Supplemental Data

WNT stimulation dissociates a Frizzled 4 inactive state complex with $G\alpha_{12/13}$

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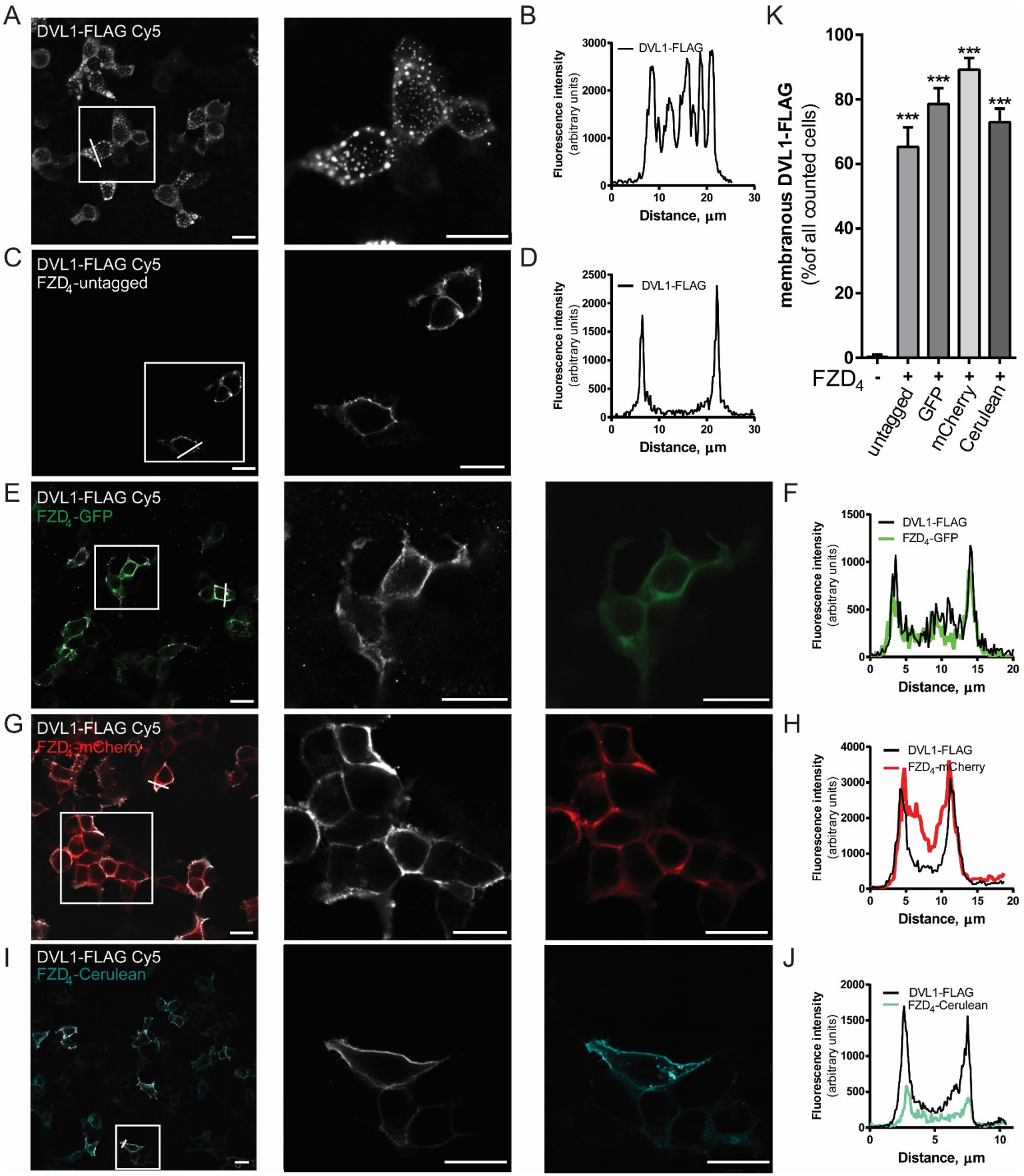
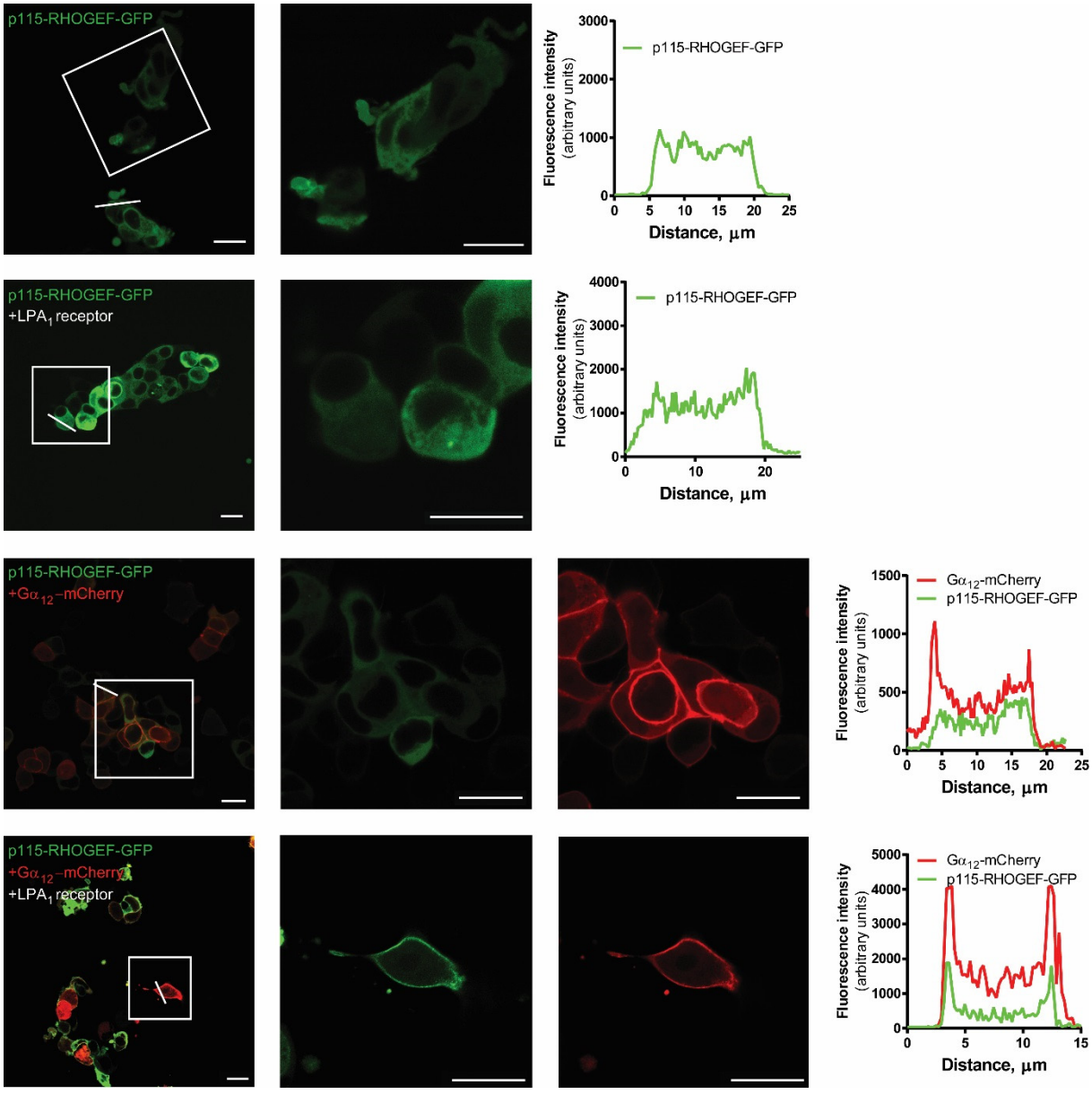
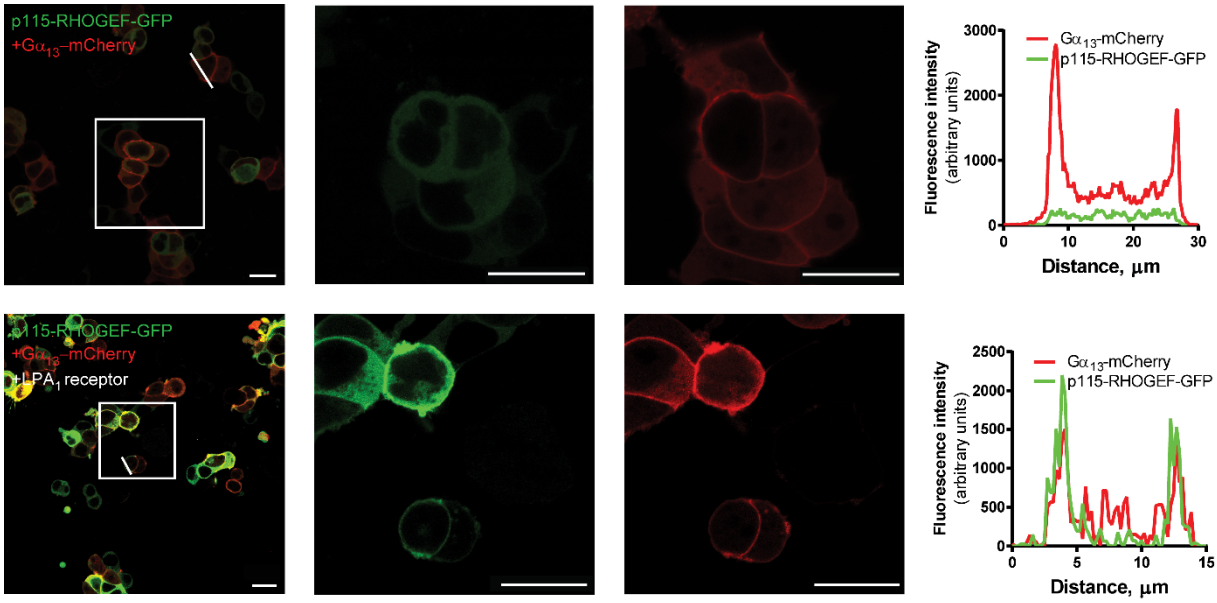
Supplemental Figure 1: Functional validation of C terminally-tagged FZD_4-GFP, FZD_4-mCherry and FZD_4-Cerulean in comparison to untagged FZD_4 by DVL recruitment. HEK293 cells were transfected with DVL1-FLAG alone (**A, B**) or in combination with human FZD_4-untagged, FZD_4-GFP, FZD_4-mCherry or FZD_4-Cerulean at a 3:1 DNA ratio receptor:DVL (**C-J**). After 24 h, cells were fixed in 4% PFA and stained by indirect immunocytochemistry with an anti-FLAG primary antibody and a Cy5-conjugated secondary antibody. Similar to what is known from other Class Frizzled receptors (Tauriello et al., 2012) and the C terminally tagged mouse FZD_4-GFP (Bryja et al., 2007), the C terminally-tagged versions of human FZD_4 used in this study efficiently recruited DVL1-FLAG from intracellular aggregates (see punctate pattern of DVL1-FLAG in absence of FZD coexpression) to a clear membrane localization. Since the C terminal KTxxxW sequence and the flanking regions of the third intracellular loop are involved in FZD-mediated DVL recruitment (Tauriello et al., 2012), this assay validates both membrane integration and functionality of the engineered human FZD_4. The white square marks the magnified area shown in the single-channel images. Size bars 20 µm. The ZEN2013 software was used to generate intensity profiles of the present fluorophores along a line passing through a single cell (**B, D, F, H, J**) . Profiles were created spanning from membrane to membrane crossing the cytosol but excluding the nucleus (white line across cell). The experiments were done three times and the results are summarized in the bar graph in (**K**). Between 12 and 296 cells per condition and independent experiment were counted by an observer blinded to the experimental condition Data are presented as mean±SEM. *** $p<0.001$ (all columns compared to control without FZD_4 expression). For figure see next page.


A
DVL1-FLAG Cy5
B
DVL1-FLAG
Fluorescence intensity (arbitrary units)
Distance, µm
K
membranous DVL1-FLAG (%of all counted cells)
FZD4 - + + + +
untagged
GFP
mCherry
Cerulean
C
DVL1-FLAG Cy5
FZD4-untagged
D
DVL1-FLAG
E
DVL1-FLAG Cy5
FZD4-GFP
F
DVL1-FLAG
FZD4-GFP
G
DVL1-FLAG Cy5
FZD4-mCherry
H
DVL1-FLAG
FZD4-mCherry
I
DVL1-FLAG Cy5
FZD4-Cerulean
J
DVL1-FLAG
FZD4-Cerulean

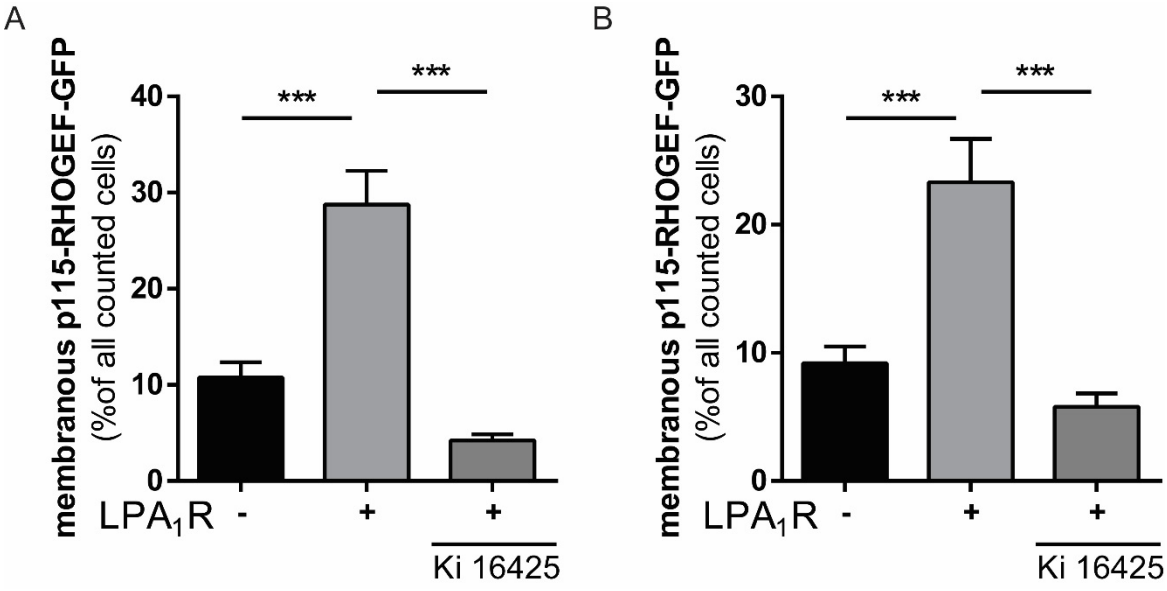
Supplemental Figure 2: Functional validation of N terminally-tagged $G\alpha_{12}$-mCherry employing p115-RHOGEF recruitment. HEK293 cells were expressed with p115-RHOGEF-GFP, LPA_1 receptor (untagged), and N terminally-tagged $G\alpha_{12/13}$-mCherry either alone or in combination (indicated in the figure). In all conditions, untagged $\beta\gamma$ subunits were coexpressed. p115-RHOGEF-GFP shows an even cytosolic distribution, which is not affected by cotransfection with LPA_1 receptor or with $G\alpha_{12}$-mCherry. However, the combination of $G\alpha_{12}$-mCherry and LPA_1 receptor induced a strong recruitment of p115-RHOGEF-GFP to the membrane-localized $G\alpha_{12}$-mCherry. Both the strict membrane localization and the effective p115-RHOGEF-GFP membrane recruitment underline that correct processing and functionality of the N terminally-tagged $G\alpha_{12}$-mCherry used in this study. The white square marks the magnified area shown in the single-channel images. Size bars 20 µm. N=3. Data quantification is presented in Supplemental Figure 4A. The ZEN2013 software was used to generate intensity profiles of the present fluorophores along a line passing through a single cell (white line across cell).



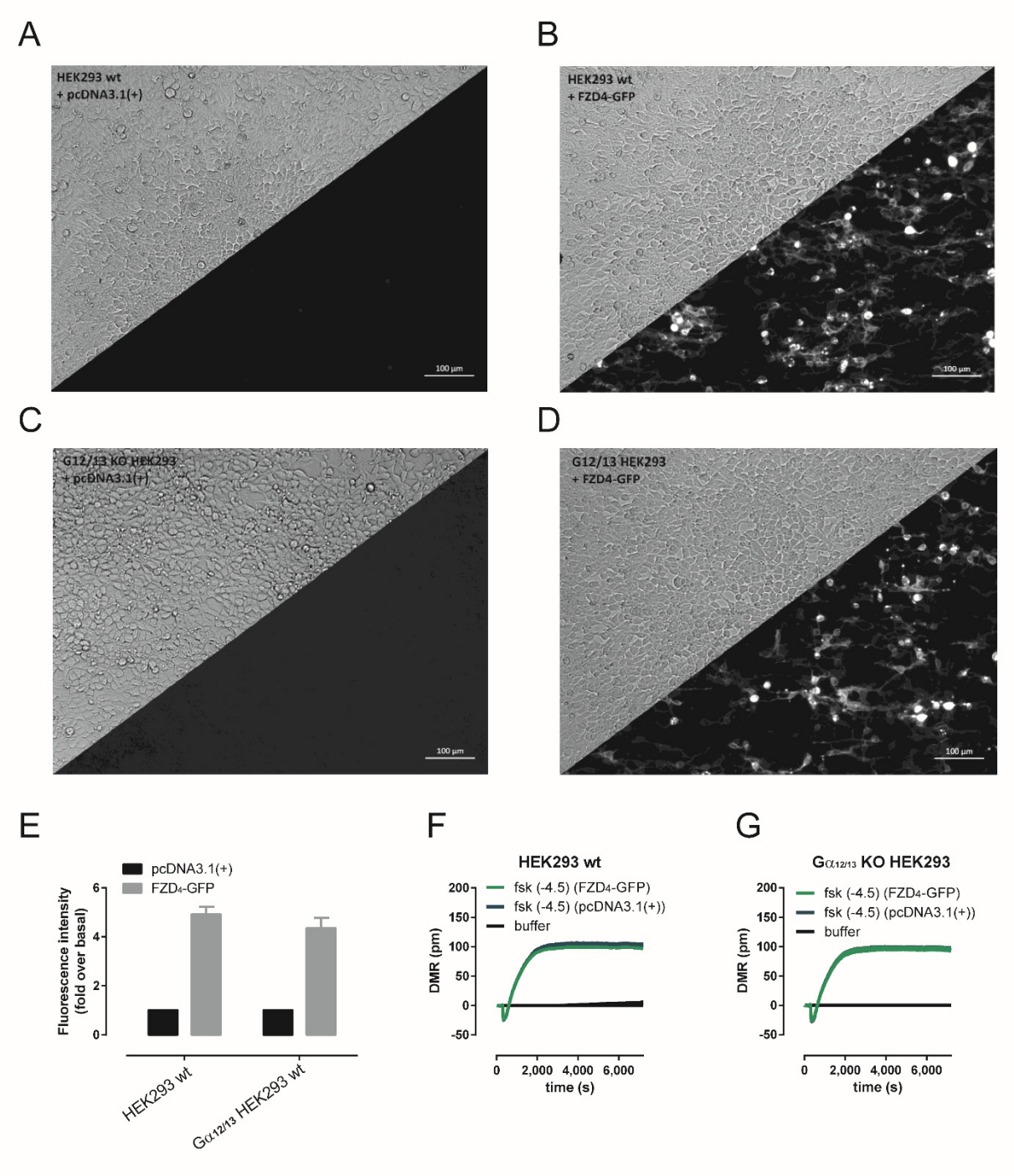
Supplemental Figure 3: Functional validation of N terminally-tagged Gα$_{13}$-mCherry employing p115-RHOGEF recruitment. HEK293 cells were expressed with p115-RHOGEF-GFP, LPA$_1$ receptor (untagged), and N terminally-tagged Gα$_{13}$-mCherry. In all conditions, untagged βγ subunits were coexpressed. p115-RHOGEF-GFP shows an even cytosolic distribution, which is not affected by cotransfection with LPA$_1$ receptor or with Gα$_{13}$-mCherry (see Supplemental Figure 2). However, the combination of Gα$_{13}$-mCherry and LPA$_1$ receptor induced a strong recruitment of p115-RHOGEF-GFP to the membrane-localized Gα$_{13}$-mCherry. Both the strict membrane localization and the effective p115-RHOGEF-GFP membrane recruitment underline that correct processing and functionality of the N terminally-tagged Gα$_{13}$-mCherry used in this study. The white square marks the magnified area shown in the single-channel images. Size bars 20 µm. N=3. Data quantification is presented in Supplemental Figure 4B. The ZEN2013 software was used to generate intensity profiles of the present fluorophores along a line passing through a single cell (see arrow).



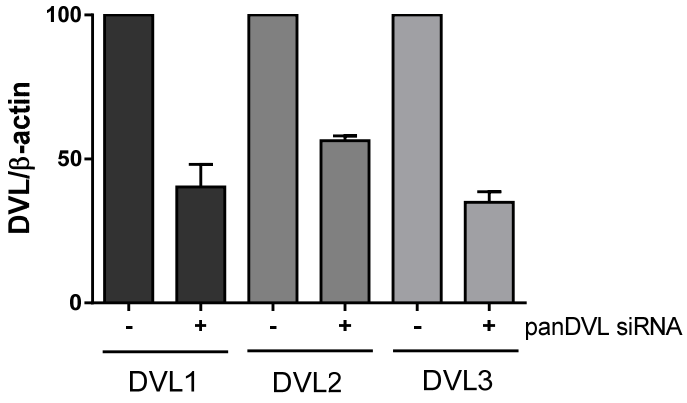
Supplemental Figure 4: Quantification of LPA_1 receptor-mediated p115-RHOGEF-GFP recruitment. Bar Graphs provide quantitation of the data presented in Supplemental Figure 2 and 3. HEK293 cells were transfected with p115-RHOGEF-GFP, LPA_1 receptor (untagged), either N terminally-tagged $G\alpha_{12}$ (**A**) or $G\alpha_{13}$-mCherry (**B**). In all conditions, untagged $\beta\gamma$ subunits were coexpressed. Cells were left either untreated or treated with 10 μM of the LPA_1R selective antagonist/inverse agonist Ki 16425. Data from 3 independent experiments are summarized in bar graphs (mean±SEM). More than 60 cells/condition and independent experiment were counted by a blinded observer. ** p<0.01; *** p<0.001.



Supplemental Figure 5: Expression levels of FZD_4-GFP and forskolin-induced DMR responses in wt and $G\alpha_{12/13}$-knock out HEK293 cells. (**A-D**) Brightfield and GFP channel images of HEK293 wild-type (**A,B**) or $G\alpha_{12/13}$ KO HEK293 cells (**C,D**) transfected with pcDNA3.1(+) or FZD_4-GFP. Scale bar indicates 100µm. (**E**) Quantification of FZD_4-GFP expression by measuring fluorescence intensity of GFP-tagged receptor constructs. (**F,G**) Forskolin (fsk)-induced DMR response of HEK293 wild-type (**F**) and $G\alpha_{12/13}$ KO HEK293 cells (**G**) either transfected with empty vector or FZD_4-GFP. Shown are representative traces, buffer corrected and measured in triplicates +SEM.



Supplemental Figure 6: Expression levels of DVL1, 2, 3 in HEK293 cells treated with ctrl and panDVL siRNA. The bar graph summarizes three independent experiments (corresponding to Fig. 5B), where DVL1, 2, 3 expression was quantified by densitometry of an immunoblot analysing cell lysates from ctrl siRNA and panDVL siRNA treated HEK293 cells. Endogenous levels of DVL1, 2, 3, were detected with anti-DVL1, 2 or 3 antibodies and quantified. DVL values were normalized to β-actin expression. Graph presents mean±SEM.



References

Bryja V, Gradl D, Schambony A, Arenas E and Schulte G (2007) beta-arrestin is a necessary component of Wnt/beta-catenin signaling in vitro and in vivo. *P Natl Acad Sci USA* **104**(16): 6690-6695.

Tauriello DV, Jordens I, Kirchner K, Slootstra JW, Kruitwagen T, Bouwman BA, Noutsou M, Rudiger SG, Schwamborn K, Schambony A and Maurice MM (2012) Wnt/beta-catenin signaling requires interaction of the Dishevelled DEP domain and C terminus with a discontinuous motif in Frizzled. *Proc Natl Acad Sci U S A* **109**(14): E812-820.