21}. Hardly any change in YFP/CFP ratio of the DORA Rac biosensor was observed after stimulation of cells transfected with either p63RhoGEF⁵⁸⁰ or p63RhoGEF⁶¹⁹ compared to control cells transfected only a cytosolic RFP (Fig. 3B). Interestingly, we found that cells transfected with either p63RhoGEF⁵⁸⁰ or p63RhoGEF⁶¹⁹ showed a robust reversible increase in YFP/CFP ratio of the DORA Cdc42 biosensor after stimulation with histamine and mepyramine. Control cells expressing the DORA Cdc42 sensor and a cytosolic RFP did not show any change in YFP/CFP ratio upon stimulation with histamine and mepyramine (Fig. 3C). Overnight incubation with *Pertussis Toxin* (PTX)³⁶ to inhibit G α i activity did not change the response in cells transfected with the DORA Cdc42 biosensor and p63RhoGEF⁶¹⁹, excluding a G α i mediated effect (Supplemental Fig. 3A). Incubation of cells transfected with the DORA Cdc42 biosensor and p63RhoGEF⁶¹⁹ with G α q inhibitor FR900359 abrogated the histamine mediated response on the DORA Cdc42 biosensor, showing that this effect is mediated by the activation of p63RhoGEF⁶¹⁹ via G α q (Supplemental Fig. 3B).

Hence, despite the difference in their subcellular locations, p63RhoGEF⁵⁸⁰ and p63RhoGEF⁶¹⁹ show surprisingly similar activation profiles on the Rho GTPase they can exert their GEF activity on.

p63RhoGEF⁶¹⁹ is recruited to the plasma membrane after activation of a GPCR. Since we measured similar Rho GTPase activation profiles with a cytosolic (p63RhoGEF⁶¹⁹) and plasma membrane (p63RhoGEF⁵⁸⁰) located variant of the same GEF, we set out to explore the mechanism behind this unexpected observation in more detail. It is well known that LARG, PDZ-RhoGEF and p115RhoGEF, RhoGEFs that are activated via direct interaction with G $\alpha_{12/13}$ ³⁷, relocate to the plasma membrane upon activation³⁸. To investigate whether p63RhoGEF⁶¹⁹ also relocates to the plasma membrane upon activation of G α q, we used real-time confocal microscopy to inspect the subcellular location p63RhoGEF⁶¹⁹ during stimulation of endogenous histamine-1-receptors. Pilot experiments indicated that the ectopic expression of p63RhoGEF⁶¹⁹ leads to a quick saturation of available binding sites of endogenously activated G α q. To overcome this, we transfected HeLa cells with p63RhoGEF⁶¹⁹-YFP, G α q-CFP and H₁R-RFP. After stimulation of the cells with histamine we observed a rapid relocation of p63RhoGEF⁶¹⁹ to the plasma membrane, which was reversed upon addition of mepyramine (Fig. 4A). This response was quantified by drawing ROIs in the cytoplasm of individual cells (Fig. 4B). Upon stimulation of cells transfected with YFP-GEFT, G α q-CFP and H₁R-RFP we observed a similar reversible relocation of GEFT to the plasma membrane, albeit with a lower amplitude (Fig. 4C, Supplemental Fig. 4A). We observed similar relocation kinetics for p63RhoGEF⁶¹⁹ and GEFT when cells were co-transfected with both constructs at the same time (RFP-GEFT, p63RhoGEF⁶¹⁹-YFP, G α q-CFP and H₁R-untagged) (Supplemental Fig. 4B). From this data we conclude that the ARHGEF25 isoform p63RhoGEF⁶¹⁹ and splice variant GEFT, which reside in the cytosol in unstimulated cells, relocate to the PM upon activation of G α q mediated signaling.

Full activation of RhoGEF activity requires heterotrimeric G-protein signaling. The observation that p63RhoGEF⁶¹⁹ relocates to the plasma membrane argues for a dual role of activated G α q where it both recruits p63RhoGEF⁶¹⁹ to the plasma membrane and relieves it from its allosteric autoinhibition by the PH domain. To separate these events we made use of the rapamycin heterodimerization system³⁹. In this approach, addition of rapamycin will recruit p63RhoGEF⁶¹⁹ to the plasma membrane without allosteric activation by G α q. Subsequent activation of a GPCR will result in allosteric activation without a contribution of relocation. By simultaneously monitoring the RhoGEF activity with the DORA RhoA biosensor we can quantify the contribution of both events to RhoGEF activity. To this end, HeLa cells transfected with the DORA RhoA biosensor, Lck-FRB-ECFP(W66A) and p63RhoGEF⁶¹⁹-fkbp12-RFP were sequentially treated with rapamycin, histamine and mepyramine (Fig. 5A). After adding rapamycin, a gradual increase in YFP/CFP ratio of the RhoA biosensor is observed, most likely due to some constitutive activity of p63RhoGEF⁶¹⁹ at the plasma membrane towards RhoA. (Fig. 5B,C red trace). Subsequent stimulation with histamine fully activates G α q, leading to the allosteric release of the inhibitory PH domain of p63RhoGEF⁶¹⁹, and rapid and large change in YFP/CFP ratio on the RhoA biosensor. The histamine-mediated activation of RhoA is then fully reversed upon addition of mepyramine (Fig. 5B, red trace, Fig. 5D). A similar experiment with the splice variant GEFT (RFP-fkbp12-GEFT) revealed an identical pattern in YFP/CFP ratio changes on the RhoA biosensor (Supplemental Fig. 5A). In contrast, when we recruited the isolated catalytic domain of ARHGEF25 (cDH-fkbp12-RFP) with rapamycin, we observed a large and fast change in ratio of YFP/CFP of the RhoA biosensor (Fig. 5B,C, black trace, Fig. 5D). The ratio did not change further upon stimulation with histamine or mepyramine, indicating that relocation of the isolated catalytic GEF domain of ARHGEF25 to the plasma membrane is sufficient for full activation of RhoA as previously reported²¹. Recruitment of an fkbp12-RFP serves as a control since this unit has no RhoGEF activity. Indeed, recruitment of fkbp12-RFP does not lead to any change in YFP/CFP ratio of the RhoA biosensor, whereas addition of histamine leads to a small reversible change in FRET ratio that can most likely be explained by the activation of the endogenous G α q mediated GEF Trio⁴⁰ (Fig. 5B, grey trace, Fig. 5C). Of note, when we compared the raw ratios of the RhoA biosensor in all conditions, we observed only an increased basal YFP/CFP ratio of the RhoA biosensor in cells transfected with cDH (Supplemental Fig. 5B). This increased basal ratio has been observed before by us²¹, and is likely due to the random interactions of the constitutive active cDH construct and the RhoA biosensor in the cytoplasm. The raw FRET ratio data also shows that the maximum YFP/CFP ratio change induced by rapamycin in the cDH condition is very comparable (25%) to the ratio change induced by rapamycin + histamine in the p63RhoGEF⁶¹⁹/GEFT conditions (Supplemental Fig. 5B).

Together, from these results we conclude that recruitment of p63RhoGEF⁶¹⁹ increases RhoGEF activity but that maximal stimulation requires allosteric activation via heterotrimeric G-protein signaling.

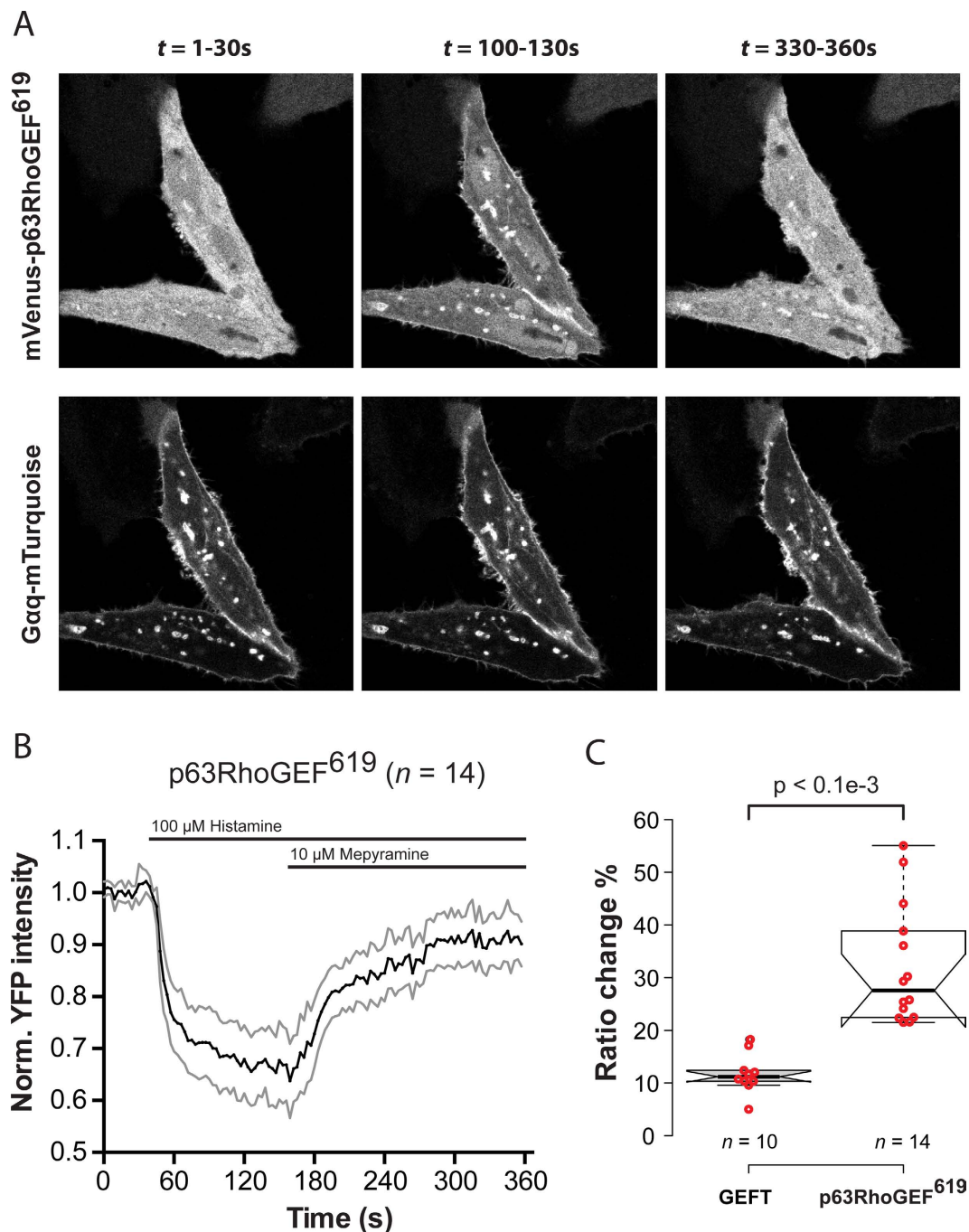


Figure 4. GPCR-Gαq mediated recruitment of p63RhoGEF⁶¹⁹ to the plasma membrane. (a) Representative images at three time intervals of p63RhoGEF⁶¹⁹-YFP (*top panels*) Relocation to the plasma membrane in HeLa cells transfected with p63RhoGEF⁶¹⁹-YFP, Gαq-CFP and H₁R-RFP (*bottom panels*; Gαq-CFP is shown for reference). (b) Quantification of the experiment shown in (a), trace shows the average normalized cytoplasmic YFP fluorescence over time (faded grey traces depict +/- 95% CI). Cells were stimulated with histamine (100 μM) at t = 42 s and the response was antagonized by the addition of mepyramine (10 μM) at t = 162 s. (c) The median amplitude change of the YFP fluorescence at t = 120 seconds is quantified for YFP-GEFT and p63RhoGEF⁶¹⁹-YFP. Centerlines of the boxplot in (c) represent the median values and notches represent the 95% confidence interval for each median. The p-values were determined by a two-tailed Mann-Whitney test. Width of the individual images in (a) is 86 μm.

Discussion

The two undisputed gene products of ARHGEF25, p63RhoGEF⁵⁸⁰ and p63RhoGEF⁶¹⁹, display obvious differences in subcellular localization when expressed in unstimulated HeLa cells. Five putative palmitoylation sites at the N-terminus of p63RhoGEF⁵⁸⁰ target this isoform to the plasma membrane^{22,23}, while p63RhoGEF⁶¹⁹ resides in the

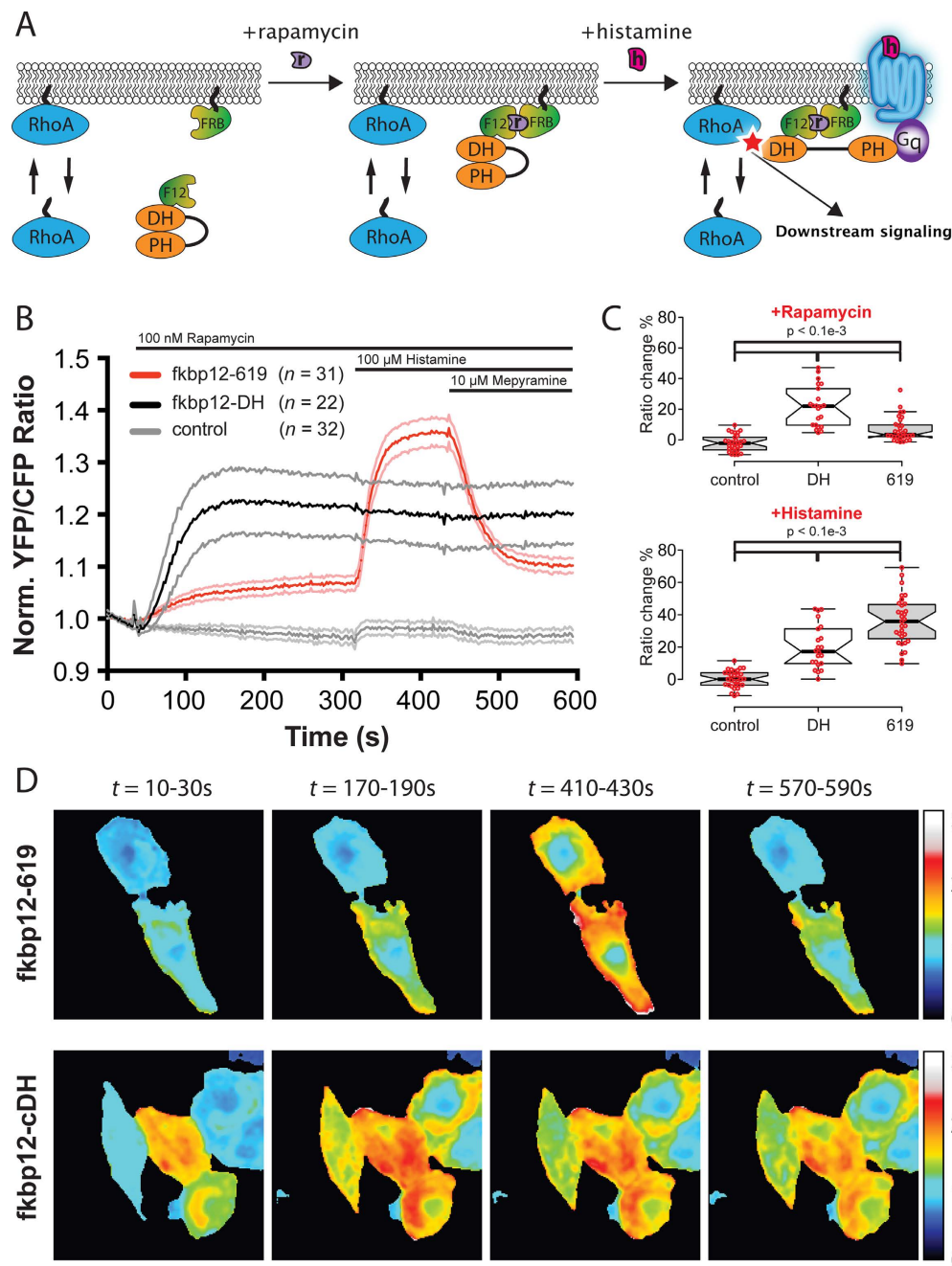


Figure 5. Activity of p63RhoGEF⁶¹⁹ towards RhoA, separating activation by recruitment to the plasma membrane from allosteric activation. (a) Schematic overview of the experiment. In the basal state, p63RhoGEF⁶¹⁹-fkbp12 is localized to the cytosol and its PH domain is folded onto the catalytic DH domain (left) (only the DH-PH domains of p63RhoGEF⁶¹⁹ are shown in the cartoon to improve clarity). Upon stimulation with rapamycin, fkbp12 dimerizes with frb, causing a relocation of p63RhoGEF⁶¹⁹-fkbp12 to the plasma membrane (middle). After stimulation with histamine, and subsequent activation of G α_q , the PH domain is released from the DH domain and p63RhoGEF⁶¹⁹-fkbp12 is fully activated, leading to the activation of RhoA. (b) Average FRET measurements of HeLa cells transfected with the DORA RhoA-biosensor, Lck-frb-CFP(w66a) and p63RhoGEF⁶¹⁹-RFP-fkbp12 ($n = 31$, red), RFP-fkbp12-cDH ($n = 22$, black) or RFP-fkbp12 transfected control cells ($n = 32$, grey). Cells were stimulated with rapamycin at $t = 32$ s, histamine at $t = 312$ s and mepyramine at $t = 432$ s. Time traces show the average ratio change of YFP/CFP fluorescence, normalized to baseline values (faded grey and pink traces depict $\pm 95\%$ CI). (c) The median amplitude of the FRET ratio change in (b) after rapamycin addition ($t = 300$) and histamine addition ($t = 420$ seconds) (bottom). (d) Examples of the experiment performed in (b) for the p63RhoGEF⁶¹⁹-RFP-fkbp12 (top) and RFP-fkbp12-cDH (bottom) condition. YFP/CFP FRET ratio images are shown of the RhoA-biosensor for four different time intervals (10–30 s, 170–190 s, 410–430 s, 570–590 s). Center lines of the boxplot in (c) represent the median values and notches represent the 95% confidence interval for each median. The p-values were determined by a two-tailed Mann-Whitney test. Width of the individual images in (d) is 115 μ m.

cytoplasm. Interestingly, despite this difference in localization, we observed no difference in basal GEF activity towards RhoA. Surprisingly, both isoforms reversibly activate RhoA with similar magnitudes upon stimulation of G α q coupled GPCRs. Closer inspection of p63RhoGEF⁶¹⁹ revealed that this isoform dynamically relocates to the plasma membrane during GPCR stimulation on a seconds timescale. Of note, the RhoGEF Trio, which has a DH/PH domain homologous to p63RhoGEF, has been shown to relocate to the plasma membrane after activation of G α q at much slower timescales⁴¹. These observations resemble the well-known relocation mechanism of the G α _{12/13} mediated RhoGEFs p115RhoGEF, PDZ-RhoGEF and LARG³⁸.

It was previously shown that recruitment of the isolated catalytic DH domain of p63RhoGEF from the cytosol to the plasma membrane leads to robust activation of RhoA²¹. By synthetically recruiting p63RhoGEF⁶¹⁹ to the plasma membrane we could separate the effects of plasma membrane recruitment of p63RhoGEF⁶¹⁹ on the one hand, and allosteric activation by G α q on the other hand. P63RhoGEF⁶¹⁹ mediated GEF activity towards RhoA is slightly enhanced when it is artificially recruited to the plasma membrane, but full GEF activity requires stimulation by G α q. This reveals a double role for G α q in cells, where it both recruits and allosterically activates P63RhoGEF⁶¹⁹.

Interestingly, we also found GEF activity towards the Cdc42 of both p63RhoGEF isoforms, while we did not find evidence of Rac activation. Although Cdc42 and Rac activity of both p63RhoGEF^{580/42} and p63RhoGEF^{619/24} has been described before, these results are controversial^{12,20}. Our results with the G α q inhibitor FR900359 indicate that the Cdc42 activity most likely originates, directly or indirectly, from G α q mediated activity.

The ARHGEF25 gene and its protein products are only found in the vertebrate subphylum. It seems likely that ARHGEF25 gene arose from a split gene event of either Trio⁴⁰ or Kalirin⁴³, which are both found in vertebrate predecessors, and contain a DH/PH cassette that is highly homologous in sequence to ARHGEF25. Interestingly, recent efforts with proteomics approaches^{25,26} have found expression of both p63RhoGEF⁶¹⁹ and p63RhoGEF⁵⁸⁰ specifically in human tissues that also contain smooth muscle, including the brain (vasculature), fetal heart, uterus, reproductive tract, urinary tract, gastrointestinal tract and the retina. The fact that smooth muscle tissue has evolved only in vertebrates⁴⁴, and p63RhoGEF function has been extensively linked to vascular smooth muscle cell function^{16,17,28,45} and hypertension^{18,19,46,47}, provides strong evidence for co-evolution and a functional connection between p63RhoGEF and smooth muscle tissue. Which of the ARHGEF25 isoforms is expressed in vascular smooth muscle tissue, whether they can be specifically targeted and if one of isoforms is main cause of hypertensive pathogenicity, should be subject of future studies.

In conclusion, we compared subcellular localization and functional output of two isoforms of the gene ARHGEF25, p63RhoGEF⁵⁸⁰ and p63RhoGEF⁶¹⁹. Interestingly, while the two isoforms display remarkable differences in subcellular localization, p63RhoGEF⁵⁸⁰ is located at the plasma membrane, and p63RhoGEF⁶¹⁹ located in the cytosol, they activate the RhoGTPases RhoA and Cdc42 to a similar extent upon G α q mediated GPCR stimulation. We found that p63RhoGEF⁶¹⁹ relocates to the plasma membrane upon activation of G α q, very similar to the mechanisms of G α _{12/13} mediated RhoGEFs. Synthetic recruitment of p63RhoGEF⁶¹⁹ to the plasma membrane slightly increases its GEF activity towards RhoA, but full activation requires allosteric activation by G α q. The resemblance of signaling output between a cytoplasmic and membrane located GEF is striking. Future studies may reveal whether signaling output of the two variants is similar under physiological conditions.

Methods

Construction of fluorescent protein fusions. The CFP, RFP and YFP variants used in this study are mTurquoise, mCherry and mVenus, respectively. P63RhoGEF⁵⁸⁰-RFP, p63RhoGEF⁵⁸⁰-YFP, YFP-p63RhoGEF⁵⁸⁰, YFP-GEFT, RFP-GEFT, RFP- fkbp12, RFP- fkbp12-GEFT and RFP-p63RhoGEF⁵⁸⁰, RFP- fkbp12-cDH were constructed previously^{21,22}. G α q-mTurquoise and H₁R-untagged⁴⁸ were previously described. H₁R-mCherry is described elsewhere³⁴.

A membrane targeting sequence (derived from amino acid residue 1–10 of Lck; MGCVCSSNPE) was constructed by annealing⁴⁹ two oligonucleotide linkers, 5'-ctagccaccatgggctgctgtgcagcagcaacccccgagcta-3' and 5'-ccgtagctcggggtgctgctgcacacgcagccatggtgg-3', with sticky overhangs and inserting it into an mVenus-C1 plasmid cut with NheI and AgeI, resulting in Lck-mVenus. Lck-mTurquoise2 was obtained by exchanging mVenus for mTurquoise2 in the Lck-mVenus plasmid by cutting with AgeI and BsrGI.

In order to construct p63RhoGEF⁶¹⁹-RFP, a plasmid containing the following nucleotide sequence was ordered (Eurofins): 5'-ggactcagatctcagctc**caagcttc**gaattctgcagtcga cgttaccaccatgaagccccggaccgc cccgccct-ggcccgcac tgaccggatactgggggtcatgggggcatgctgcgcgcatgcgcctccctgggcaggagggggcccccaaggagaagccctctag-ggttggtgggtaccgagccagagtctgaacgtacggaggagatcacagaaggatcgcgaaatgaggtcctcgccggggctctgcagcccgatcctattccattgcggcagtgaggggagtatatcgcttctgctgcctccggtctggctgccccctctggccccagcttgccctcagcttgccctgt-tccccagggccc cccagggccctcagtgccctgaggagatg gttgatcatccaacattgtctcagtggtg aaactgaggcag**cagctggtc**aggcagga-3'. Cutting the plasmid with HindIII and PshAI (restriction sites in bold and underlined) and ligating it into a plasmid containing p63RhoGEF⁵⁸⁰-RFP, cut with the same enzymes, resulted in p63RhoGEF⁶¹⁹-RFP. P63RhoGEF⁶¹⁹-YFP was obtained by swapping the RFP for YFP with AgeI and BsrGI. p63RhoGEF⁶¹⁹-fkbp12-RFP was obtained by cutting p63RhoGEF⁶¹⁹-RFP with HindIII and BsrGI and ligating fkbp12, cut with the same enzymes from N1-fkbp12-mTurquoise2, in between the sequence for p63RhoGEF⁶¹⁹ and RFP.

The Lck-FRB-ECFP(W66A) was a kind gift from M. Putyrski³⁹. The DORA Rho GTPase sensors were described previously; DORA-RhoA^{21,50}, DORA-Rac1 sensor³² and the DORA-Cdc42 sensor³³. N1-fkbp12-mTurquoise2 was a kind gift from Kevin Batenburg. Plasmids constructed in this study will be made available through Addgene: https://www.addgene.org/Dorus_Gadella/

Cell Culture & Sample Preparation. HeLa cells (American Tissue Culture Collection: Manassas, VA, USA) were cultured using Dulbecco's Modified Eagle Medium (DMEM) supplied with Glutamax, 10% FBS,

Penicillin (100 U/ml) and Streptomycin (100 µg/ml). Cell culture, transfection and live cell microscopy conditions were performed as previously described²¹.

Widefield microscopy. Ratiometric FRET measurements were performed using a previously described wide-field fluorescence microscope²¹. Typical exposure times ranged from 50 ms to 150 ms, and camera binning was set to 4 × 4. Fluorophores were excited with 420 nm light (slit width 30 nm) and reflected onto the sample by a 455DCLP dichroic mirror and CFP emission was detected with a BP470/30 filter, and YFP emission was detected with a BP535/30 filter by rotating the filter wheel. All acquisitions were corrected for background signal and bleedthrough of CFP emission in the YFP channel (around 55% of the intensity measured in the CFP channel).

In dynamic experiments, cells were stimulated with 100 µM histamine (Sigma-Aldrich) and 10 µM Mepyramine (Sigma-Aldrich) or 100 nM Rapamycin (LC Laboratories, Woburn, USA). Where indicated, cells were incubated with 100 ng/ml Pertussis Toxin (PTX) (Sigma Aldrich) overnight. The Gαq inhibitor FR900359³⁵ was added to cells 2 hours before the measurements started at a concentration of 2 µM and was purchased from the University of Bonn (<http://www.pharmbio.uni-bonn.de/signaltransduktion>).

Confocal microscopy. Localization and relocation experiments were performed using a Nikon A1 confocal microscope equipped with a 60x oil immersion objective (Plan Apochromat VC, NA 1.4). The pinhole size was set to 1 Airy unit (<0.8 µm). 457 nm, 514 nm and 561 nm laser lines were reflected onto the sample by a 457/514/561 dichroic mirror. To avoid bleed-through, images were acquired with sequential line scanning modus. CFP, YFP and RFP emission was detected using BP482/35, BP540/30 and BP595/50 emission filters respectively.

Data Analysis. ImageJ (National Institute of Health) was used to analyze the raw microscopy images. Further processing of the data was done in Excel (Microsoft Office) and graphs and statistics were conducted using Graphpad version 6.0 for Mac, GraphPad Software, La Jolla California USA, www.graphpad.com. The non-parametric two-tailed Mann-Whitney test was used to determine p values. We list exact p-values where possible, unless p values < 0.1e-3, since GraphPad does not give exact p-values in these situations.

Boxplots were generated online, using the website <http://boxplot.tyerslab.com/>, showing individual data points, the median (center line) and 95% confidence intervals (notches). Box limits indicate the 25th and 75th percentiles as determined by R software and whiskers extend 1.5 times the interquartile range (IQR) from the 25th and 75th percentiles. The notches are defined as $\pm 1.58 \cdot \text{IQR} / \sqrt{n}$ and represent the 95% confidence interval for each median.

The p-values and 95% confidence intervals are determined to aid the interpretation and are not used as thresholds to determine biological significance⁵¹.

To compare the intensities of the GEF, PM marker and cytosolic marker, line plots in Fig. 1 were made by normalizing the fluorescent intensities in the different channels to the maximum value.

Sequence Analysis. Protein sequences from other species were obtained from - and evolutionary analysis of p63RhoGEF⁵⁸⁰ and p63RhoGEF⁶¹⁹ was performed using a combination of BLAST (NCBI), Ensembl genome browser⁵² and the UCSC human genome browser⁵³. Sequences were aligned and visualized using Jalview⁵⁴.

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Author Contributions

J.v.U., M.M., J.G. performed experiments, analyzed the data and wrote the manuscript. Y.I.W. and T.Y. designed and constructed the DORA-Rho GTPase biosensors. T.W.J.G. assisted with experimental design and interpretation of data. All authors approved the final manuscript.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

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