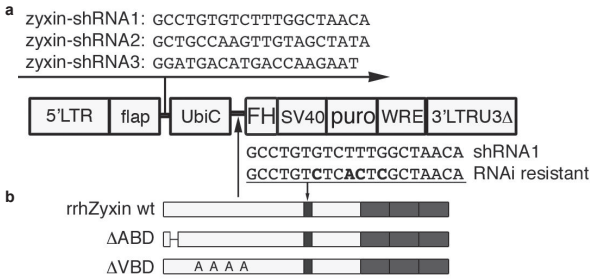
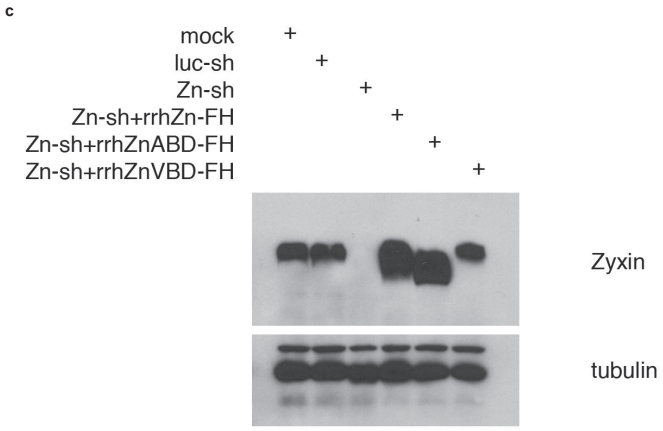
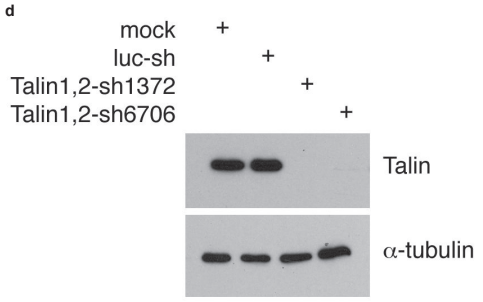
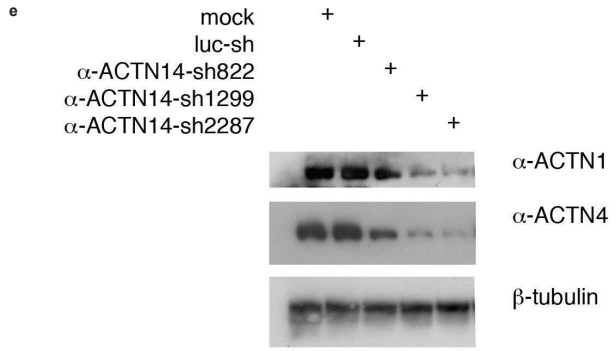
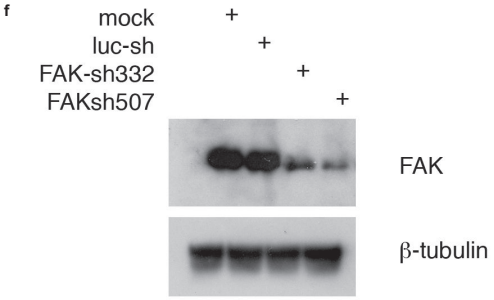
SUPPLEMENTARY INFORMATION

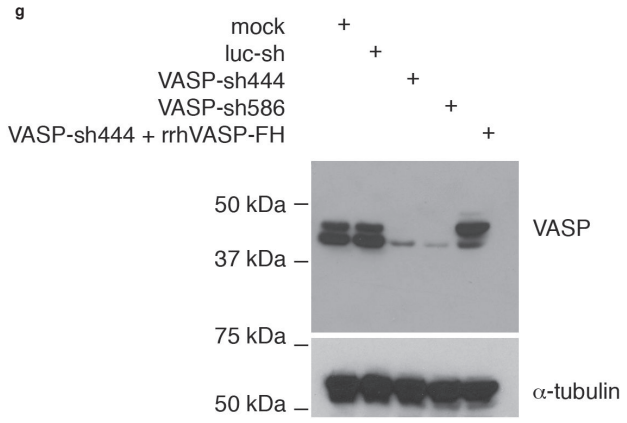


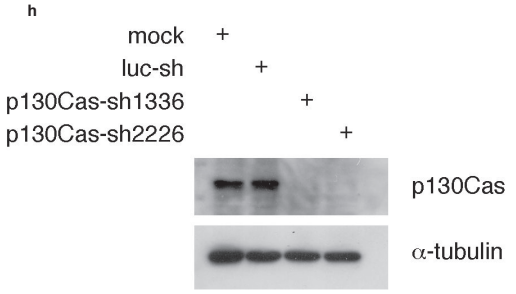




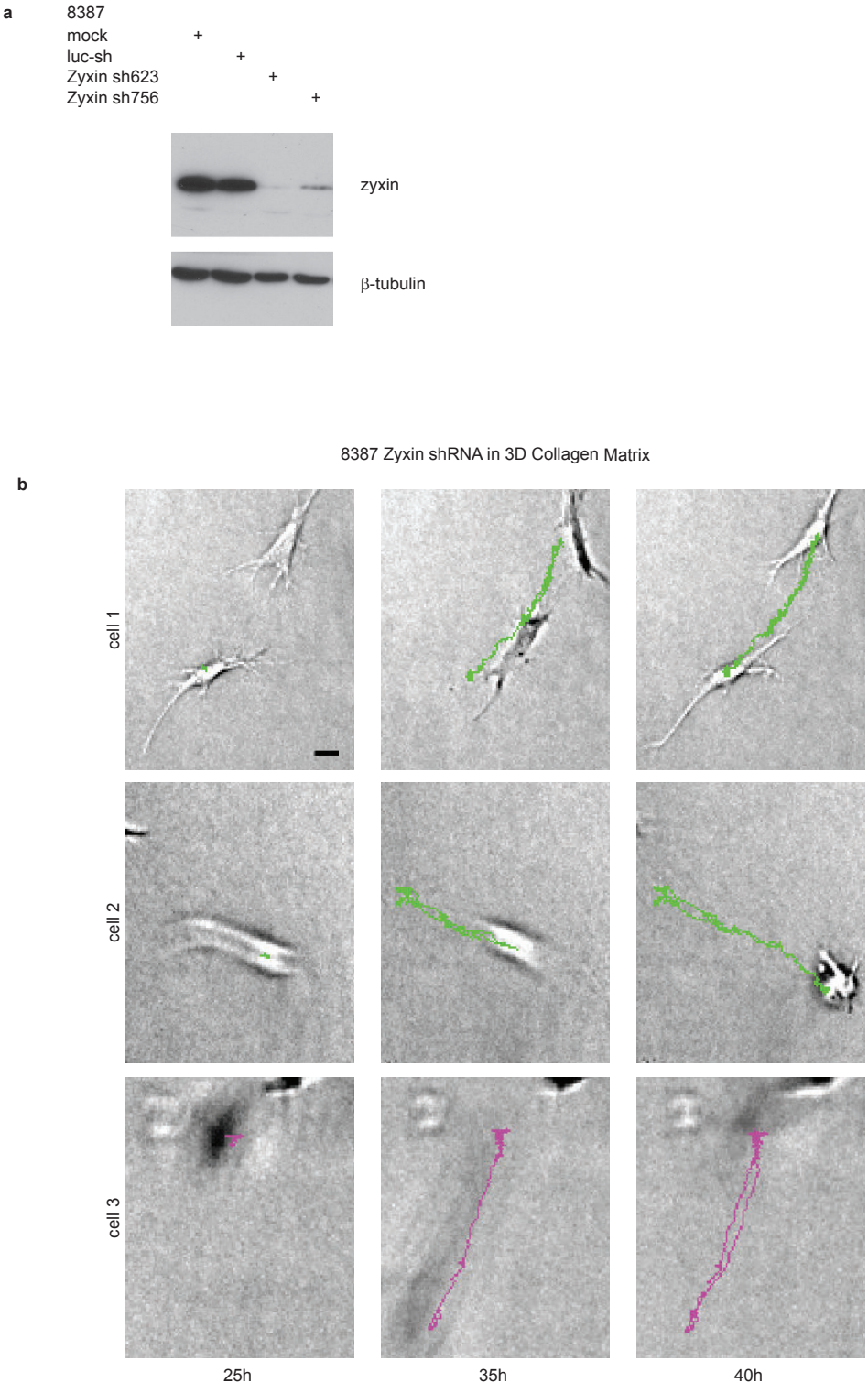






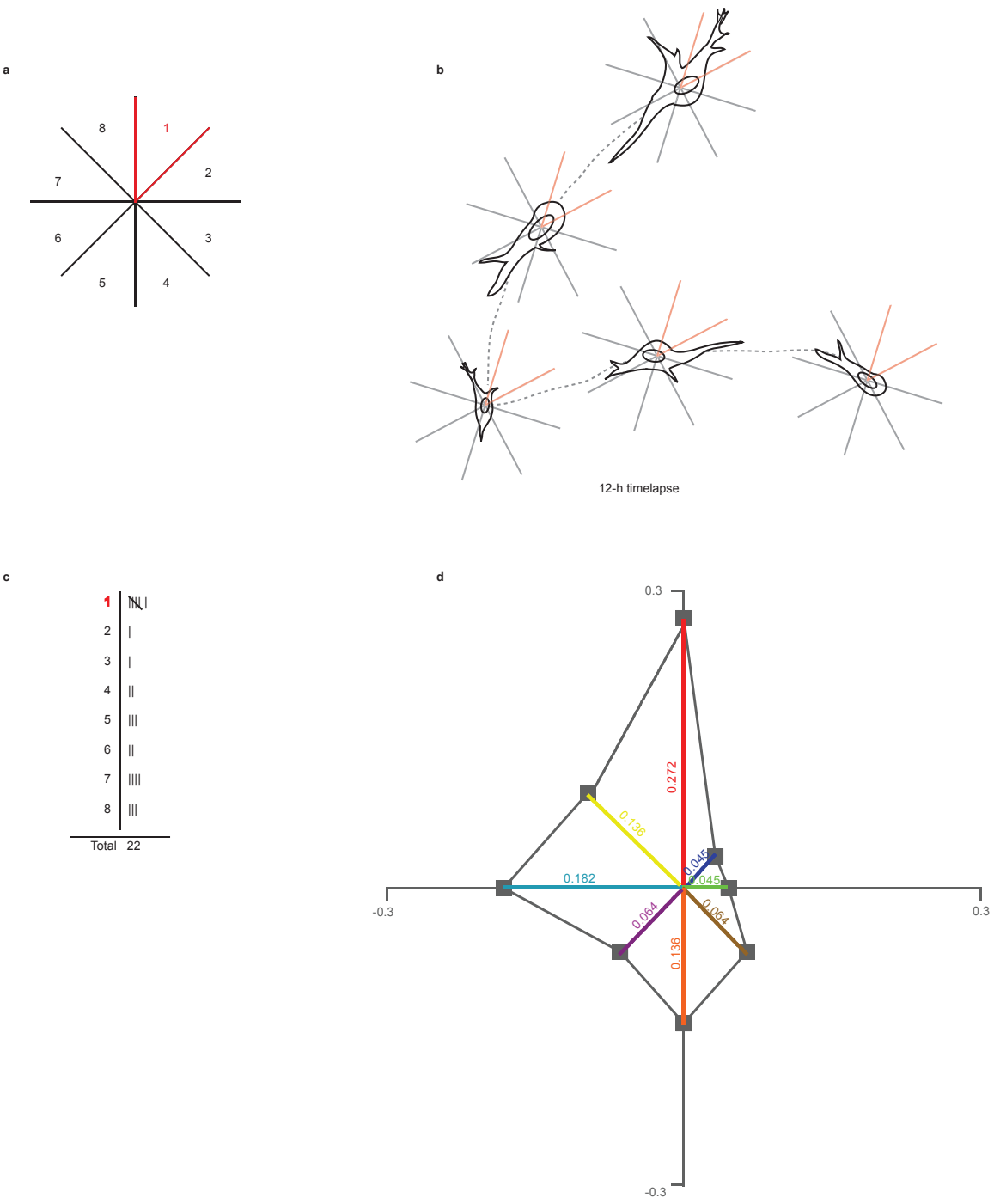


Supplementary Figure S1. Depletion of zyxin and zyxin-associated proteins in HT1080 cells and expression of zyxin mutants in zyxin-depleted HT0180 cells. A and B. Zyxin constructs used in the studies. **C.** Western blots for Zyxin and α-tubulin in HT1080 cells + mock vector, HT1080 cells + luciferase vector, HT1080 cells + Zyxin-sh756, HT1080 cells + Zyxin-sh756 + rrhZyxin-FH, HT1080 cells + Zyxin-sh756 + rrhZyxinABD-FH, and HT1080 cells + Zyxin-sh756 + rrhZyxinVBD-FH. **D.** Western blots for Talin1,2 and α-tubulin in HT1080 cells + mock vector, HT1080 cells + luciferase vector, HT-1080 cells + Talin1,2-sh1372, and HT1080 cells + Talin1,2-sh6706. **E.** Western blots for α-actinin1,4 and β-tubulin in HT1080 cells + mock vector, HT1080 cells + luciferase vector, HT1080 cells + α-actinin1,4-sh822, HT1080 cells + α-actinin1,4-sh1299, and HT1080 cells + α-actinin1,4-sh2287. **F**. Western blots for FAK and β-tubulin in HT1080 cells + mock vector, HT1080 cells + luciferase vector, HT1080 cells + FAK-sh332, and HT1080 cells + FAK-sh507. **G**. Western blots for VASP and β-tubulin in HT1080 cells + mock vector, HT1080 cells + luciferase vector, HT1080 cells + VASP-sh444, HT1080 cells + VASP-sh586, HT1080 cells + FAK-sh444+ rrhVASP-FH. **H.** P130Cas and α-tubulin in HT1080 cells + mock vector, HT-1080 cells + luciferase vector, HT1080 cells + sh-1336, and HT1080 cells + sh-2226. **G.** VASP and α-tubulin in HT-1080 cells + mock vector, HT-1080 cells + luciferase vector, HT-1080 cells + sh-444, HT-1080 cells + sh-586, HT-1080 cells + VASP-sh444 + rrhVASP-FH. See more details in the Methods section.



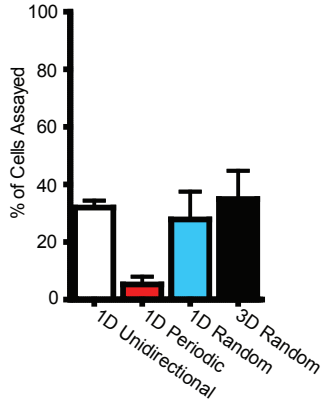
Supplementary Figure S2. 1-D oscillatory motion of zyxin-depleted human 8387 fibrosarcoma cells in 3-D matrices. **A**. Western blots for Zyxin and β-tubulin in 8487 cells + mock vector, 8487

cells + luciferase vector, and 8487 cells + Zyxin-sh623, 8487 cells + Zyxin-sh756. **B**. Trajectories of individual 8487 cell 1, cell 2, and 3 in a 2mg/ml 3-D collagen I matrix, the first day after embedding (top row) and on the second day (bottom row).



Supplementary Figure S3. Method used to compute the orientation of protrusions generated by cells inside a 3D matrix. A. Radial protrusion plot divided into 8 segments which is positioned onto the cell centroid with segment 1 (red) arbitrarily aligned with the initial major protrusion at time zero. **B**. Cartoon of a single cell migrating over twelve hours where the radial protrusion plot is aligned with the first major protrusion of the cell at time zero (top) and maintained in that alignment over the course of the timelapse. **C.** Table of number of protrusions (right column) occurring in each radial protrusion

plot segment (left column) for the single cell. **D**. A plot of the orientation summary for the single cell where the distance of each point from the origin represents the fraction of protrusions occurring in that segment of the radial plot, with the positive y-axis corresponding to segment 1 (red), etc.



Supplementary Figure S4. Effect of Rac1 inhibition on 3D cell migration. Fraction of cells showing a 1D unidirectional migration, 1D periodic motion, 1D random motion, and 3D random motion in a collagen I matrix. No significance among the bars.