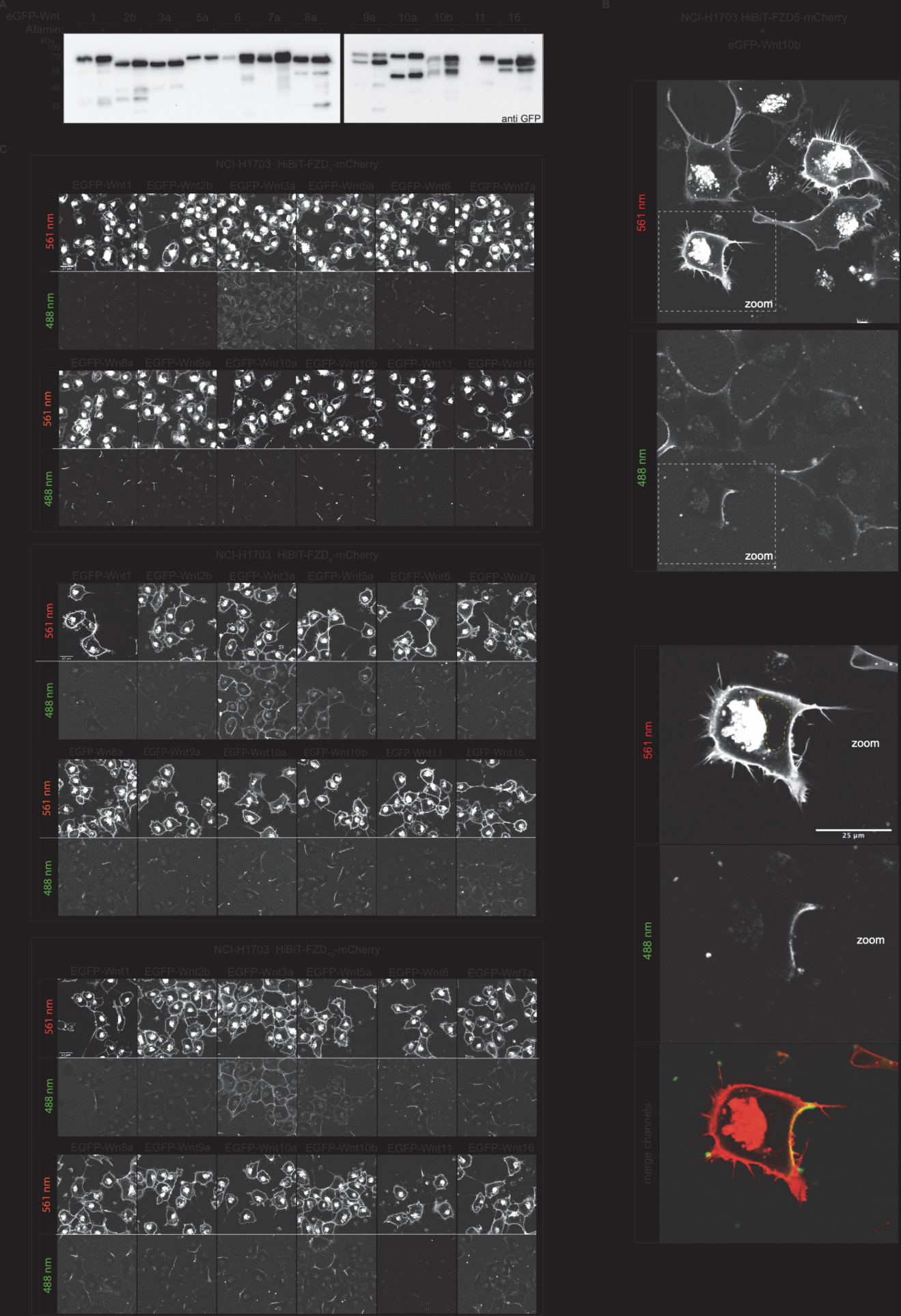
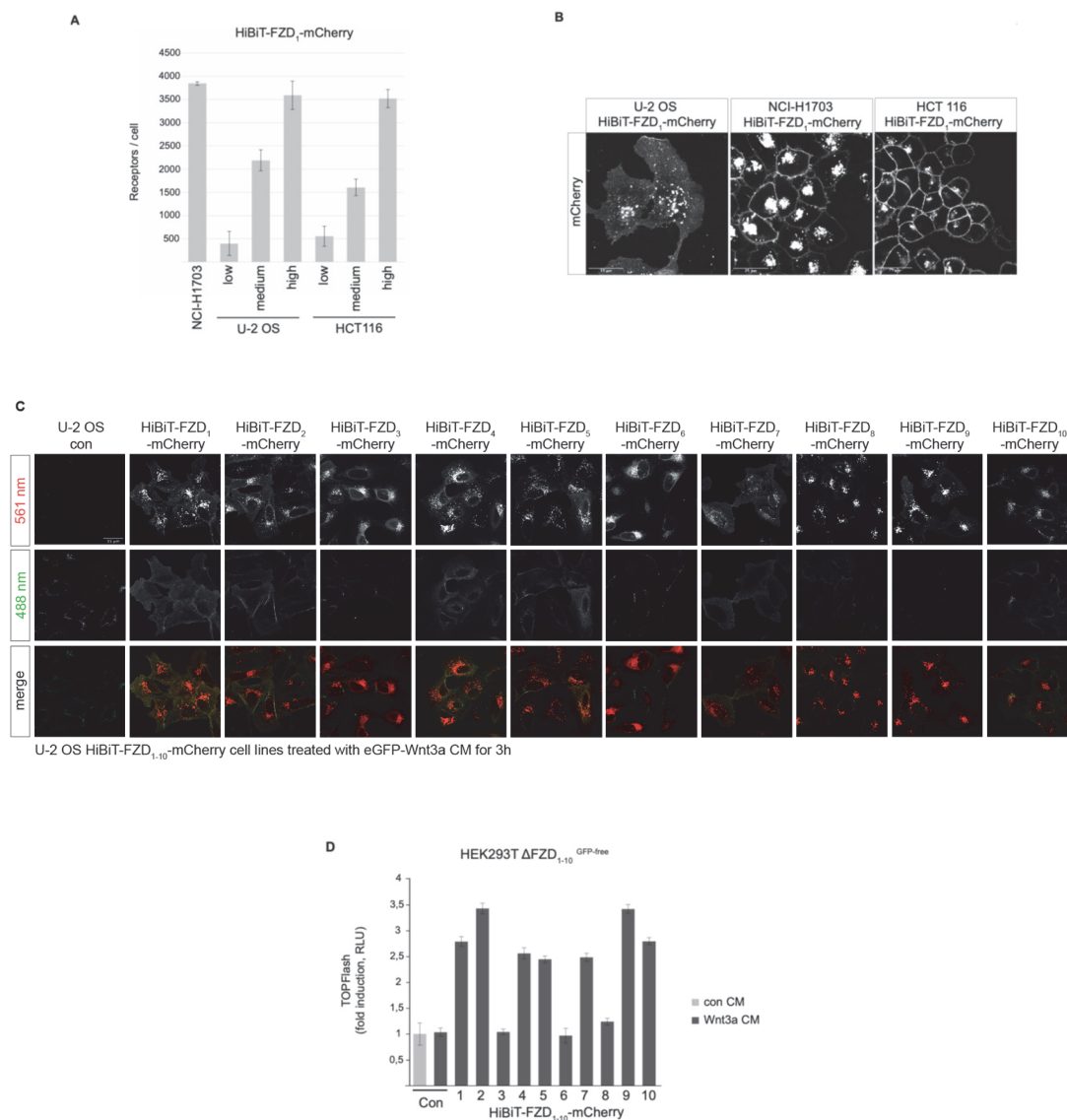


Supplementary Figure S1



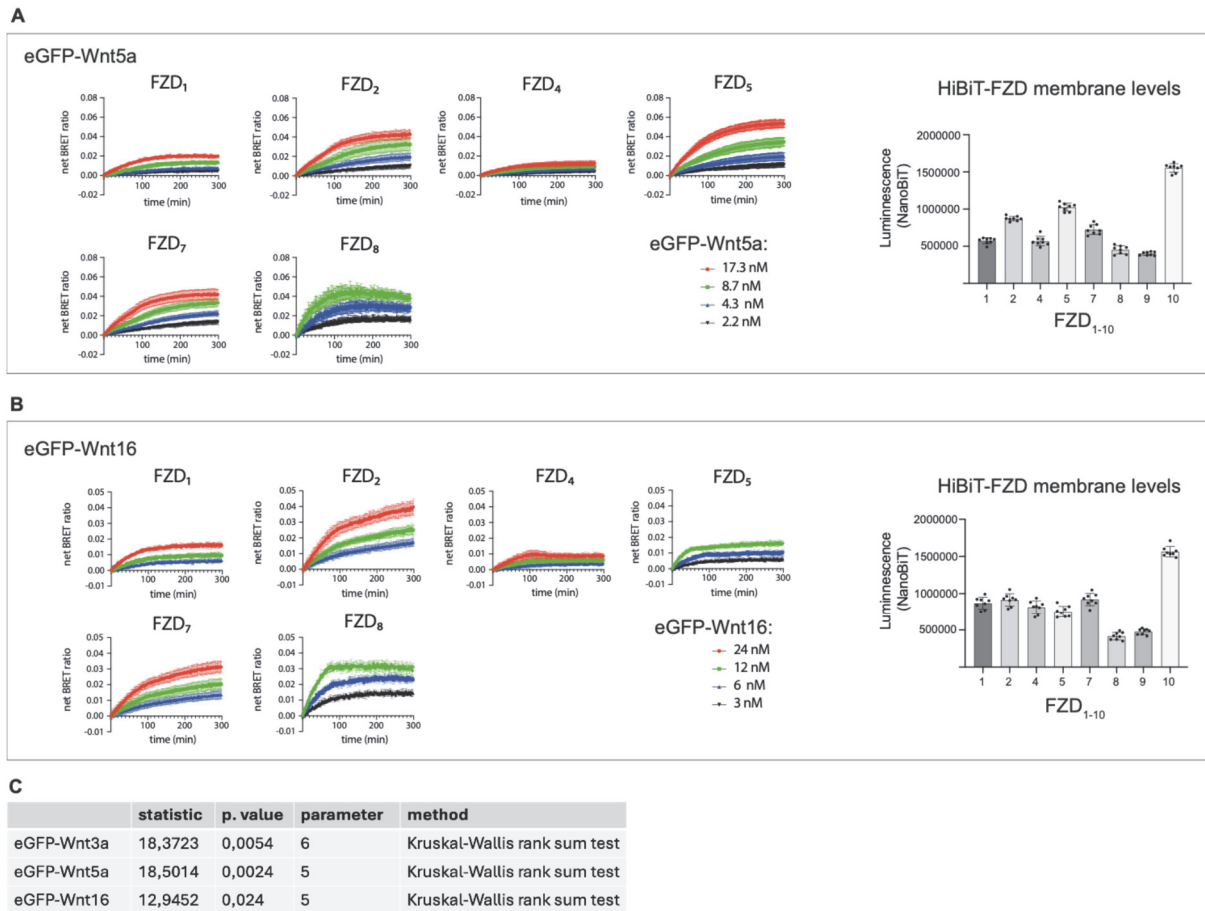
Supplementary Figure S1. Generation and testing of eGFP-Wnt fusion proteins. **A)** Western Blot (WB) of conditioned medium (CM) harvested from HEK293T cells transfected with the indicated constructs, confirming the secretion of full length eGFP-Wnt proteins with/without Afamin co-expression. **B)** Laser scanning confocal microscopy images of NCI-H1703 cells with stable integration of mouse HiBiT-FZD₅-mCherry, incubated for 3 h with eGFP-Wnt10b CM derived from Expi293TM suspension cells. The three lower images represent zoomed-in images of boxed areas shown in the upper images and highlight the asymmetric nature of how some Wnt's appear to associate to cells. **C)** Laser scanning confocal microscopy images of NCI-H1703 cells with stable integration of mouse HiBiT-FZD_{4,8,10}-mCherry, incubated for 3 h with the indicated eGFP-Wnt CM derived from Expi293TM suspension cells.

Supplementary Figure S2



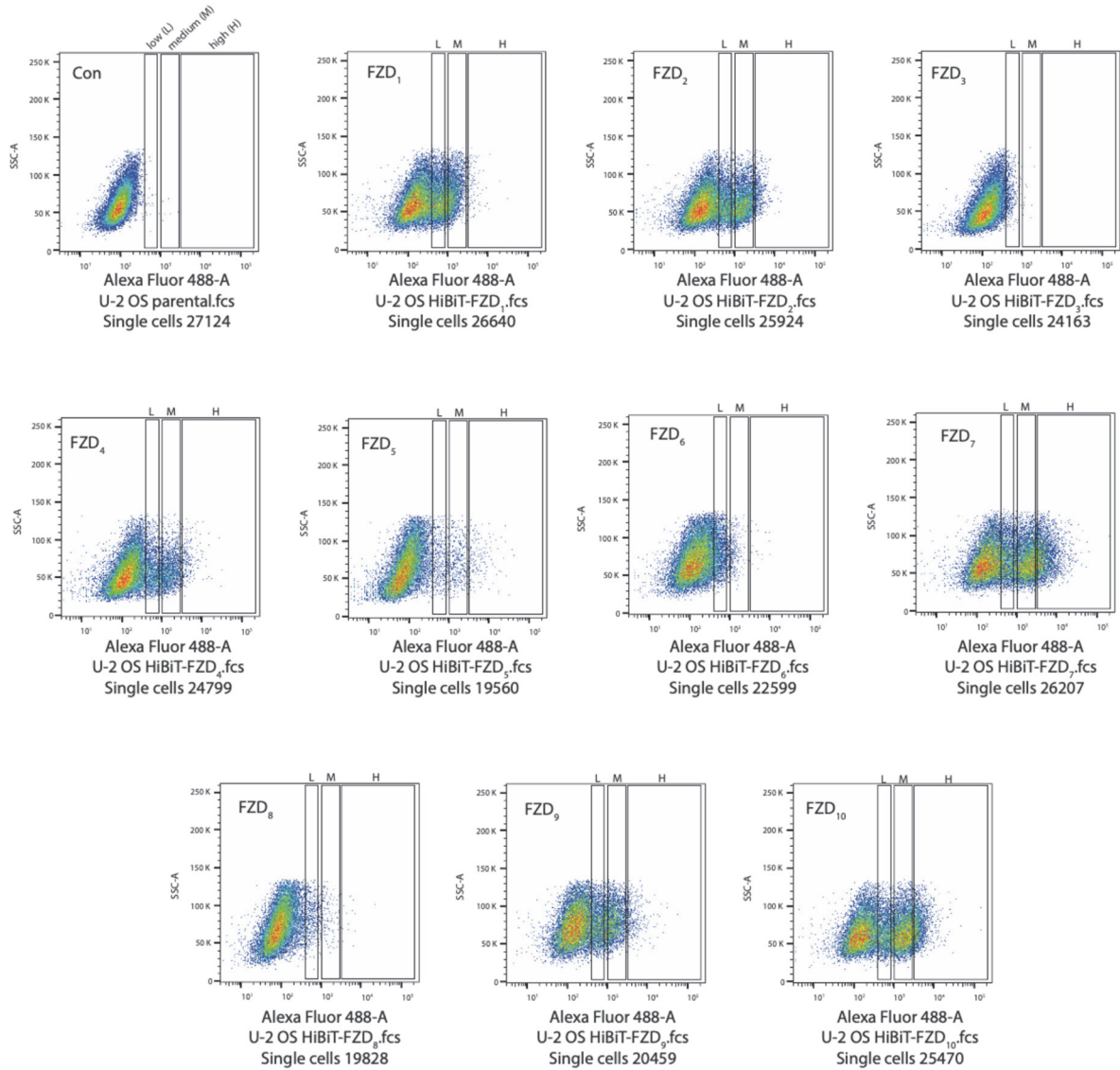
Supplementary Figure S2. Comparison of stable expression levels of HiBiT-FZD₁ in NCI-1703 U-2 OS and HCT116 cells. **A)** Indirect quantification of HiBiT-FZD₁-mCherry at the cell surface of the indicated FACS sorted stable cell lines. **B)** Laser scanning confocal microscopy images comparing the expression of HiBiT-FZD₁-mCherry in the indicated FACS sorted cells. **C)** Laser scanning confocal microscopy images of living U-2 OS cells with stable integration of mouse HiBiT-FZD-mCherry constructs as indicated. Cells were incubated for 3 h with eGFP-Wnt3a condition medium (CM) derived from Expi293TM suspension cells. **D)** TOPFlash reporter assay in Δ FZD₁₋₁₀^{GFP-free} HEK293 cells, showing the relative Wnt/ β -catenin signaling activity of the indicated mouse HiBiT-FZD-mCherry receptors. Error bars represent $\pm$ S.D. from means of 4 independent biological samples.

Supplementary Figure S3



Supplementary Figure S3. Comparison of Wnt5a and Wnt16 binding to multiple FZD expressing cell lines. Association kinetics of eGFP-Wnt5a (**A**) and eGFP-Wnt16 (**B**) with U-2 OS cells expressing the indicated HiBiT-FZD proteins. Binding curves were generated over time using 2.2, 4.3, 8.7, 17.3 nM of eGFP-Wnt5a or 3, 6, 12, 24 nM of eGFP-Wnt16. BRET ratios were measured every 80 s for a total of 5 h. Raw data were fitted to the 'two or more hot concentrations model' and are presented as mean $\pm$ SEM from $n=3-4$ individual experiments. Estimation of relative HiBiT-FZD cell surface levels in FACS-sorted U-2 OS cells used for each assay are shown in graphs on right. C) The open source software R was used for Kruskal-Wallis rank sum test statistics to determine significance between different FZD samples within experiments.

Supplementary Figure S4



Supplementary Figure S4. FACS sorting of U-2 OS cells stably expressing HiBiT-FZD₁₋₁₀. Single cell sorting of U-2 OS cells stably expressing HiBiT-FZD-mCherry at the cell surface. U-2 OS cell suspensions were incubated with mouse anti-HiBiT antibody following by labelling with anti-mouse Alexa 488 antibody. All cells were analyzed on one dimensional plots of the green channel- 488 nm for HiBiT positive cells. Gated in black as low (L), medium (M) and high (H) are cell populations with different expression levels of HiBiT-FZD at the cell surface. Parental U-2 OS cells were used as background control. Cells gated for low, medium and high were single cell sorted in 96 well plate.