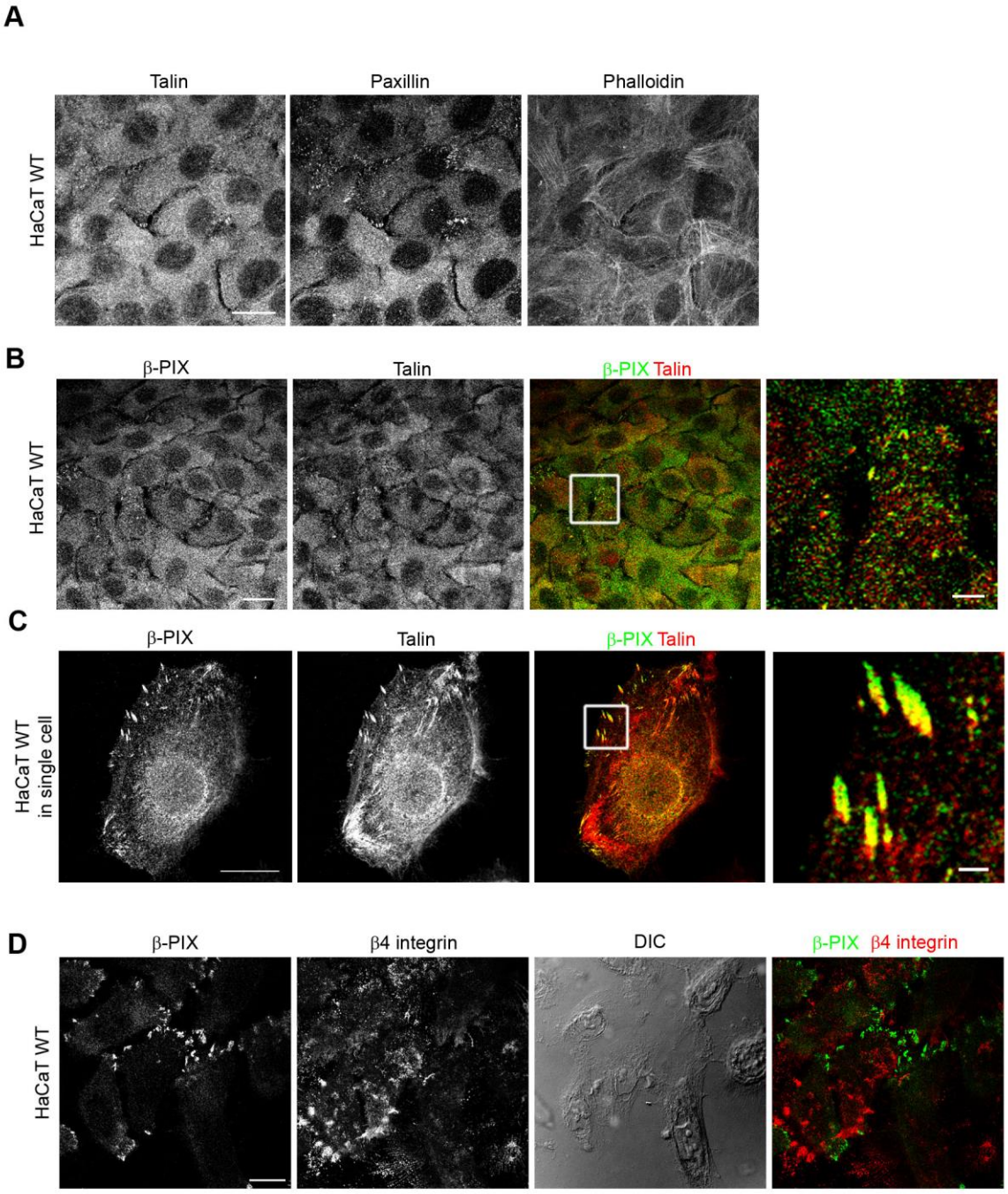

Fig. S1. **FA and hemidesmosome protein localization in HaCaT cells.** (A) Talin, paxillin, and F-actin (using phalloidin) localization in a non-scratch wounded monolayer cell sheet of HaCaT cells. Scale bar, 20 μm. β-PIX and talin localization in a non-scratch wounded monolayer sheet of HaCaT cells (B) and single HaCaT cell (C). (B,C) The third panels from the left show overlays of the two stains (green, β-PIX; red, talin). Boxed areas are shown at high magnification in the fourth panels. Scale bars, 20 μm (left panels), 5 μm (fourth panel in B), and 2 μm (fourth panel in C). (D) β-PIX and β4 integrin localization in HaCaT cells. The third panels from the left shows a differential interference contrast (DIC) image of the cells. The forth panel from the left shows overlays of the two stains (green, β-PIX; red, β4 integrin). Scale bar, 20 μm.

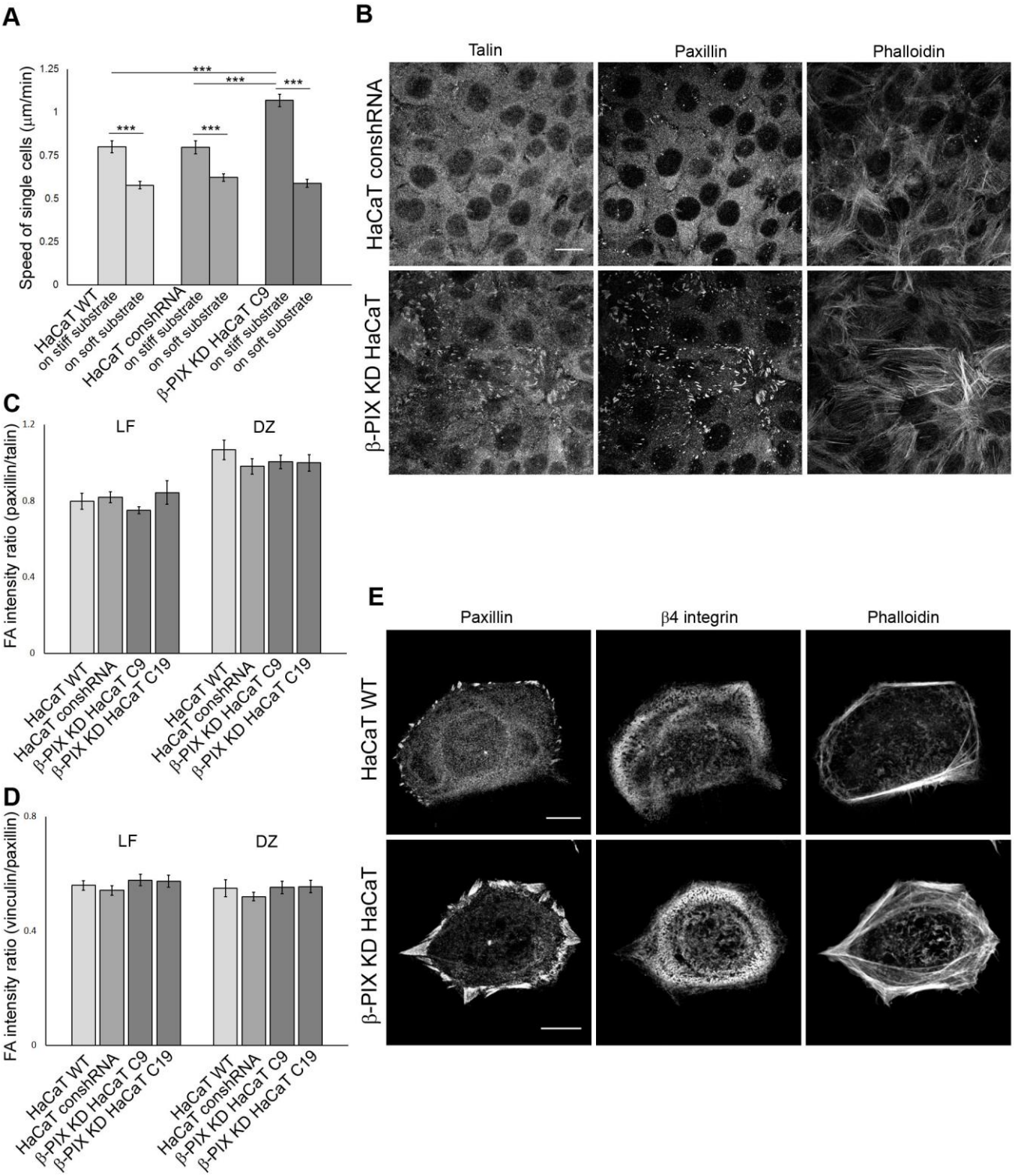

Fig. S2. **Cell motility on gel substrates and cell-matrix adhesion protein organization in β-PIX KD HaCaT cells.** (A) Speed of single HaCaT WT, HaCaT conshRNA, and β-PIX KD HaCaT cells on stiff or soft gel substrates (n ≥ 82 cells each). (B) Talin, paxillin, and F-actin (using phalloidin) localization in intact sheets of HaCaT conshRNA and β-PIX KD HaCaT cells as indicated. Scale bar, 20 μm. Ratios of paxillin to talin (C) and vinculin to paxillin (D) intensities quantified from double-stained scratched monolayer cell sheets (n ≥ 30 FAs each). (E) Paxillin, β4 integrin, and F-actin localization in a single HaCaT WT and β-PIX KD HaCaT cell. Scale bars, 20 μm. (A,C,D) Values are means ± SEM. *** p<0.001. Student's t test.

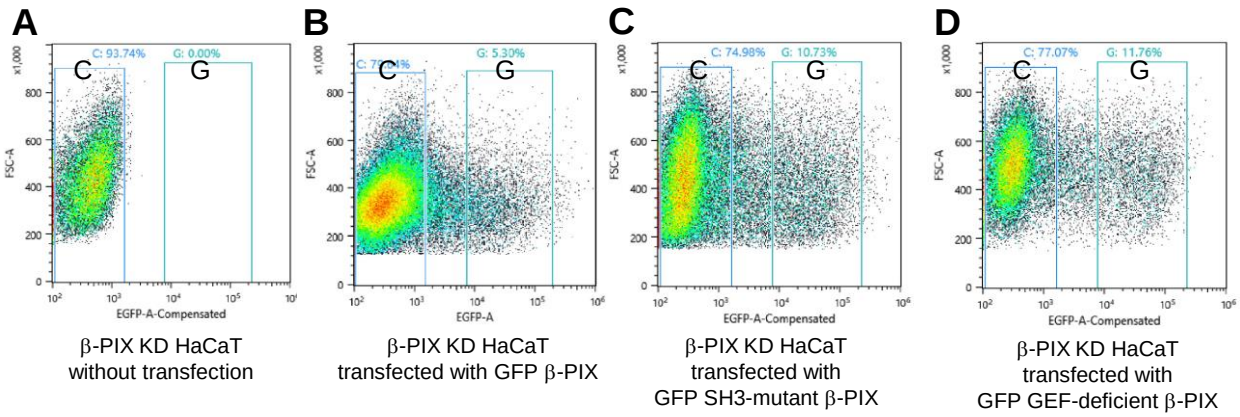

Fig. S3. **FACS sorting of β-PIX KD HaCaT cells transfected with GFP β-PIX constructs.** (A) FACS analyses of non-transfected β-PIX KD HaCaT cells (negative control). Following transfection with GFP-tagged wild type β-PIX (B), SH3-mutant β-PIX (C), or GEF-deficient β-PIX (D), β-PIX KD HaCaT cells were FACS sorted according to GFP intensity. Cells included in the squares marked G in B, C, and D were used for subsequent studies. Cells included in the squares marked C in each plot were considered as GFP(-) and used as negative controls for subsequent studies. x axis, forward scatter: y axis, GFP intensity.

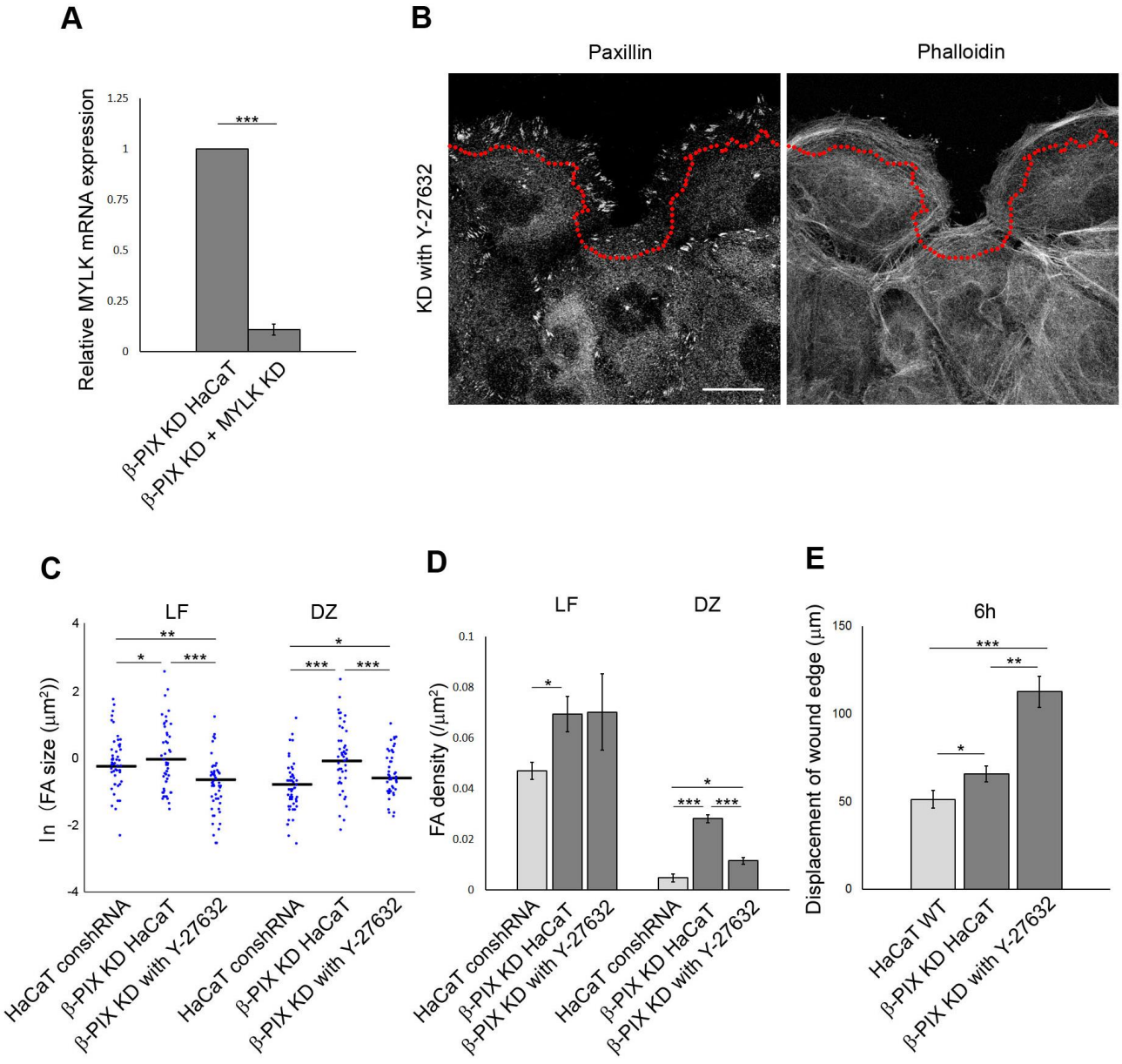

Fig. S4. **MYLK mRNA expression in β-PIX KD HaCaT cells expressing MYLK siRNA and motility, FA size, and FA density in β-PIX KD HaCaT cells treated with Y-27632.** (A) Quantification of relative mRNA level of MYLK in β-PIX KD HaCaT cells with or without transfection of siRNA targeting MYLK. (B) Paxillin and F-actin (using phalloidin) localization in a scratch wounded monolayer of β-PIX KD HaCaT cells treated with the ROCK inhibitor Y-27632. The red dotted line marks the arbitrary border of the LF and DZ. Scale bar, 20 μm. (C) Logarithmic transformation of FA sizes in the LF and DZ of HaCaT conshRNA treated with DMSO (n = 143 in LF, n = 119 in DZ), β-PIX KD HaCaT C9 treated with DMSO (n = 253 in LF, n= 892 in DZ), and of β-PIX KD HaCaT treated with Y-27632 (n = 98 in LF, n= 123 in DZ) cells is presented graphically. 40 randomly selected FA sizes are shown in each case but the means (horizontal bars) are of all FAs assayed. (D) FA density in the LF and DZ of HaCaT conshRNA treated with DMSO, β-PIX KD HaCaT C9 treated with DMSO, and of β-PIX KD HaCaT treated with Y-27632 cells. (C,D) A minimum of 4 assays were undertaken in 3 independent studies. (E) Quantification of the displacement of the wound edge at 6 hours after scratching of the indicated monolayers (n = 3 independent assays with a minimum of 4 measurements per wound). (D,E) Values are means ± SEM. (C-E) *P<0.05, **P<0.01, *** p<0.001. Student's t test.

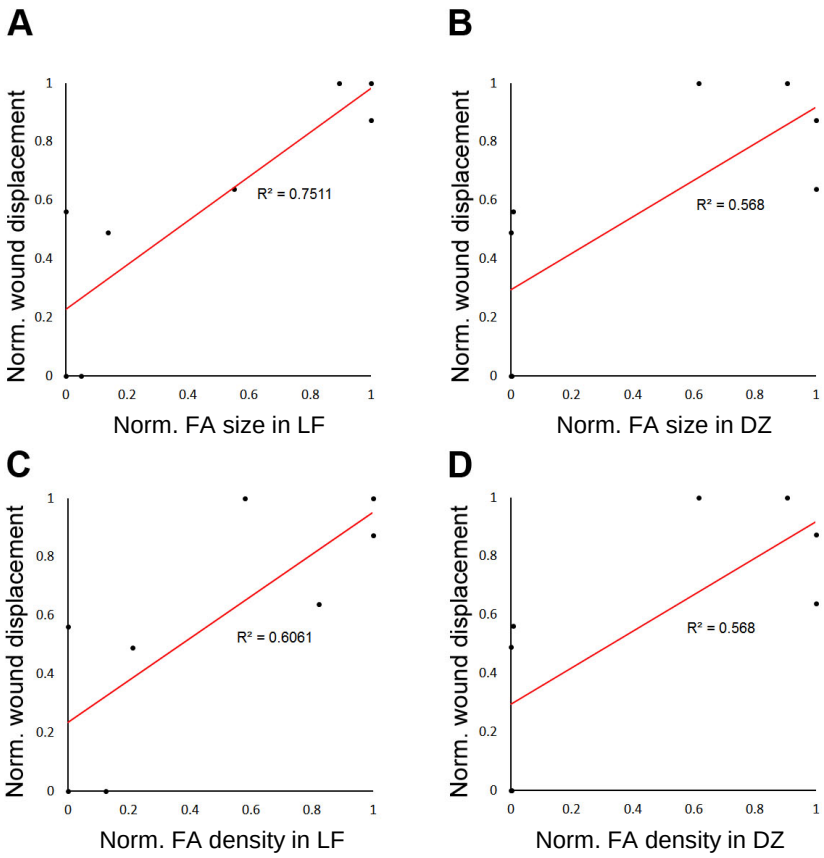

Fig. S5. **Relationship of FA size and density to cell motility during collective cell migration.** Each scatter plot shows the normalized displacement of the wound edge in relation to FA size in LF (A), FA size in DZ (B), FA density in LF (C), and FA density in DZ (D). Each mean value was obtained from experiments performed using HaCaT WT, HaCaT conshRNA, β-PIX KD HaCaT C9, β-PIX KD HaCaT C19, β-PIX KD HaCaT with ML-7, N/TERT WT, β-PIX KD N/TERT C2, and β-PIX KD N/TERT C3. All mean values were normalized using a formula $(x\text{-}x_{min})/(x_{max}\text{-}x_{min})$ for each cell type. Black dots and red lines indicate normalized mean values and regression lines, respectively.

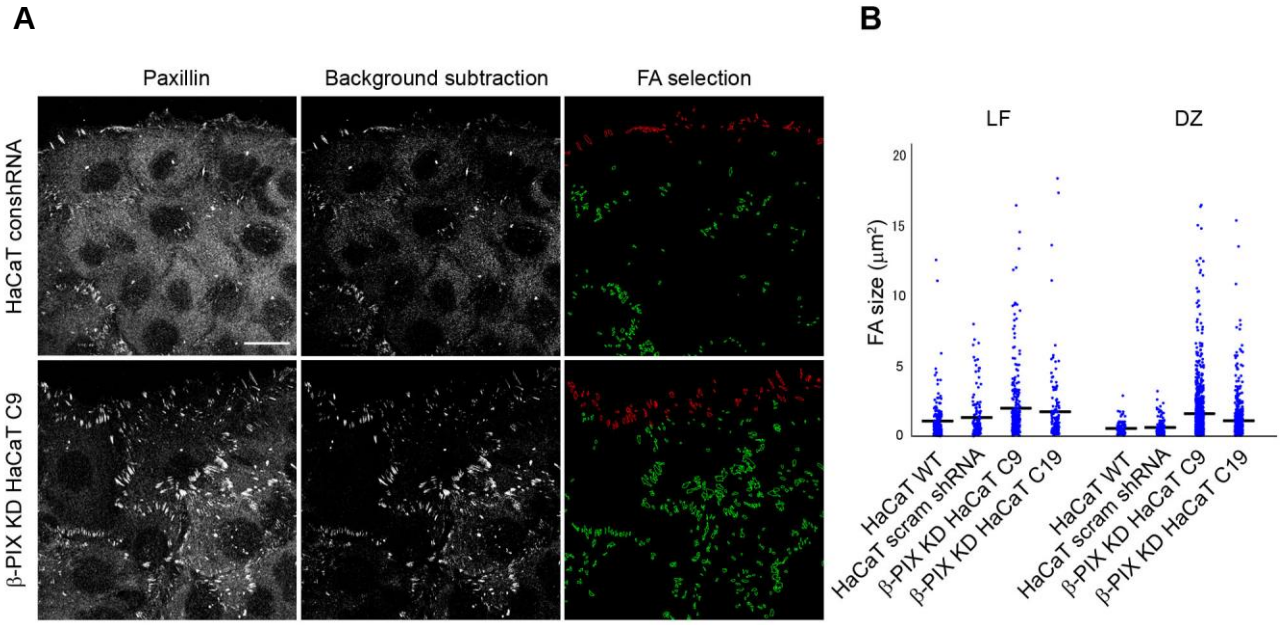

Fig. S6. **Background subtraction, FA selection, and FA size quantification.** (A) Background signals in images of paxillin staining (left panels) were subtracted using ImageJ (second panels). FAs were selected with the wand tool (third panels). Selected FAs were categorized as belonging to the LF (red) or DZ (green). After the quantification of FA size (B), the data were logarithmically transformed, as shown in Fig 2E, in order to fit the data to a normal distribution.

Product name	Sequence
ARHGEF7 MISSION shRNA Lentiviral Transduction Particles TRCN0000047596	CCGGGAAGTTAAGTTCAGCAAACATCTCGA GATGTTTGCTGAACTTAACTTCTTTTTG
MISSION pLKO.1-puro Non-Target shRNA Control Plasmid DNA	GCGCGATAGCGCTAATAATTT

Table. S1. **shRNA and control shRNA used in this study**