Figure S1

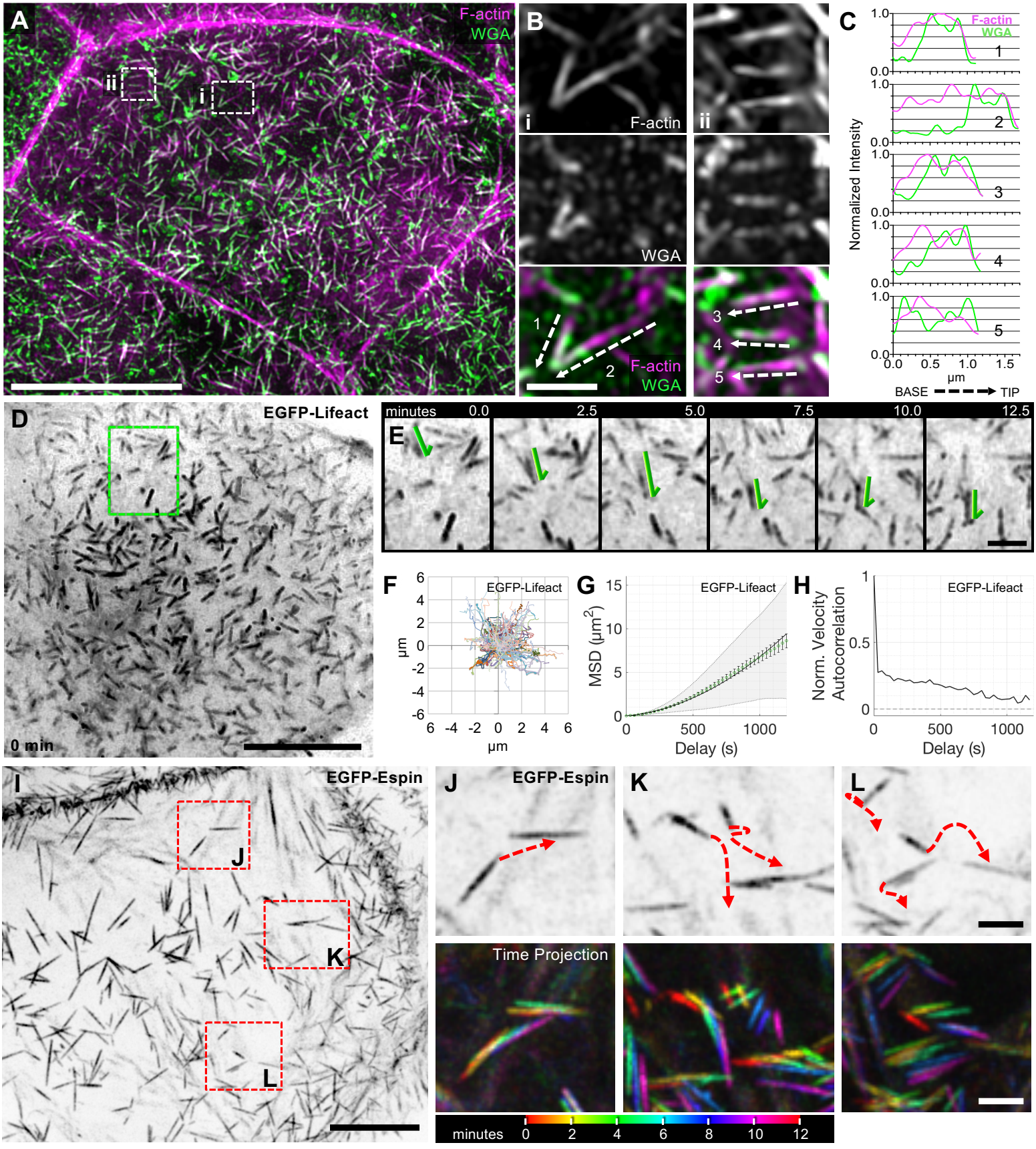

A
F-actin
WGA
ii
i
B
i
F-actin
ii
WGA
1
2
F-actin
WGA
3
4
5
C
F-actin
WGA
1
2
3
4
5
1.0
0.0
Normalized Intensity
0.0
0.5
1.0
1.5
μm
BASE
TIP
D
EGFP-Lifeact
0 min
E
minutes
0.0
2.5
5.0
7.5
10.0
12.5
F
EGFP-Lifeact
μm
G
EGFP-Lifeact
MSD (μm²)
Delay (s)
H
EGFP-Lifeact
Norm. Velocity Autocorrelation
Delay (s)
I
EGFP-Espin
J
K
L
J
EGFP-Espin
K
L
Time Projection
minutes
0
2
4
6
8
10
12

Figure S2

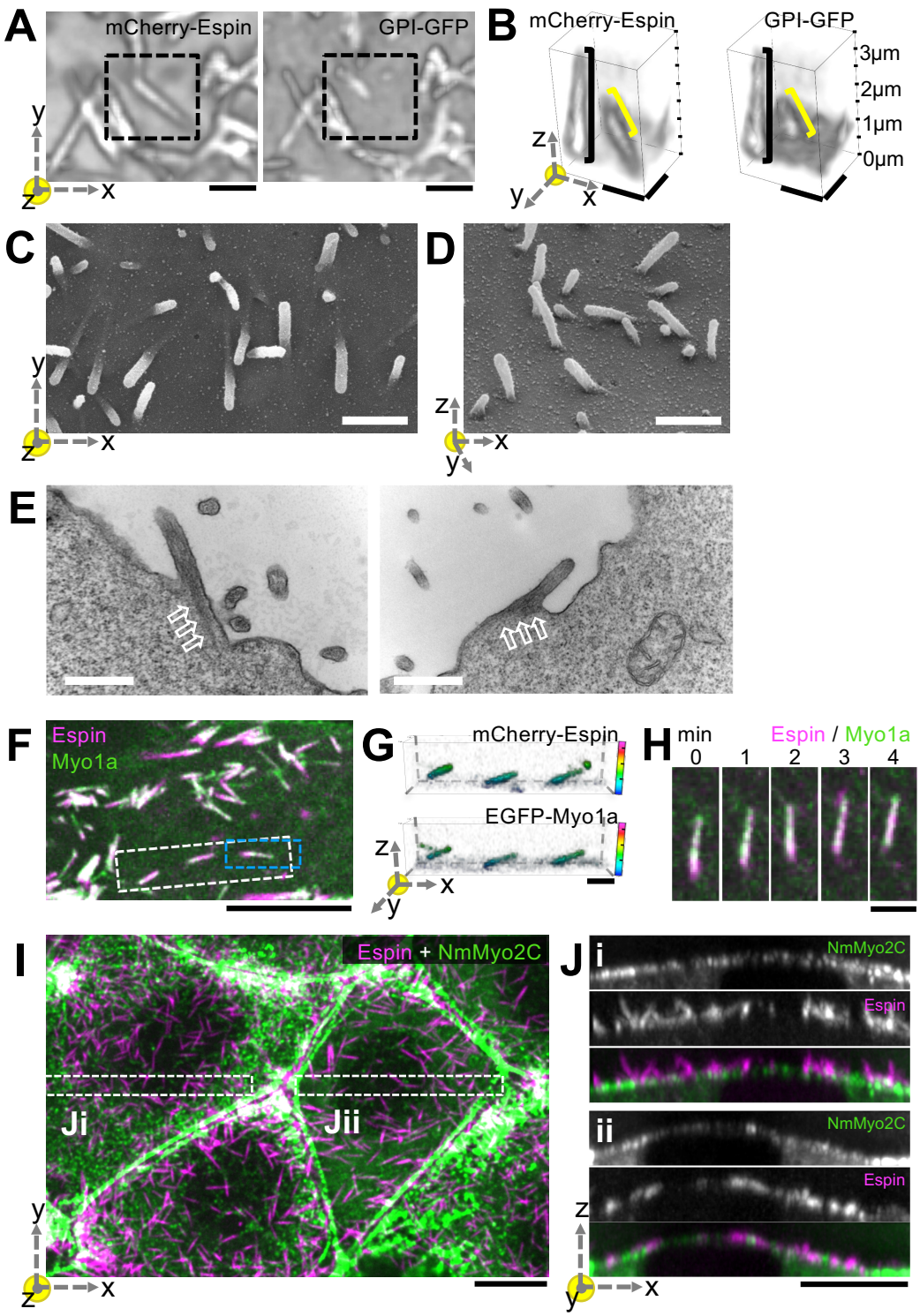
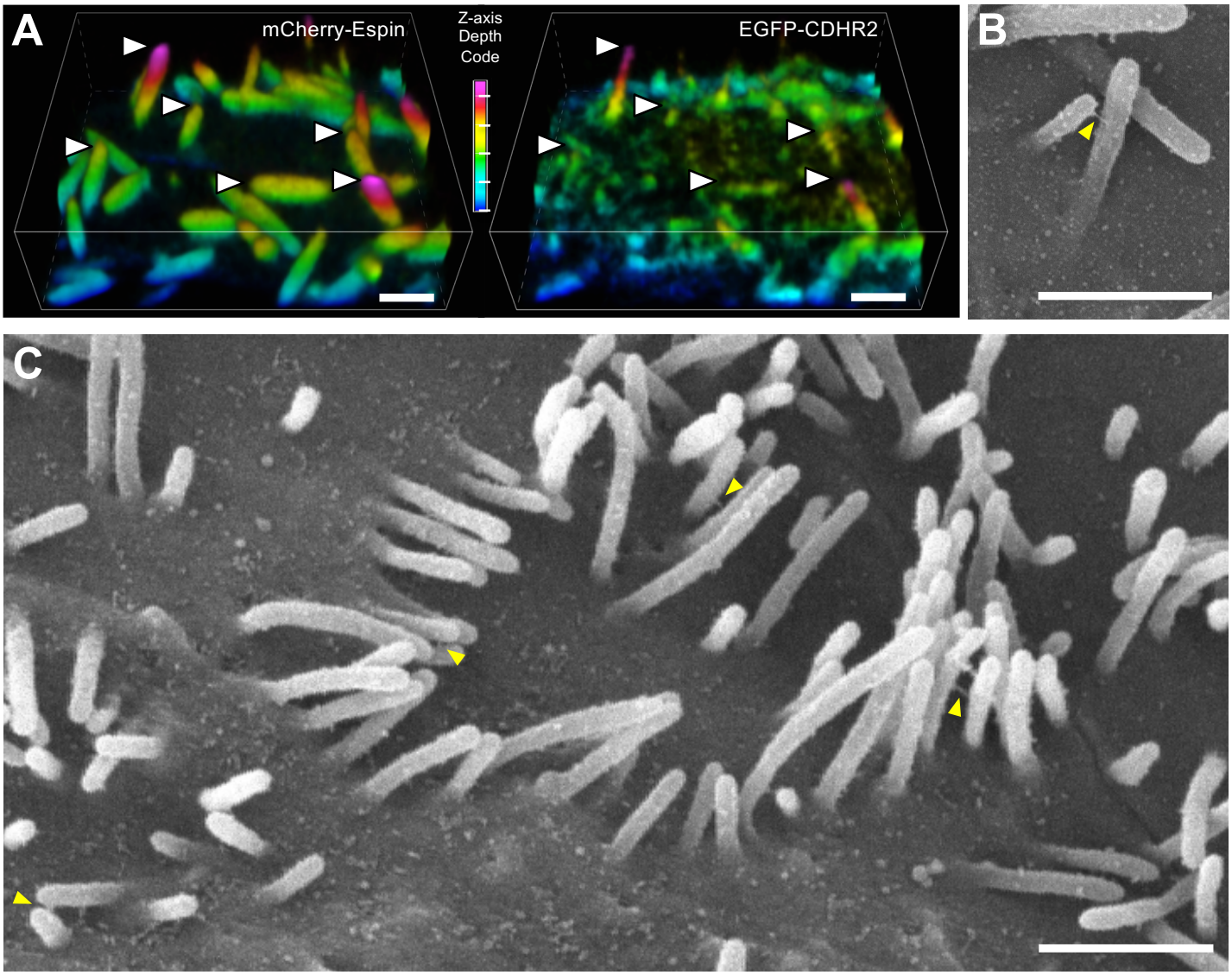

A
mCherry-Espin
GPI-GFP
B
mCherry-Espin
GPI-GFP
3μm
2μm
1μm
0μm
C
D
E
F
Espin
Myo1a
G
mCherry-Espin
EGFP-Myo1a
H
min
0
1
2
3
4
Espin / Myo1a
I
Espin + NmMyo2C
Ji
Jii
J
i
NmMyo2C
Espin
ii
NmMyo2C
Espin

Figure S3



SUPPLEMENTAL INFORMATION

Figure S1. Visualization and characterization of nascent microvilli, Related to Figure 1. (A) Fixed CL4 cell stained with Phalloidin (magenta) and wheat germ agglutinin (WGA, green) to mark F-actin and membrane, respectively. Boxed areas correspond to B. Scale bar is 10 μm. (B) Enlarged images highlight individual microvilli with distal tip membrane coverage (i) or more complete membrane coverage (ii). Scale bar is 1 μm. (C) Line scan intensity plots of phalloidin and WGA signal along the microvillar axis; arrows in B indicate line scan position and orientation. (D) SDCM of the apical surface of a CL4 cell transiently expressing EGFP-Lifeact. Scale bar is 10 μm. (E) Time series montage of boxed area in D; arrows highlight a microvillus translocating across the cell surface. Scale bar is 2 μm. (F) Rose plot of trajectories calculated from the tips of microvilli from D (n = 102). (G) Microvillar trajectories from F were subject to MSD analysis; data were well fit to an active movement model (black line) where D = 0.000361 $\mu m^2/s$ and V = 0.14 μm/min. (H) Microvillar trajectories from F were subject to normalized velocity autocorrelation analysis. The dotted line at zero indicates the velocity autocorrelation for random diffusive movement. Similar trajectories, MSD and autocorrelation results were observed across replicate cells (n = 81 microvilli from 7 cells). (I) SDCM of the apical surface of a Caco-2_{BBE} cell transiently expressing EGFP-Espin, viewed as a maximum intensity z-projection. Scale bar is 10 μm, red dashed boxes correspond to J-L. (J-L) Enlarged images from I (top) with time projections (bottom) showing microvillar movement over 12 minutes. Scale bars are 2 μm.

Figure S2. Motile microvilli are wrapped in membrane and interact with myosins, Related to Figure 1D and Figure 2. (A) SDCM of a CL4 cell expressing mCherry-Espin and GPI-GFP. A single z-stack was deconvolved and viewed as a 3D alpha-blended composite. Scale bar is 1 μm. (B) Volume from boxed area in A was cropped and rotated to emphasize two membrane-wrapped microvilli. One microvillus extends ~3 μm from the cell surface (black bracket), the other shorter structure is nearly parallel to the cell surface (yellow bracket). Brackets indicate the extent of the membrane signal. Scale bars are 1 μm. SEM of CL4 cells expressing mCherry-Espin viewed *en face* (C) or rotated 35˚ for an oblique view (D). Scale bars are 1 μm. (E) TEM of CL4 cells expressing mCherry-Espin.

Arrows highlight where the F-actin core extends into the cytoplasm. Scale bars are 500 nm. (F) SDCM of a CL4 cell expressing mCherry-Espin and EGFP-Myo1a. A single z-stack at time = 0 was deconvolved and viewed as a 3D alpha-blended composite. Scale bar is 10 μm. White and blue boxed areas correspond to G and H respectively. (G) Volume from F rotated to highlight microvilli extending above the cell surface at a low angle contain Myo1a. Scale bar is 2 μm. (H) Time series of a single microvillus from F showing Myo1a is present in motile microvilli. Scale bar is 2 μm. (I) SDCM of a CL4 cell expressing mCherry-Espin and EGFP-NmMyo2C. A single z-stack was deconvolved and viewed as a maximum intensity z-projection. Scale bar is 10 μm. Boxed areas correspond to Ji-ii. (J) Sections through the central portion of the cell are viewed as an xz-projection to visualize the localization of NmMyo2C at the apical surface in proximity to microvilli rootlets. Scale bar is 10 μm.

Figure S3. Motile microvilli contain CDHR2 and can form intermicrovillar adhesive links, Related to Figure 5. (A) SDCM of the apical surface of a CL4 cell expressing mCherry-Espin and EGFP-CDHR2. A single z-stack was deconvolved and viewed as 3D depth color coded alpha-blended composite. Scale bars are 2 μm, z-axis depth color code (center) with tick marks at 1 μm intervals. Arrow heads indicate tips of microvilli (Espin) that colocalize with CDHR2. (B-C) SEM of CL4 cells expressing mCherry-Espin showing threadlike linkages (yellow arrowheads) between two individual microvilli (B) or clusters of microvilli (C). Scale bars are 1 μm.