Supplementary figure legends

Fig. S1: Functional analysis and subcellular localization of DLC3-WT and -K725E in HeLa cells. (A, B) HeLa cells transiently expressing GFP alone, GFP-DLC3-K725E or GFP-DLC3-WT (green) were stained for phalloidin (red) and nuclei were counterstained with DAPI (blue). Representative maximum projections are shown and imaging parameters were identical. (B) Quantification of the mean fluorescence intensity of phalloidin per cell. Data show the mean ± s.e.m. (n=3, N≥25 cells); ns, not significant; **P=0.0021 (one-way ANOVA followed by Tukey´s post-test). (C) HeLa cells were co-transfected overnight with the RhoA WT biosensor pTriEx-RhoA FLARE.sc and empty FLAG vector, FLAG-DLC3-K725E or FLAG-DLC3-WT, and on the next day, cells were stained for FLAG. FRET efficiencies were measured over the whole cell. Results of the cells from two independent experiments are depicted as the mean ± s.e.m. (N≥40 cells); ns, not significant; ***P<0.0001 (one-way ANOVA followed by Tukey´s post-test). (D) HeLa cells transiently expressing GFP-DLC3-K725E (green) were either stained for Rab6 or TGN46 (red). In the case of RhoB, cells were co-transfected with vectors encoding mCherry-DLC3-K725E (depicted in green) and CFP-RhoB (depicted in red). Scale bars: 10 µm.

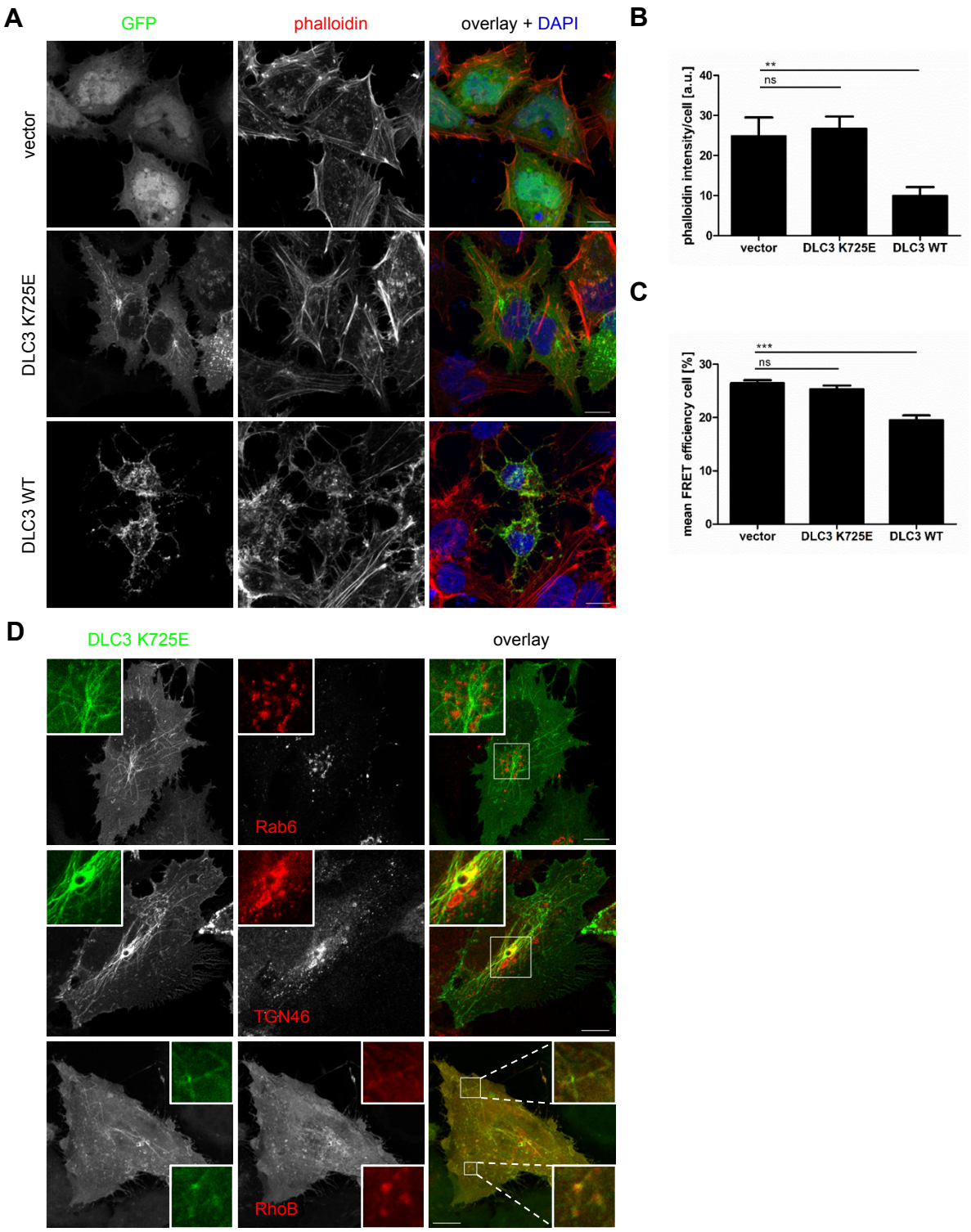
Fig. S2: Validation of perinuclear vesiculation and RhoA activation using an independent siRNA targeting DLC3. (A–E) HeLa cells were transfected with control (spControl) and DLC3-specific (siDLC3/spDLC3) siRNAs. (A) Three days post siRNA transfection, cells were stained for Rab8 (green) and nuclei were counterstained with DAPI (blue). The number of cells containing Rab8-positive tubules was counted and normalized to the total number of analyzed cells. Data show the mean ± s.e.m. (n=3, N≥100 cells); *P=0.0306 (paired Student´s t-test). Whole cell lysates were analyzed by western blotting with anti-DLC3 and anti-tubulin antibodies. (B) Three days post siRNA transfection, cells were stained for giantin (green) and nuclei were counterstained with DAPI (blue). Golgi fragmentation was calculated for the Golgi markers giantin, GM130 and p230. Values of one representative experiment were normalized to the mean Golgi compaction index of control cells. Data show the mean ± s.e.m. (N≥20 cells); **P<0.01; ***P<0.001 (two-way ANOVA followed by Bonferroni post-test). (A, B) Scale bars: 10 µm. (C, D) Two days post siRNA transfection, cells were transfected with the pTriEx-RhoA WT FLARE.sc biosensor and, on the next day, stained with giantin-specific primary and Alexa Fluor 633-conjugated secondary antibodies. (C) FRET acceptor photobleaching was performed as described in Fig. 4. Scale bars: 5 µm. (D) The mean FRET efficiencies within the

cell and at the Golgi of one representative experiment are plotted as the mean ± s.e.m. (N≥12 cells); upper graph, *P=0.0329, lower graph, *P=0.0325 (unpaired Student´s t-test). (E) Two days post siRNA transfection, cells were transfected with vectors encoding the DLC3 GAP domain fused to DLC3-SAM (SAM-GAP) or DLC3-SAM alone (SAM). Cells were fixed and stained for giantin 24 h later. The Golgi compaction index was calculated for at least three independent experiments. Values were normalized to untransfected control cells. Data show the mean ± s.e.m. (n=3-6, N≥50 cells); ns, not significant; **P<0.01; ***P<0.001 (two-way ANOVA followed by Bonferroni post-test).

Fig. S3: Cytoskeletal changes in cells lacking DLC3. HeLa cells were transfected with control (spControl) and DLC3-specific (spDLC3) siRNAs. Three days post transfection, cells were stained for paxillin (green) and phalloidin (red), nuclei were counterstained with DAPI (blue). Scale bars: 10 µm.

Fig. S4: DLC3 depletion alters EGFR trafficking and prolongs EGFR signaling. (A) HeLa cells were transfected with control (spControl) or DLC3-specific (spDLC3) siRNAs. Three days post siRNA transfection, cells were incubated with EGF-555 (red) for 120 min and either stained for EEA1 or Rab7 (green). Nuclei were counterstained with DAPI (blue). Scale bars: 10 µm. (B) MCF7 cells were transfected with control (spControl) or DLC3-specific (spDLC3) siRNAs. Two days post siRNA transfection, cells were serum-starved overnight and, prior to lysis, stimulated with 50 ng/ml EGF for the indicated times. Whole cell lysates were analyzed by western blotting with anti-pAKT (Thr308; arrow), anti-AKT, anti-pERK (Thr202/Tyr204), anti-ERK and anti-tubulin antibodies. One representative western blot out of two independent experiments is shown.

Fig. S1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GFP
phalloidin
overlay + DAPI
vector
DLC3 K725E
DLC3 WT
B
phalloidin intensity/cell [a.u.]
**
ns
vector
DLC3 K725E
DLC3 WT
C
mean FRET efficiency cell [%]
***
ns
vector
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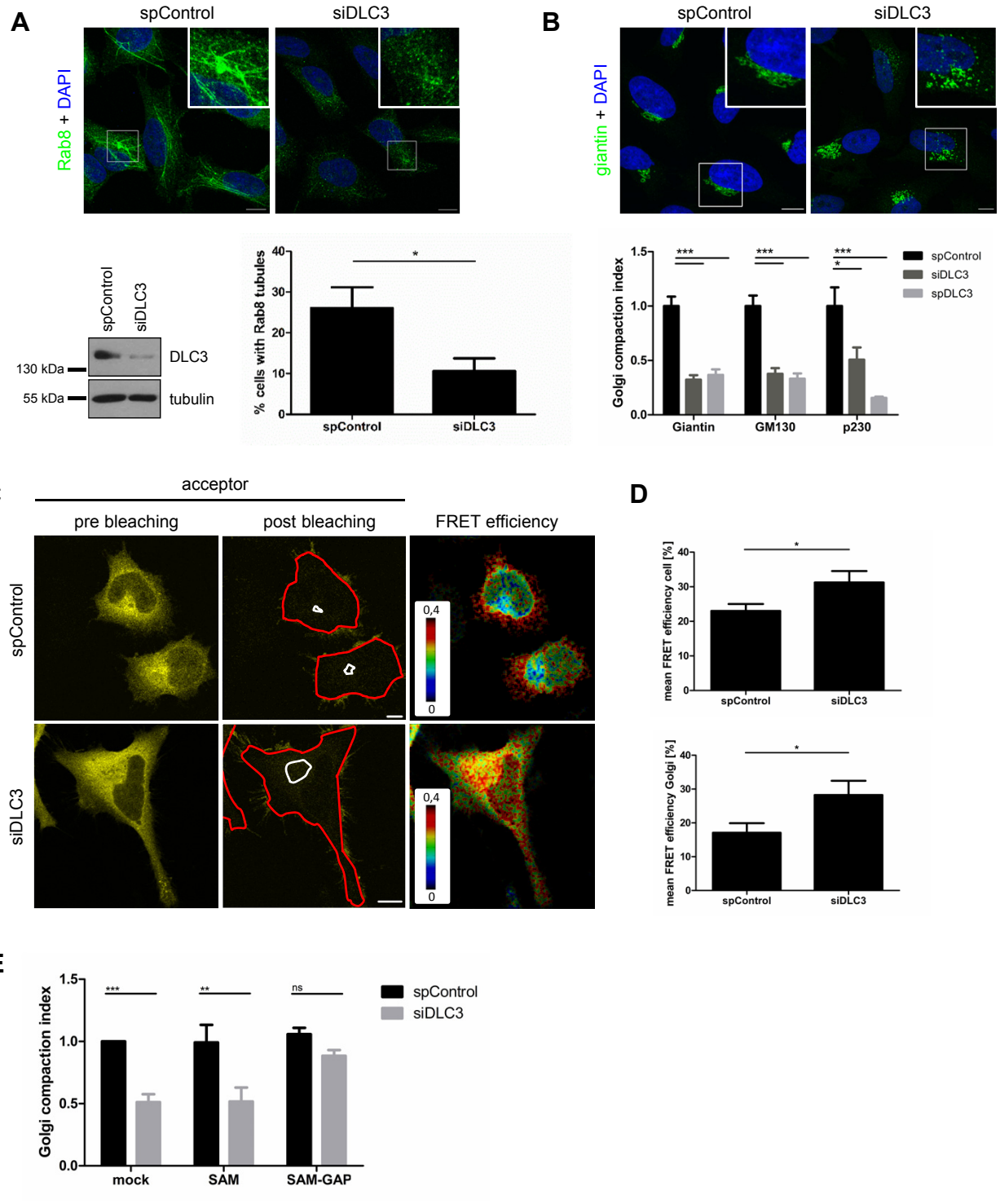
DLC3 WT
D
DLC3 K725E
overlay
Rab6
TGN46
RhoB

Fig. S2


A
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Rab8 + DAPI
spControl
siDLC3
130 kDa
55 kDa
DLC3
tubulin
% cells with Rab8 tubules
spControl
siDLC3
B
spControl
siDLC3
giantin + DAPI
Golgi compaction index
Giantin
GM130
p230
spControl
siDLC3
spDLC3
C
acceptor
pre bleaching
post bleaching
FRET efficiency
spControl
siDLC3
0,4
0
D
mean FRET efficiency cell [%]
spControl
siDLC3
mean FRET efficiency Golgi [%]
spControl
siDLC3
E
Golgi compaction index
mock
SAM
SAM-GAP
spControl
siDLC3

Fig. S3

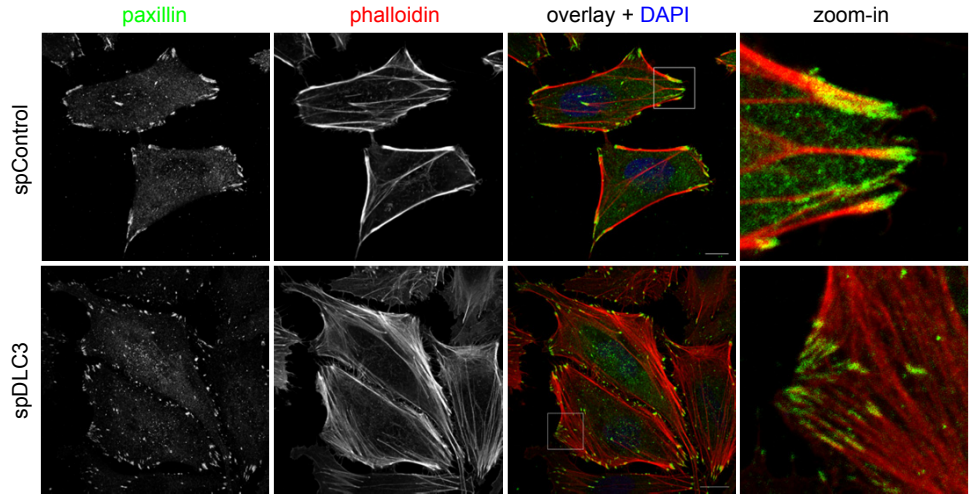
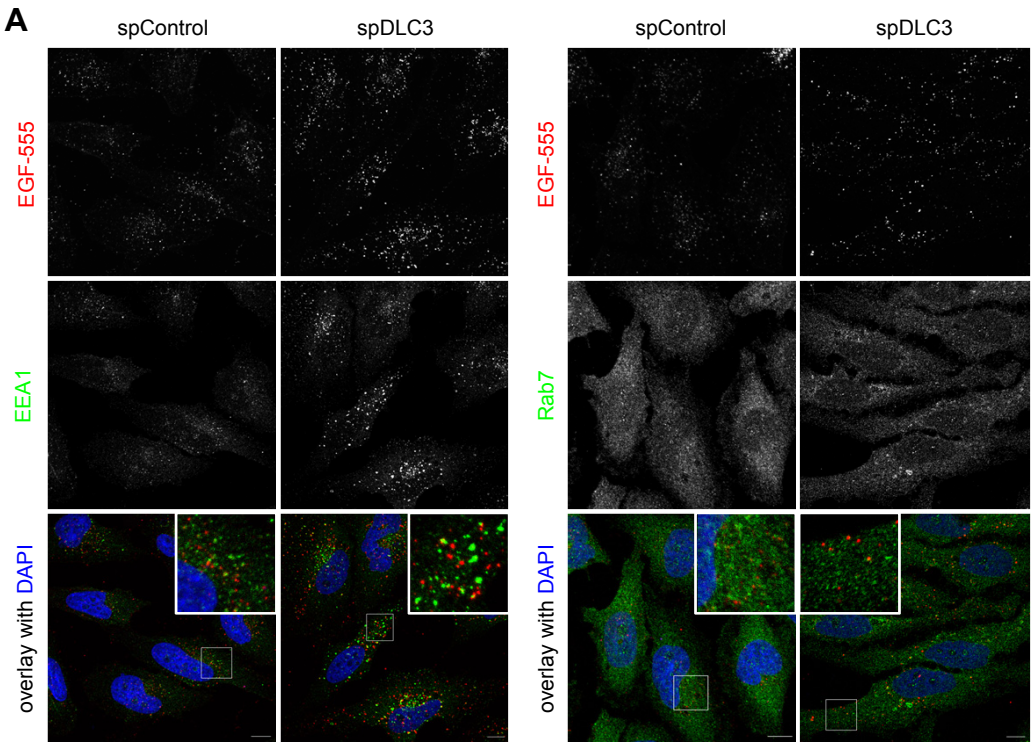

paxillin
phalloidin
overlay + DAPI
zoom-in
spControl
spDLC3

Fig. S4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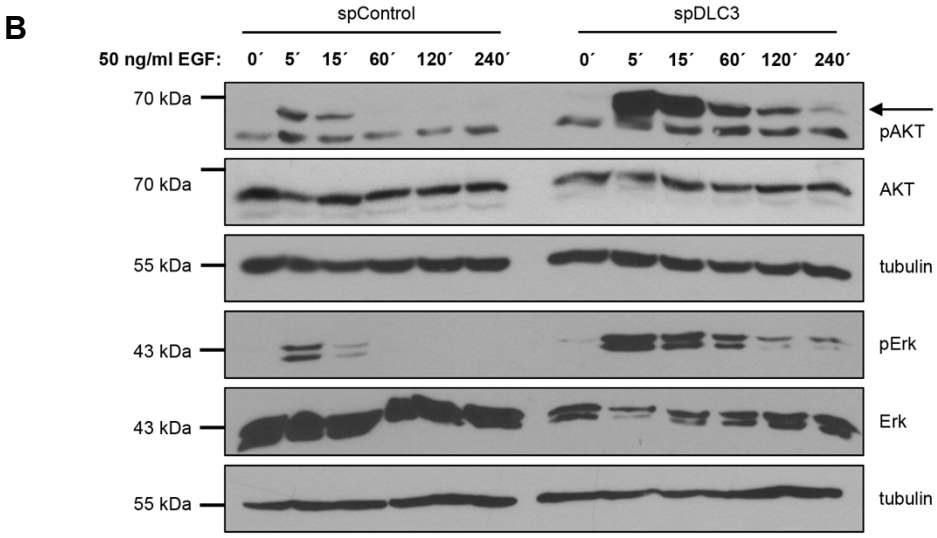
spDLC3
spControl
spDLC3
EGF-555
EEA1
overlay with DAPI
EGF-555
Rab7
overlay with DAPI


B
spControl
spDLC3
50 ng/ml EGF:
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0´ 5´ 15´ 60´ 120´ 240´
70 kDa
pAKT
70 kDa
AKT
55 kDa
tubulin
43 kDa
pErk
43 kDa
Erk
55 kDa
tubulin