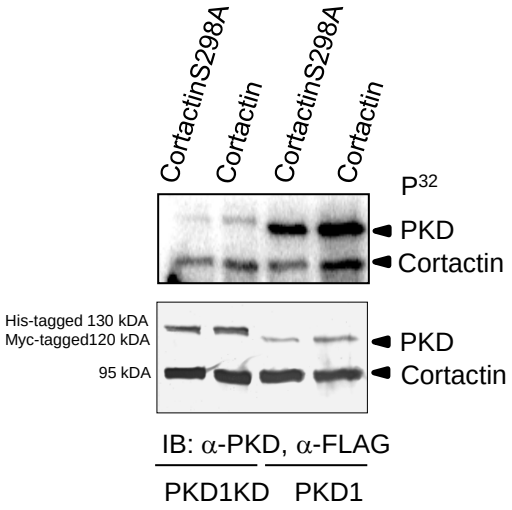
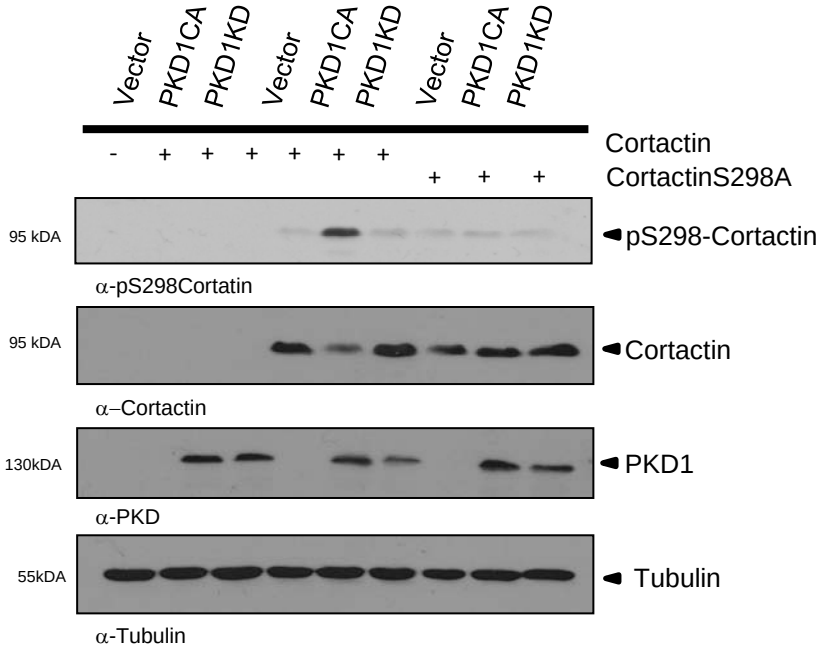
Supplemental data:

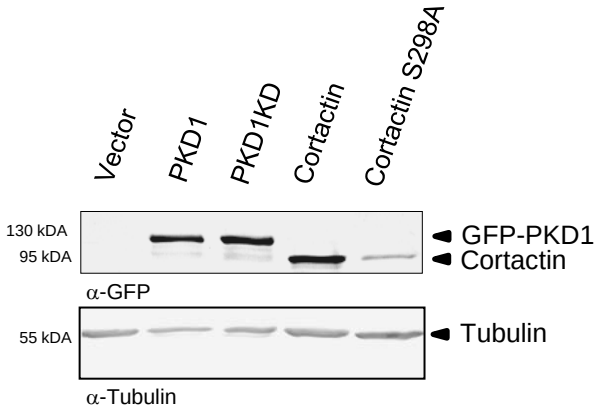
S1A



S1B

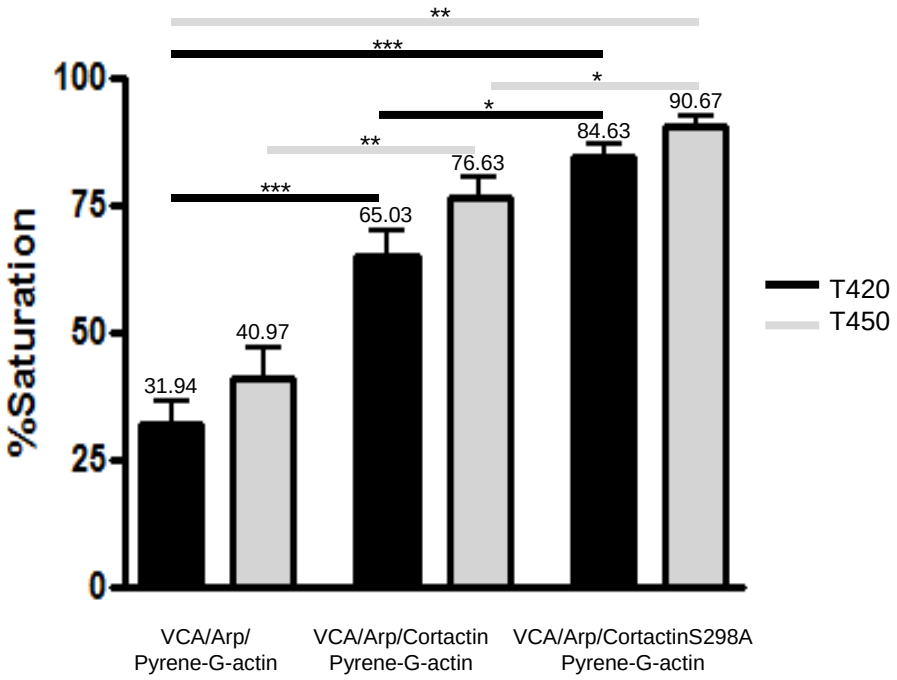


S 2

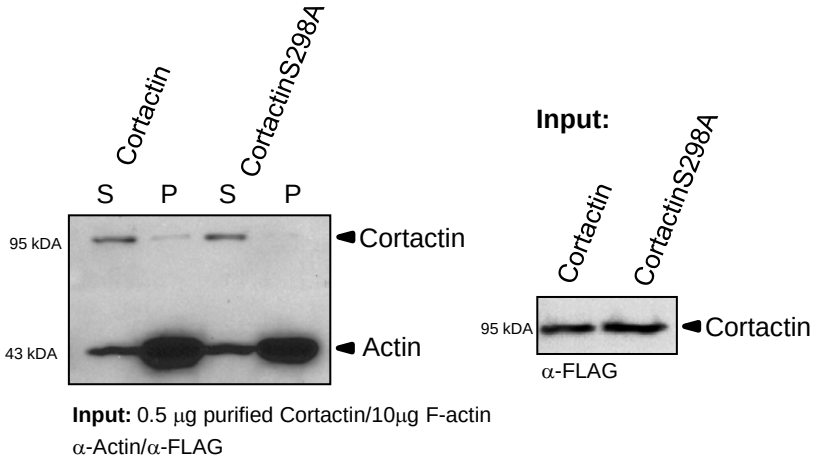

Vector
PKD1
PKD1KD
Cortactin
Cortactin S298A
130 kDA
95 kDA
GFP-PKD1
Cortactin
α-GFP
55 kDA
Tubulin
α-Tubulin

S 3

3A



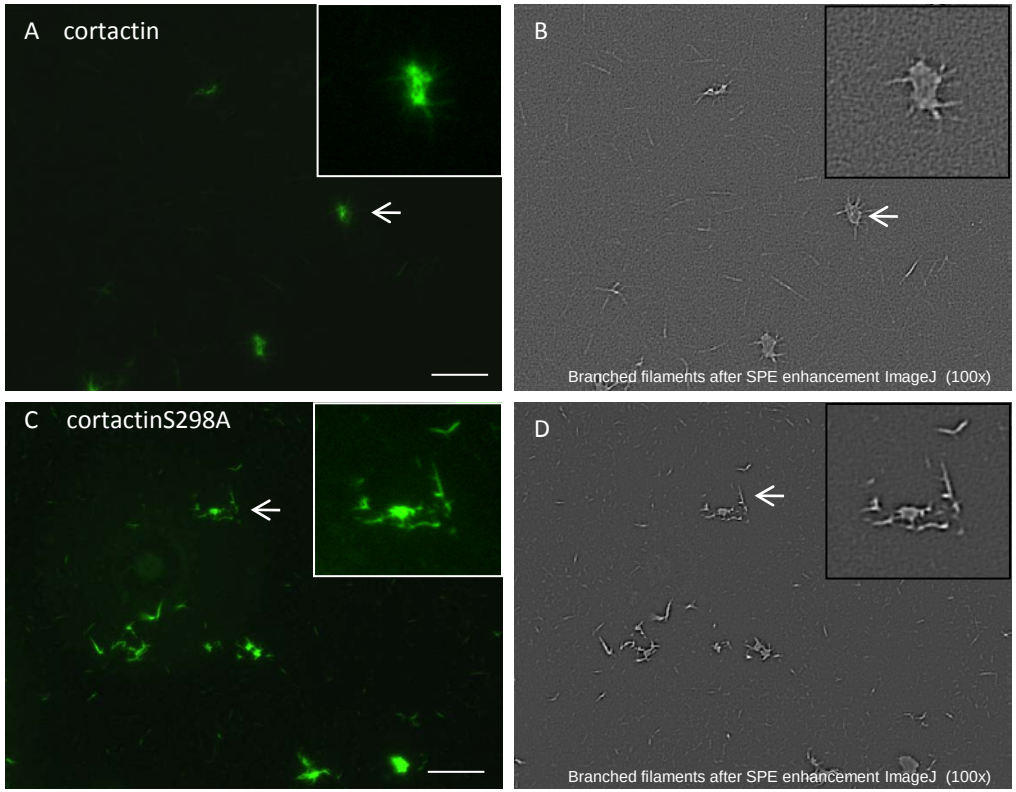
3B



Input: 0.5 μg purified Cortactin/10μg F-actin

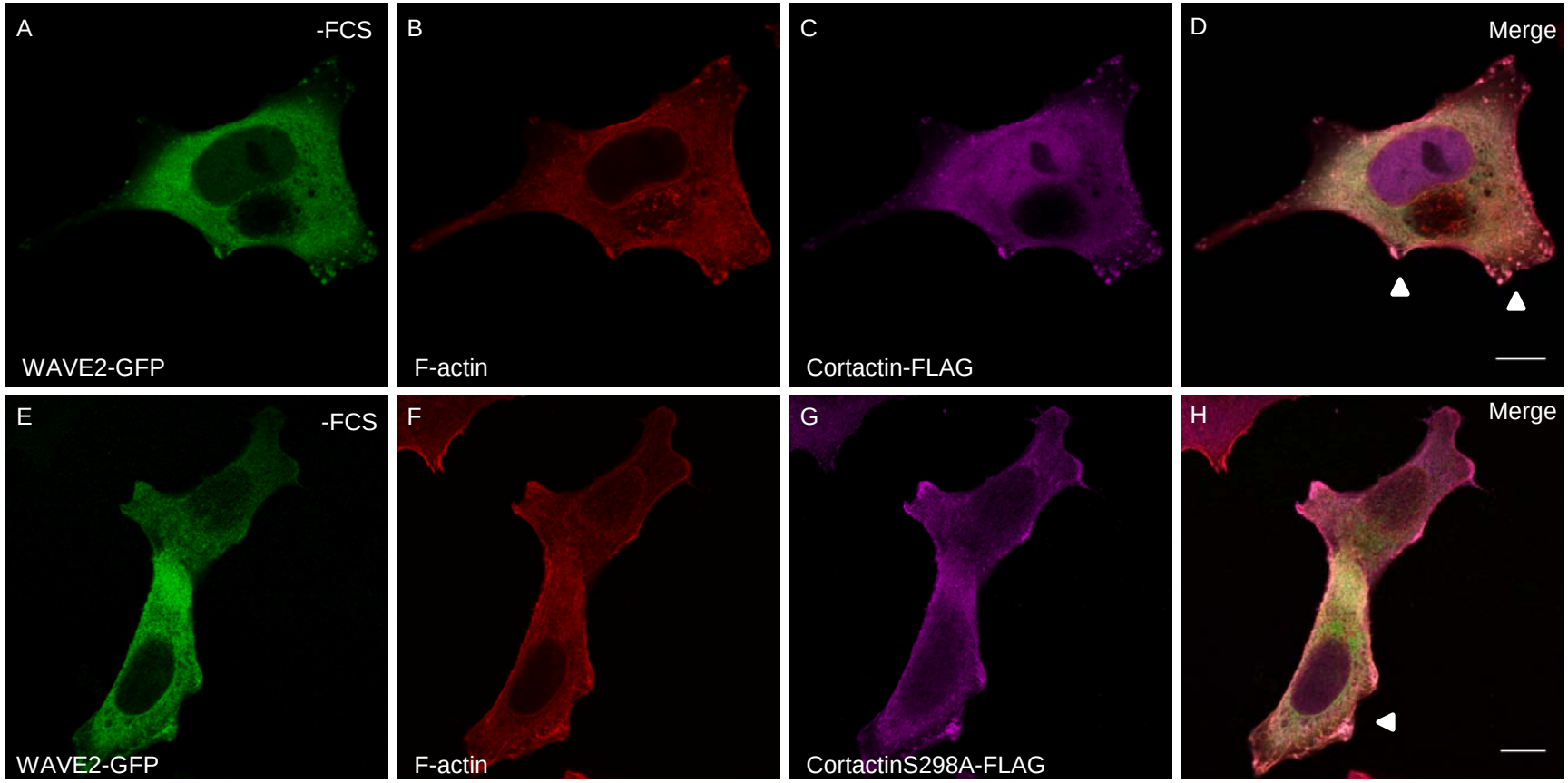
α-Actin/α-FLAG

3C

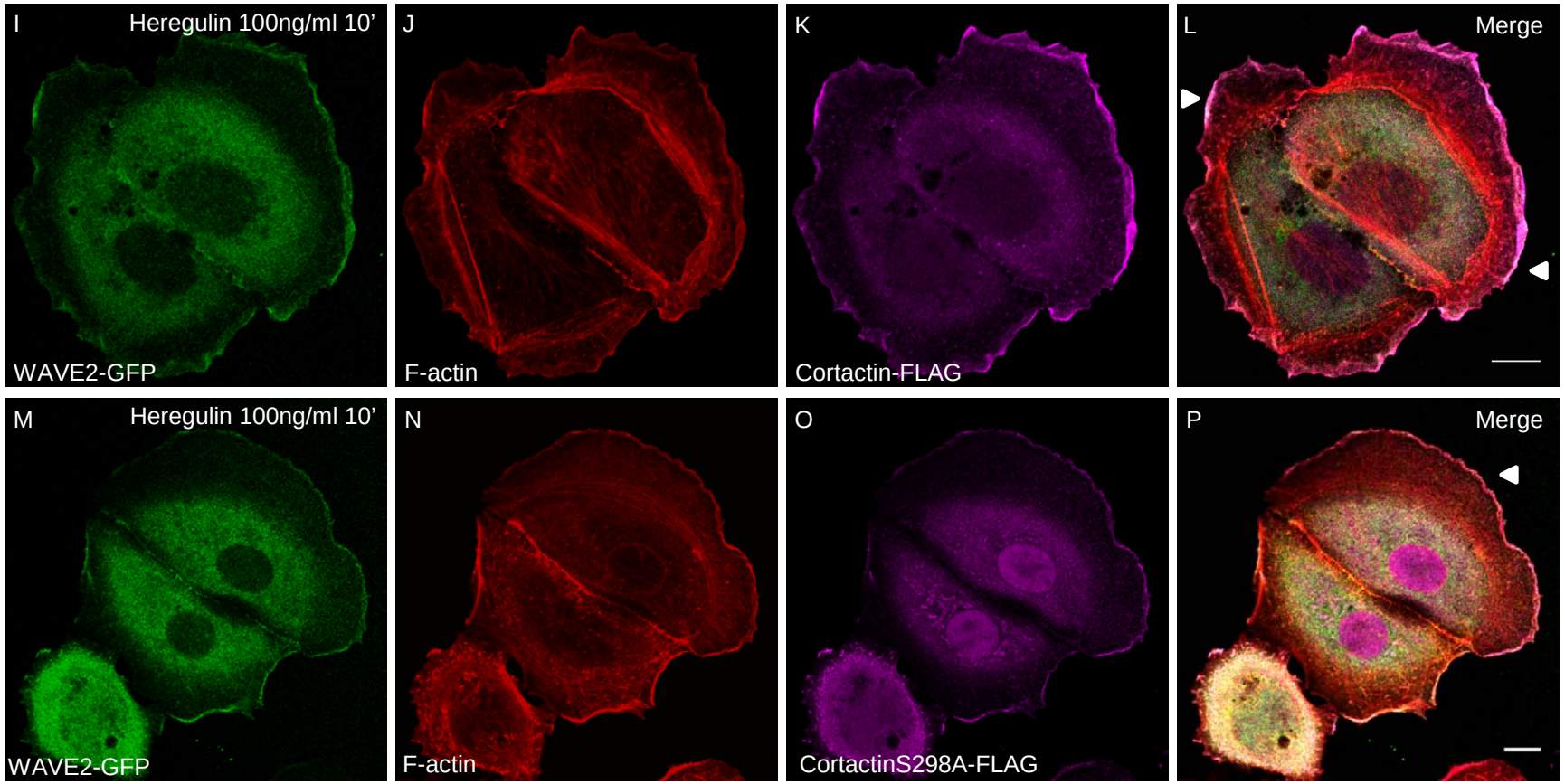


protein	cortactin	cortactinS298A
Filaments (n=5 images)	813	1743
branches >2	39	60
Ratio: filament/branches	20.85	29.05
% branches	**4.8**	**3.4**

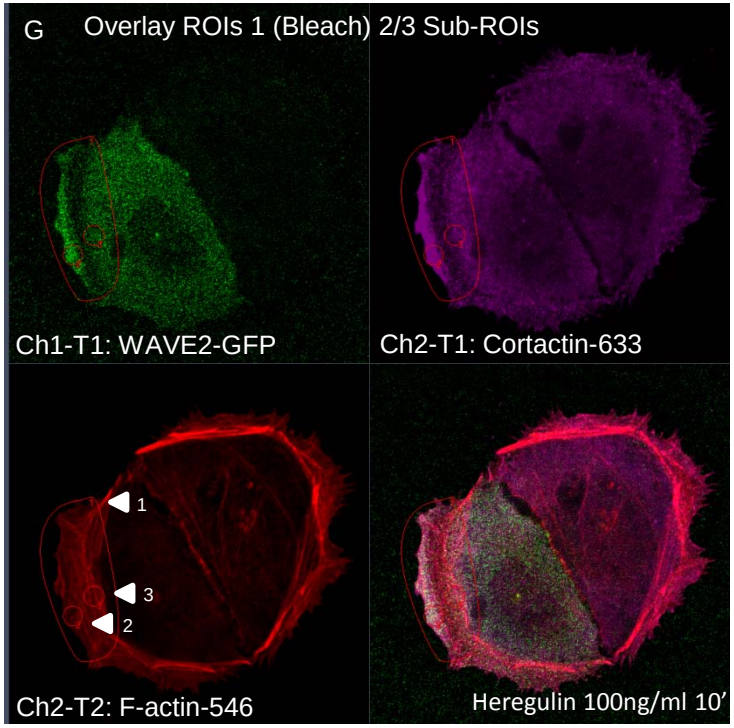
S 4A


A
-FCS
WAVE2-GFP
B
F-actin
C
Cortactin-FLAG
D
Merge
E
-FCS
WAVE2-GFP
F
F-actin
G
CortactinS298A-FLAG
H
Merge

S 4B

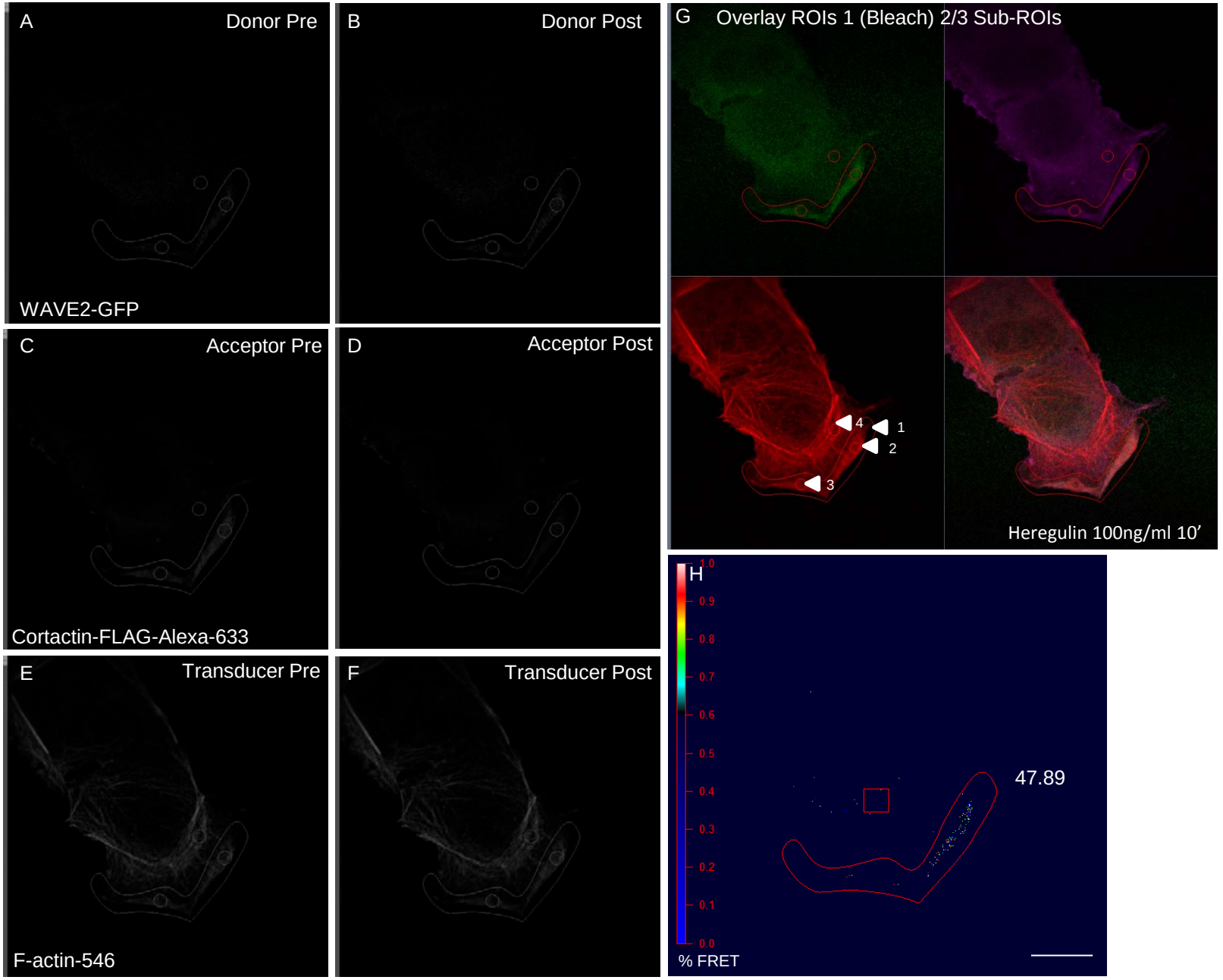

I
Heregulin 100ng/ml 10’
WAVE2-GFP
J
F-actin
K
Cortactin-FLAG
L
Merge
M
Heregulin 100ng/ml 10’
WAVE2-GFP
N
F-actin
O
CortactinS298A-FLAG
P
Merge

S 4C

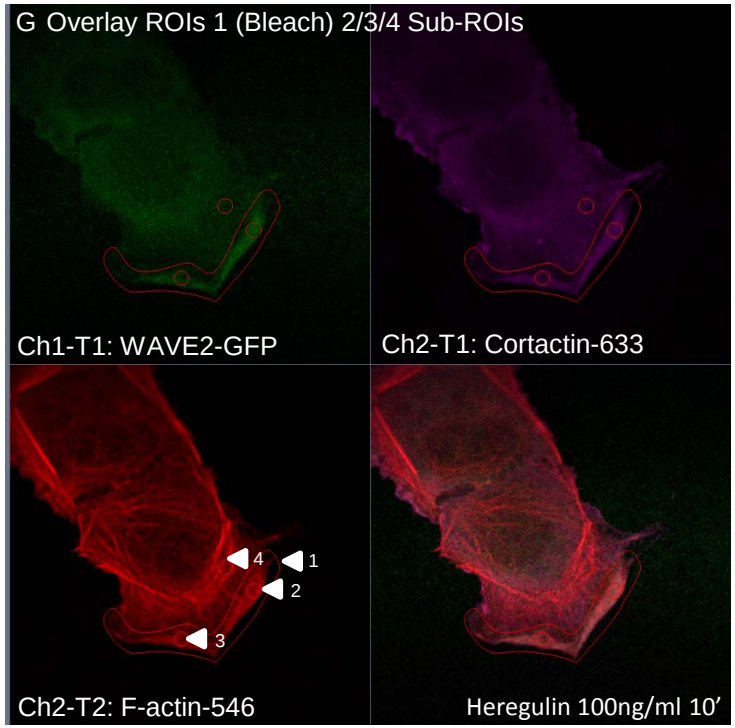


Intensity Mean of ROI	Roi1 Ch1-T1	Roi1 Ch2-T1 Bleach	Roi1 Ch2-T2	Roi2 Ch1-T1	Roi2 Ch2-T1 Bleach	Roi2 Ch2-T2	Roi3 Ch1-T1	Roi3 Ch2-T1 Bleach	Roi3 Ch2-T2
Pre	11.4	39.9	9.6	14.5	60.9	19.1	10.5	36.7	30.5
Post	14	36.7	10	17.1	23.2	19.2	11.2	16	30.5
% FRET	Donor Ch1-T1: 18.57 Donor Ch2-T2: 4			Donor Ch1-T1: 15.2 Donor Ch2-T2: 0.52			Donor Ch1-T1 No FRET Donor Ch2-T2 No FRET		

S 4D

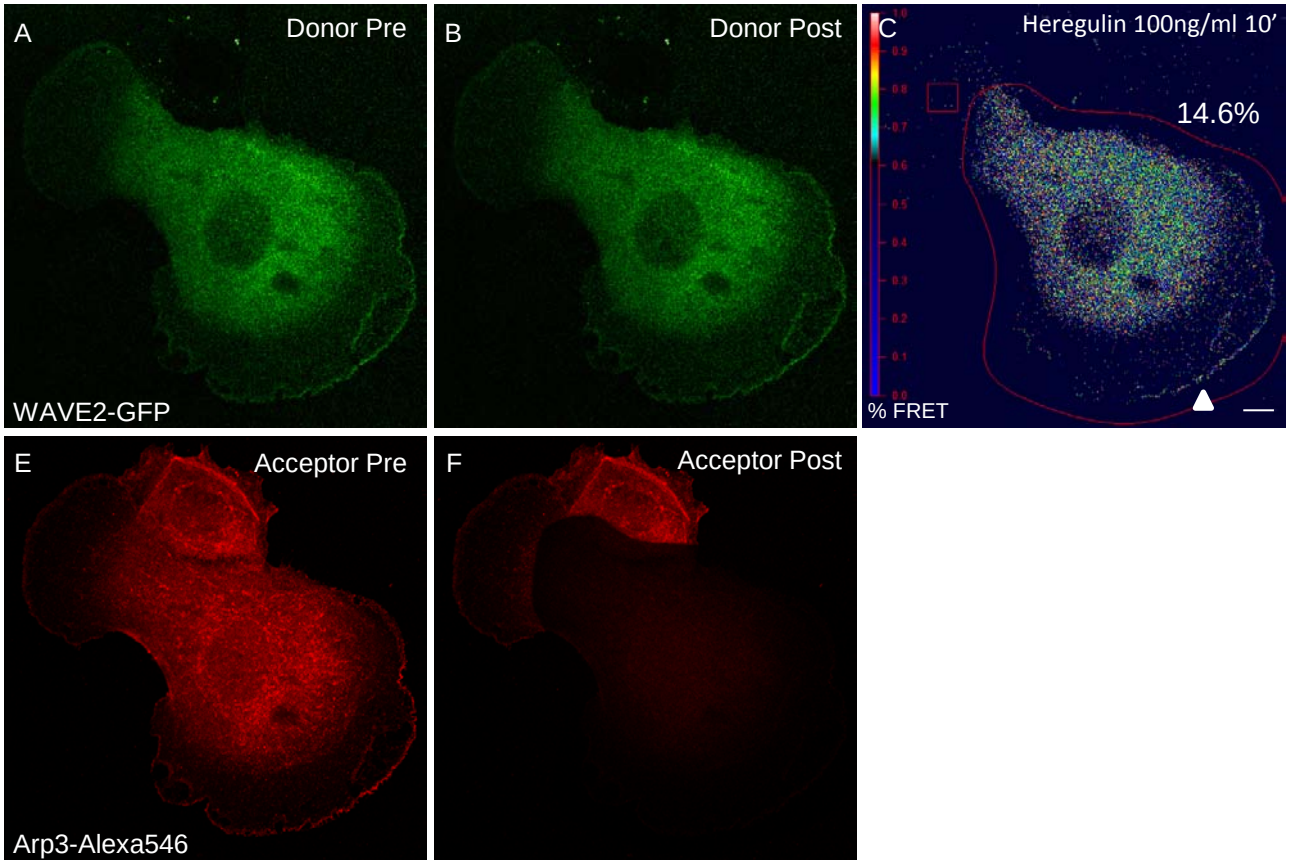

A
Donor Pre
WAVE2-GFP
B
Donor Post
C
Acceptor Pre
Cortactin-FLAG-Alexa-633
D
Acceptor Post
E
Transducer Pre
F-actin-546
F
Transducer Post
G
Overlay ROIs 1 (Bleach) 2/3 Sub-ROIs
4
1
2
3
Heregulin 100ng/ml 10’
H
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
% FRET
47.89

S 4D

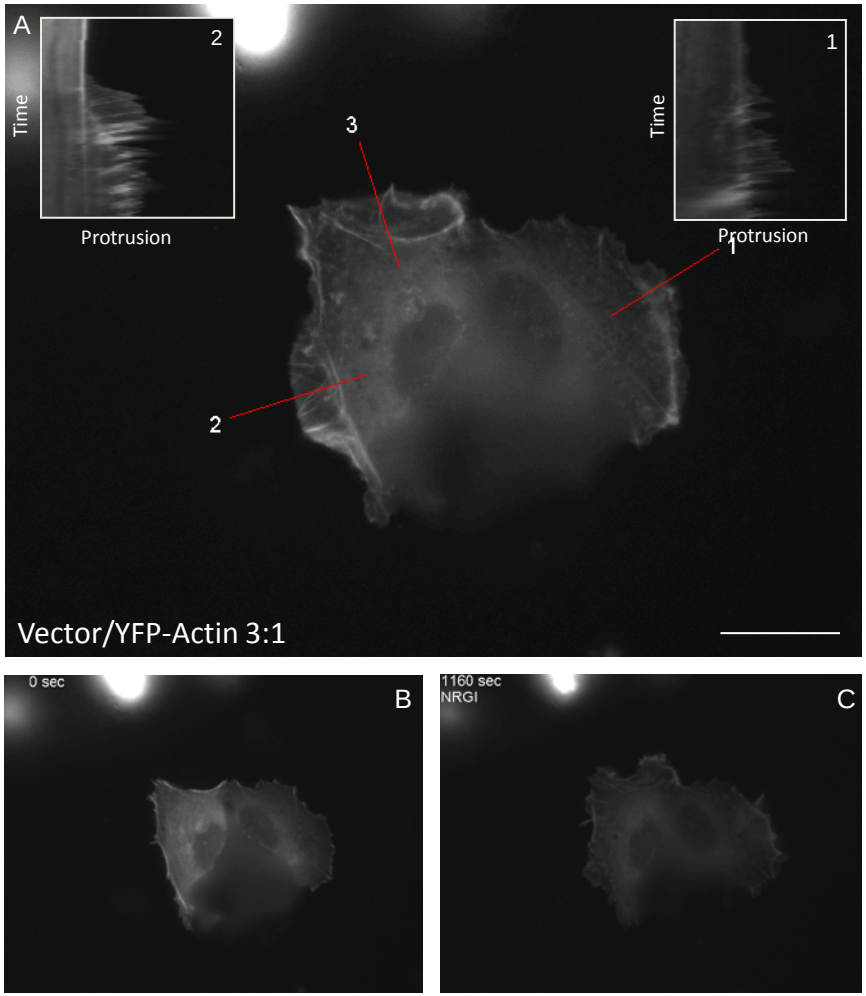


Intensity Mean of ROI	Roi1 Ch1-T1	Roi1 Ch2-T1 Bleach	Roi1 Ch2-T2	Roi2 Ch1-T1	Roi2 Ch2-T1 Bleach	Roi2 Ch2-T2	Roi3 Ch1-T1	Roi3 Ch2-T1 Bleach	Roi3 Ch2-T2	Roi4 Ch1-T1 Off bleach area	Roi4 Ch2-T1	Roi4 Ch2-T2
Pre	10.7	32.3	46.9	27.6	115.2	91.7	16.2	66.9	64.7	11.5	32.1	97.6
Post	11.5	25.6	45.2	30.8	56.1	95.7	15.7	32.3	60.7	11.7	30.8	95.2
% FRET	Donor Ch1-T1: 6.96 Donor Ch2-T2: No FRET			Donor Ch1-T1: 10.39 Donor Ch2-T2: 4.18			Donor Ch1-T1 No FRET Donor Ch2-T2 No FRET			Donor Ch1-T1 BG: 1.71 Donor Ch2-T2 No FRET		

S4E


A
Donor Pre
WAVE2-GFP
B
Donor Post
C
Heregulin 100ng/ml 10’
14.6%
% FRET
E
Acceptor Pre
Arp3-Alexa546
F
Acceptor Post

S 5


A
2
Time
Protrusion
3
1
Time
Protrusion
1
2
Vector/YFP-Actin 3:1
0 sec
B
1160 sec
NRGI
C

Supplemental figures:

Figure S1: PKD phosphorylates cortactin at S298 *in vitro*. *In vitro* kinase assays with purified PKD1 (myc-tagged) and FLAGM2-affinity-purified cortactin, FLAG-cortactinS298A as well as kinase-inactive PKD1 (His-tagged) as control. Autoradiography of the kinase assay is shown in the upper panel, protein levels are depicted below. **(B)** Characterization of the pS298-cortactin phosphorylation following co-expression of active and kinase-inactive PKD1 with FLAG-cortactin or FLAG-cortactinS298. Tubulin was used as a loading control.

Figure S2: Transgene expression of stable Panc89 cells expressing GFP-Vector, PKD1-GFP, PKD1KD-GFP, cortactin-GFP, cortactinS298A-GFP. Expression of transgenes was probed with α-GFP antibody, tubulin was used as a loading control.

Figure S3: (A) Quantification of actin polymerization. %saturation of the polymerization reactions are depicted in the graph for at T420s and T450s time points. Saturation values for reaction were calculated as follows. Asterisks indicate statistical significance calculated by student t-test ($p<0.0001$ ***; $p<0.005$ **; $p<0.05$ *). **(B)** High-speed F-actin binding assays with purified FLAG-cortactin and FLAG-cortactinS298A. 0.5µg of purified cortactin and cortactinS298A were incubated with 10µg *in vitro* polymerized, purified F-actin. Supernatant and 100,000 x g pellet fractions were analyzed by 10% SDS page and visualized by blotting and subsequent detection with α-FLAG and α-actin antibodies. Input levels of proteins are shown at the right hand side of the figure. **(C)** *In vitro* branching assays performed in parallel to the *in vitro* actin polymerization assays **(Fig. 5A)**. 3µM G-actin was polymerized in the presence of an aequimolar amount Alexa-488-Phalloidin with 50nM Arp complex, 400nM GST-VCA and 1000nM FLAG-purified cortactin or cortactinS298A for 10 min. 1:10 dilutions of terminated reactions were mounted on slides and filaments were visualized at 100x magnification by a Leica widefield fluorescence microscope. The sale bar represents 5µM. 5 images each (cortactin (A-B)/cortactinS298A (C-D) were brightness and contrast enhanced, SPE-processed (B, D), segmented, and subjected to automatic detection by a 20-inf filter (NIH ImageJ) after branched filament clusters with >2 branches (white arrows) were singled out manually and excluded from automatic detection. Quantified was the ratio of the total number of filaments (5 images)/highly-branched filament clusters with branches >2 and % branched filaments.

Figure S4: (A) (A-H) WAVE2-GFP, F-actin and FLAG-cortactin (A-D) or FLAG-cortactinS298A (E-H) co-localize in dot-like structures and in membrane ruffles of MCF-7 serum-starved control cells. Additional co-localization of WAVE2-GFP, F-actin and FLAG-cortactin (I-L) or FLAG-cortactinS298A (M-P) in membrane ruffles of MCF-7 lamellipodia as well as in perinuclear regions. As shown in **Fig. 3B** cells were stimulated with Heregulin. Depicted are single confocal sections. Respective relevant structures are highlighted with arrowheads. The scale bar represents 10µm. **(C)** G, overlay images of bleach ROI and Sub-ROIs for quantification of RAW data and calculation of RAW %FRET signals by Mean of ROI analysis **(Fig. 3C)**. Sub-ROI2 within bleach ROI1 has been placed at dynamic ruffling structures in the periphery of Heregulin-stimulated lamellipodia of MCF-7 cells, whereas Sub-ROI3 marks non-dynamic actin arc filaments. Depicted are single confocal sections. Respective relevant structures or ROIs (G) are highlighted with arrowheads. The scale bar represents 10µm. **(D)** Additional 3-dye 'daisy-chain' acceptor-photobleach-FRET experiment with WAVE2-GFP as primary FRET-donor, FLAG-cortactin stained with anti-FLAG M2-Alexa-633 secondary antibody as final FRET-acceptor and F-actin (Phalloidine-Alexa546) as FRET-signal "transducer". Pre- and post-bleach images of donor (A, B), acceptor (C, D) and transducer (E, F) are depicted. FRET was quantified by acquiring a time-bleach image series of cells. %FRET images (H) for the primary donor WAVE2-GFP were generated using ZEN-Software of LSM 710 confocal microscope (Zeiss, Jena). The Bezier bleach ROI and rectangle threshold ROI are

marked by red lines. The scale bar represents 10µm. G, overlay images of bleach ROI and Sub-ROIs for quantification of RAW data and calculation of RAW %FRET signals by Mean of ROI analysis. **(E)** WAVE2-GFP and Arp3 interact at the edge of lamellipodia and in the cytoplasm of Heregulin-stimulated MCF-7 cells. Exemplary acceptor-photobleach-FRET experiment with WAVE2-GFP as FRET-donor and endogenous Arp3 stained with a monoclonal primary and Alexa-546 secondary antibody as FRET-acceptor. Respective pre- and post-bleach images of donor (A, B) and acceptor (C, D) are depicted. FRET was quantified by acquiring a time-bleach image series of cells. %FRET images (E) were generated using ZEN-Software of LSM 710 confocal microscope (Zeiss, Jena). The Bezier bleach ROI and rectangle threshold ROI are marked by red lines. The scale bar represents 10µm.

Figure S5: (A-C) Vector controls (Supplemental Fig.7) showed in line with (16) reduced protrusion persistence when compared to cortactin and cortactinS298A cells. Plotted in the figure are overlay images with kymography lines and samples of respective kymograms. The scale bar represents 10 µM. The reduced persistence does not allow for a similar quantification of protrusion velocity.