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Supplementary Information for

Macropinocytosis-Mediated Membrane Recycling Drives Neural Crest Migration By Delivering F-actin to the Lamellipodium

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SI Materials and Methods

Molecular Cloning and Viral Production

Faenesylated-YFP, Faenesylated-scarlet, Faenesylated-Dendra2, Utrophin-scarlet, 2G4-GFP, EB3-scarlet, Actin-scarlet, Cortactin-scarlet, Rab11-DsRed, DN-Rab11-2A-mCherry were cloned into the replication incompetent retroviral (RIA) vector. Recombinant RIA plasmids were cotransfected with Envelop A plasmid into chick DF1 cells (ATCC, Manassas, VA; #CRL-12203, Lot number 62712171, Certificate of Analysis with negative mycoplasma testing available at ATCC website) in 15 cm culture dishes using standard transfection protocol (Li et al., 2017). When the cells were confluent, the cell culture medium was harvested once per day for three days, and was concentrated at 26,000 rpm for 1.5 hr. The pellet was dissolved in a minimal volume of DMEM.

Viral Infection

Fertilized chicken eggs were obtained from AA Lab Eggs (Westminster, CA) and incubated at 38°C for 44 hours or until embryos reached stages HH11+/12- (Hamburger and Hamilton, 1951). Eggs were windowed and injected with 5% Indian ink in Ringer's underneath the embryo disc and Ringer's solution was used to keep the embryos humid. To achieve efficient transfection of the neural tube and neural crest cells, concentrated virus (10^6 - 10^7 pfu/ml) were injected into the posterior neuropore, filling the entire tube (about 0.5 μ l per embryo). Injected embryos were incubated at 38°C for 24 hours, collected with filter paper carriers and washed in Ringer's solution.

Explant Culture of Chick Embryos

Transverse cuts through posterior sclerotomes of the forelimb region were made every 2-somites with a Sharp point Stab Knife, 22.5° Straight (Fine Science Tools). Next, the knife was used to cut through lateral tissue. Once freed of neighboring tissue, the slices were briefly washed in Ringer's solution and immediately put into fluorodish (World Precision Instruments) containing prewarmed Neurobasal media (Gibco) supplemented with glutamine and penicillin/streptomycin.

Nocodazole was dissolved in the medium with the final concentration of 4 μ g/ml to inhibit MT polymerization. Texas red conjugated Dextran was dissolved in the medium with the final concentration of 15 μ g/ml for macropinocytosis engulfment assay.

Image Acquisition

The whole fluorodish was transferred into the incubation chamber (37°C and 5% CO₂) of a Zeiss LSM 800 inverted microscope for time-lapse imaging. For all imaging experiments, optical sectioning was achieved at 1 μ m intervals.

For long-term imaging of Faenesylated-YFP expressing cells to detect phagocytosis, 20 \times /0.8 NA objective was used and digital amplification was set to 0.6. The samples were excited by 488 nm laser with 0.8% relative power and imaged at 2 minutes intervals for 12 hours.

For imaging MT and EB3 dynamics, 63 \times /1.4 NA objective lens was used and digital amplification was set to 3. The samples were imaged at 5 seconds intervals. 2G4-GFP and EB3-Sarlet were excited by 488 nm laser with 0.4% relative power and 561 nm laser with 0.7% relative power, relatively.

For all other imaging analysis, 63 \times /1.4 NA objective lens was used and digital amplification was set to 1. The samples were imaged at 22 seconds intervals. 488 nm laser with 0.4% relative power was used to image Faenesylated-YFP; 561 nm laser with 0.4% relative power was used to image Faenesylated-Scarlet and Utrophin-Scarlet; 488 nm laser with 0.4% relative power was used to image Actin-YFP and Cortactin-YFP.

Image Segmentation and Quantitation

The 4D images were imported into IMARIS 9.3. 3D surface segmentation of cells, 3D spot segmentation and 4D tracking of vesicles were performed. The resultant parameters including vesicle speed, position, displacement length relative to nuclei movement were used for quantitative analysis in MATLAB R2010b. Displacement lengths of vesicles were calculated by subtracting the coordinates at time $t = 0$ and a given time.

Analysis of Lipid Addition to Cell Membrane

Corresponding to Fig. 2H, the last frame of every vesicle was identified. The field of view was rotated so that the membrane nearest the last observed position of the vesicle is oriented vertically downwards (i). Thus the last observed vesicle position is at the center and the nearest membrane is at the bottom. The average across all vesicles is shown in Fig. 2I. The inside of the cell is delineated by the solid red line marking average membrane intensities above 43 (arbitrary units). The resulting membrane extension due to vesicle fusion (J), calculated by the difference between last frame in which the vesicle was observed and the next frame. The green contour shows the largest changes in the membrane, which are mainly localized to the extracellular region (red contour).

Analysis of EB3 Flow

Corresponding to Fig. 3E-G, for the region of interest in the image (E), 2D fast Fourier transform (FFT) representation was calculated. The rotation of the FFT image is correlated with the orientation of the features in the real image (27). To determine this direction, the cross-correlation between FFT image and vertical line pattern was measured, rotated at different angles. The tuning of FFT image is defined as the angle of pattern at which cross-correlation is maximized (dashed line). 5 ROIs were selected for two time points (frames 30 and 35). The ROIs were split into two groups: around the front of the cell, and around the cell body/nucleus. Collected tuning angles for each ROI in top (cell front) or bottom (cell rear) sets were collected, combined for both frames. Tuning curves for top and bottom ROIs were shown. ROIs in cell front are more tightly tuned to angle in $-60..-45$ range. ROIs around cell body are not displaying directionality tuning.

Analysis of Lamellipodial Orientation

Cells were automatically segmented after training a classifier (Trainable Weka Segmentation, ImageJ) on a few cells, one classifier for each cell category. Lamellipodium were manually selected for all forty five frames of each time lapse of a cell. The angle formed by the line from the center of mass of the cell to the center of mass of its podia was computed and values in the $[0, 360]$ degrees range reported.

Phalloidin Staining

A slice (2 somite and 500 μm in thickness) of stage HH18-19 chick embryos was made through the forelimb level. The slice was fixed in 4% PFA at 4 degree for 30 min. Frozen tissues sections were permeabilized with blocking buffer (1xPBS with: 10% vol/vol normal goat serum, 1% BSA, 0.1% vol/vol Triton X-100,), stained with phalloidin (1:500 dilution, Molecular Probes).

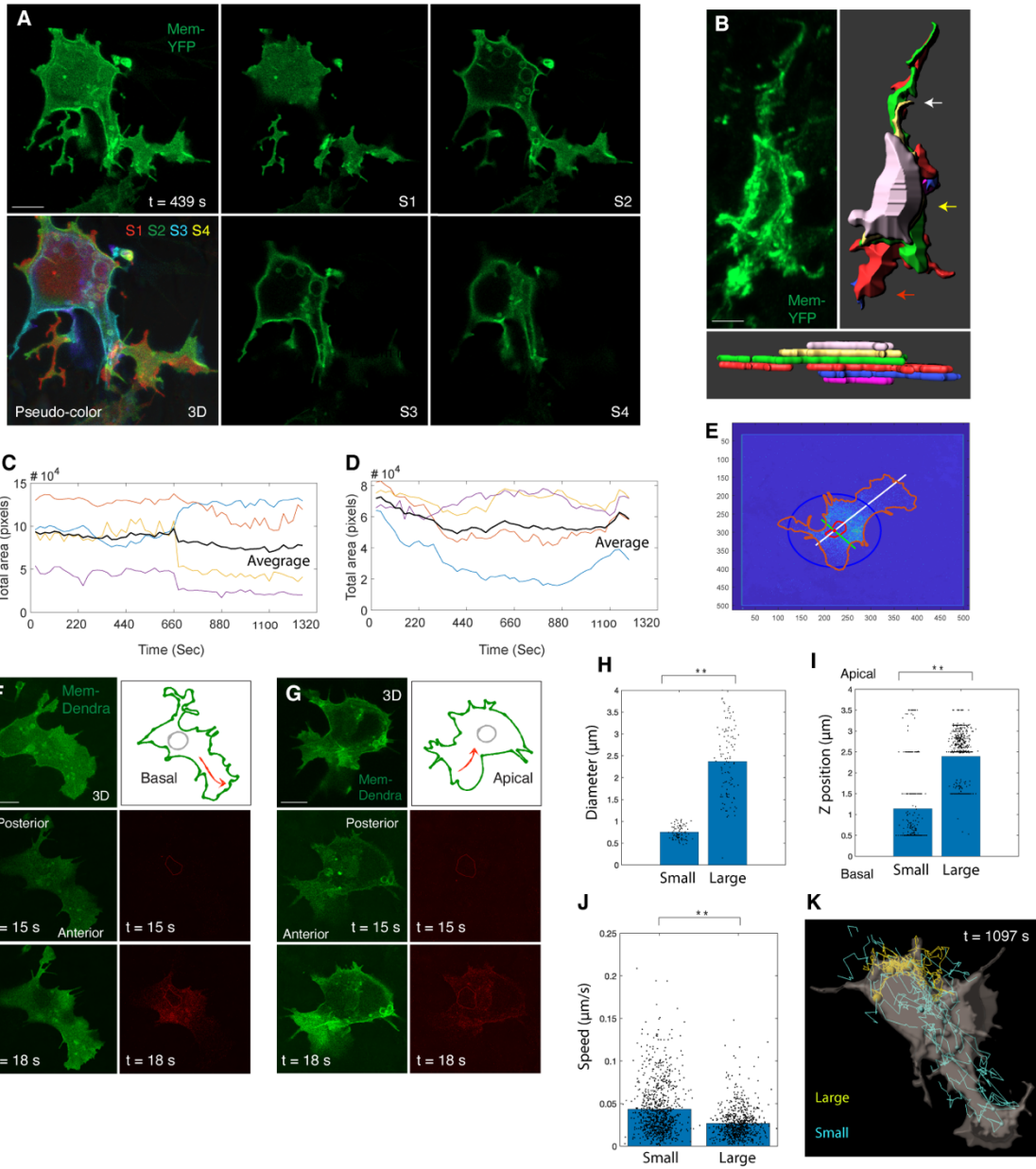


Fig S1. Morphological Changes of Migrating Cells

(A) Dissecting a 3D image along the apical-basal axis allows for distinguishing membrane and vesicles. S1, S2, S3, S4 corresponds to individual optical slice from basal to apical side, respectively. 3D image is color-coded according to individual slices. Scale bar: 4 μm .

(B) A neural crest cell in the frozen sections show a typical migrating configuration including the lamellipodium (red arrow), the cell body (yellow arrow), and the tail (white arrow). Scale bar: 4 μm .

(C and D) The surface area of individual optical slices (colored lines) change while the average value (black line) remains constant over time.

(E) The principle of analyzing photo-conversion data. The red polygon and the white line denotes the photoconverted region and the orientation of cell migration, respectively. Green line is the border between the anterior and posterior regions for quantifying photoconversion signal (red fluorescence). Blue circle is the region for normalizing signal.

(F and G) Photo-conversion of the selected region (red polygons) on the cell's basal (F) and apical (G) side reveals anterograde and retrograde membrane flow, respectively. In the basal side (F), after 405 nm laser excitation ($t = 15\text{s}$), more red fluorescence appears in the anterior end of the cell ($t = 18\text{s}$); in the apical side (G), more fluorescence signal is observed in the posterior side. The schematic summarizes the flow direction of the membrane. Scale bar: 5 μm .

(H-K) Vesicles display distinct sizes and mobilities. H: Quantifying the sizes of small ($n = 23$) versus large ($n = 10$) vesicles (Ranksum test, $p < 0.001$, $n = 3$ cells). I: Along apical-basal axis, small vesicles ($n = 23$) are more widely distributed than large ones ($n = 10$) (Ranksum test, $p < 0.001$, $n = 3$ cells). J: Speed analysis shows that small vesicles ($n = 23$) move faster than large ones ($n = 10$) (Ranksum test, $p < 0.001$, $n = 3$ cells). K: Trajectories of small and large vesicles. Compared with small vesicles, large ones mainly remain in the apical side of the cell with minimal movement. Scale bar: 7 μm .

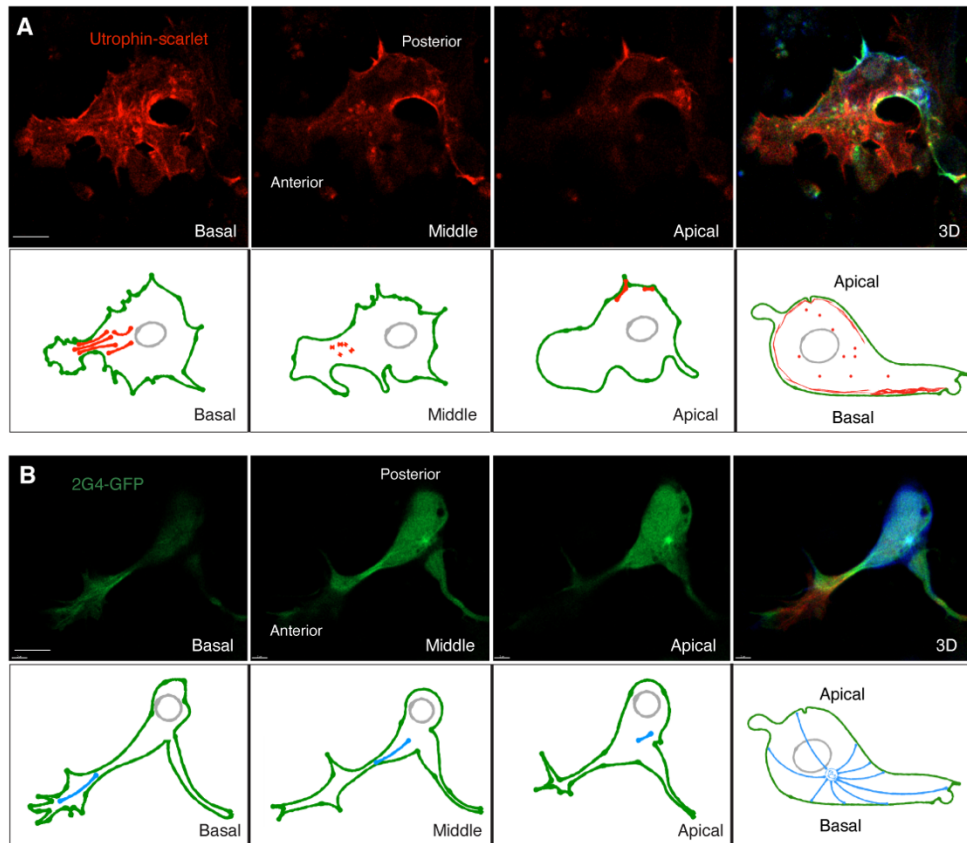


Fig S2. Spatial organization of F-actin and MTs

(A) Spatial organization of F-actin. Three individual slices of the cell (from basal to apical side) expressing Utrophin-scarlet (top view); the schematic below individual images shows actin alignment. In the 3D image, individual slice is pseudo-colored; the schematic below shows actin organization from lateral view.

(B) Spatial organization of MTs. Three individual slices of the cell (from basal to apical side) expressing 2G4-GFP from top view; the schematic below individual images shows MT alignment. The brightest spot in the apical region is the microtubule organizing center (MTOC). In the 3D image, individual slice is pseudo-colored; the schematic below shows MT organization from lateral view.

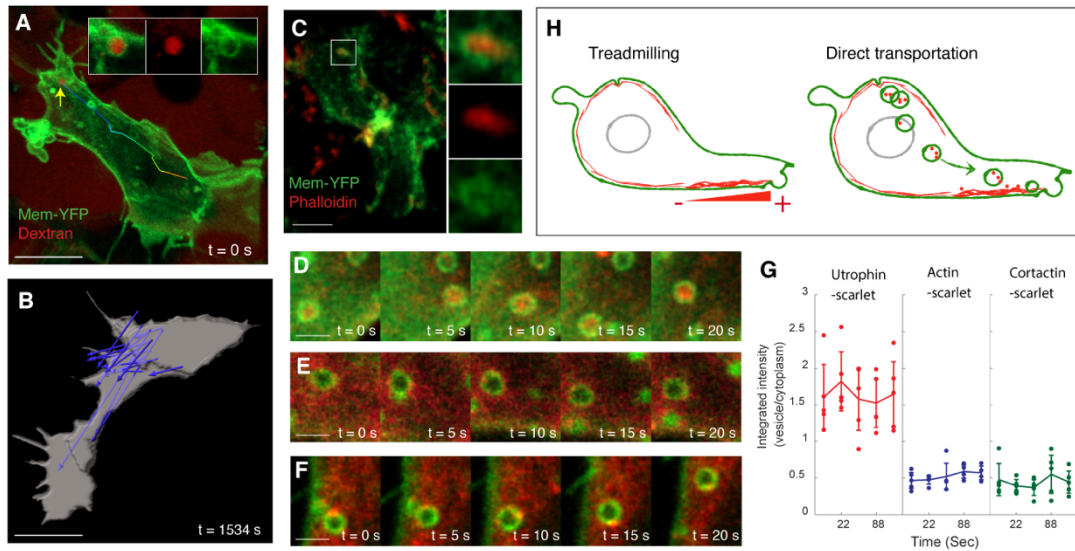


Fig S3. Characterization of Type I Vesicles

(A) Dextran dissolved in the culture media is engulfed by a vesicle (arrow) and transported to cell front (the trajectory of the vesicle color-coded relative to time.). Box shows the magnified view of the vesicle. Scale bar: 7 μm .

(B) Net displacement vectors of the F-actin containing vesicles corresponding to the snapshots in Fig. 4C, confirming their anterograde movements. Scale bar: 7 μm .

(C) Phalloidin staining shows encapsulation of endogenous F-actin by lipid vesicles. Scale bar: 4 μm .

(D-F) Live imaging to show non-canonical macropinosomes wrapping large amount of F-actin (D), but low amount of total actin (E) and cortactin (F). D: one vesicle expressing Mem-YPF (green) and Utrophin-scarlet (red); E: one vesicle expressing Mem-YFP (green) and Actin-scarlet (red); F: one vesicle expressing Mem-YFP (green) and Cortactin-scarlet (red). Scale bars: 1 μm .

(G): Fluorescence intensity analysis of D-F. Based on the same principle in Fig. 7B, the ratio of the fluorescence intensity inside and outside vesicles is plotted. $n = 3$ cells for each experiments.

(H) Schematic diagrams of the “Treadmilling” model versus the “Direct transportation” model.

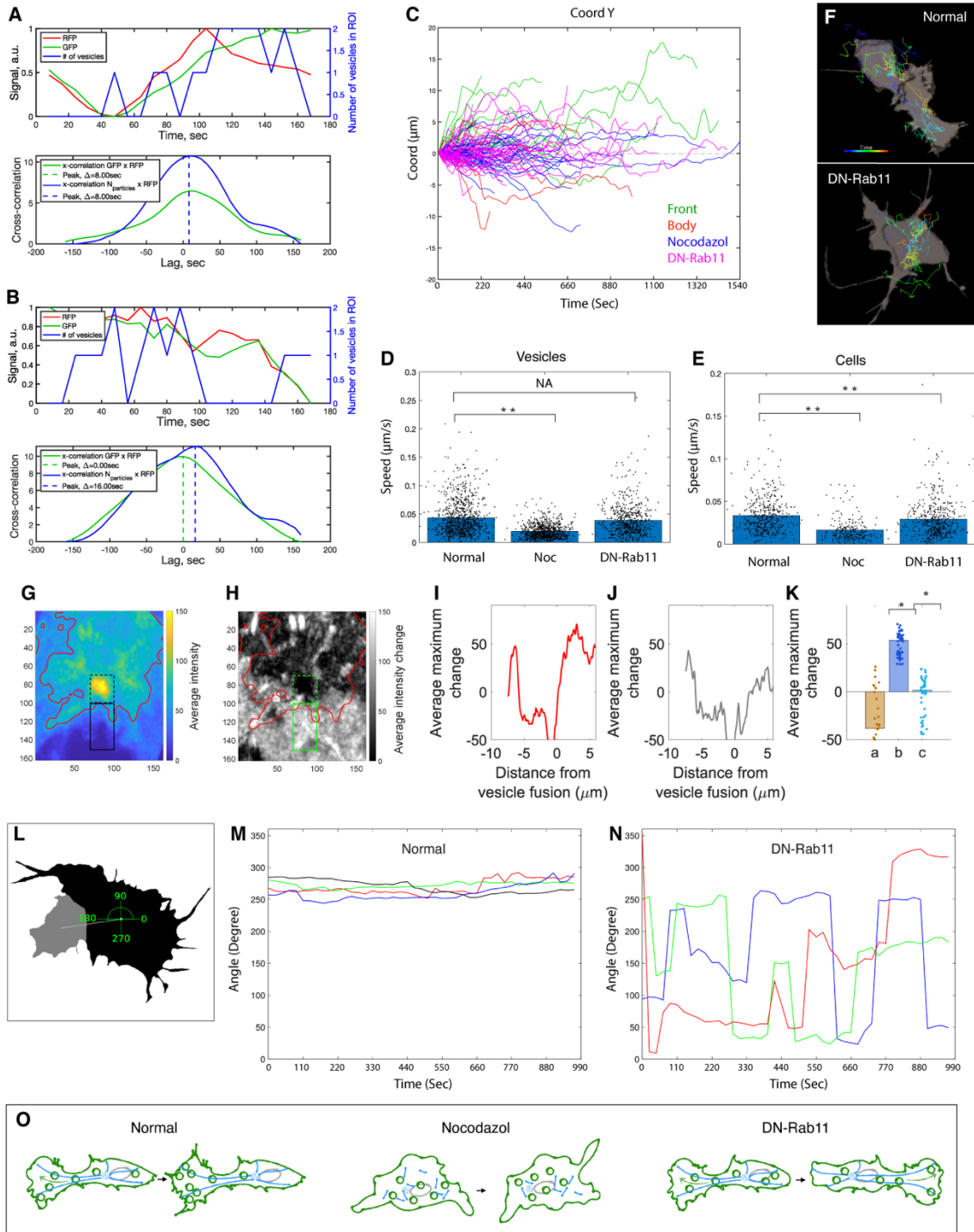


Fig S4. Perturbing Vesicle Mobility Impacts Cell Morphology and Migration

(A) Quantitative analysis to show that membrane motion is followed by the invasion of MTs and vesicles.

(B) Negative control shows that in a window randomly placed inside the cell, the sequential order of membrane and vesicle flow is not observed.

(C) Comparison of the anterior-posterior displacements of the vesicles the normal (n = 33 vesicles, n = 3 cells), nocodazole treated (n = 35 vesicles, n = 3 cells), and DN-Rab11 positive cells (n = 33 vesicles, n = 3 cells). Many vesicles in normal cells move to the front end (upward shifts of many green lines).

(D) Speed analysis confirms that vesicles in the nocodazole treated cells (n = 35 vesicles, n = 2 cells) move more slowly than in the normal cells (n = 33 vesicles, n = 3 cells) (Ranksum test, $p < 0.001$). However, vesicles in the DN-Rab11 expressing cells move in a normal speed (n = 35 vesicles, n = 2 cells)

(E) Speed analysis shows that both of the nocodazole treated cells (n = 5) and the DN-Rab11 expressing cells (n = 5) move more slowly than normal (n = 5) (Ranksum test, $p < 0.001$).

(F) Vesicle trajectories in a normal cell and a DN-Rab11 expressing cell.

(G-K) Morphological analysis reveals lamellipodial expansion by vesicles in the DN-Rab11 expressing cells. (Ranksum test, $P < 0.001$, n = 31 pixels representing the average of 6 vesicles). Scale bar: 4 μm .

(L-N) Lamellipodial extension in the DN-Rab11 expressing cells are not sustained. L: The principle of analyzing lamellipodium orientation. The lamellipodium (grey) and the cell body (black) are segmented. The white line is drawn to connect the centroids of both parts to calculate the orientation of the principle lamellipodium (the largest one), which is further plotted against time (M, N); each line represents orientation progression of the lamellipodium in one cell.

(O) Schematic comparison of vesicle behaviors and cell morphologies under different circumstances. In normal condition, continuous vesicle flow toward cell front end establishes and sustains the lamellipodium. When cells are treated with nocodazole, vesicles do not migrate close to cell periphery to establish the lamellipodium. When the normal activity of Rab11 is perturbed, vesicles move to both anterior and posterior ends, and the lamellipodium forms in both directions.

Legends for Movies

Movie S1: Neural Crest cells (Membrane-YFP) migrate with lamellipodial extension in the anterior and cell body retraction in the posterior. Time unit (minute : second). Relating to Figure 1D.

Movie S2: Photo-conversion to show membrane flow in basal (left) and apical (right) region. The cells expressing Farnesylated-Dendra2 are illuminated with 405 nm laser (red polygon). Consequently, green fluorescence turns into red, which is monitored to assess the orientation of membrane flow. On the basal side, there appears to be more red signal in the anterior; in contrast, in the apical side, there appears to be more red signal at the posterior. Time unit (minute : second). Left and right movies relating to Figure S1F and S1G, respectively.

Movie S3: A segmented view of vesicle movement after computational motion correction. The vesicles are computationally segmented and distinct groups of vesicles are assigned distinct colors. Yellow labels large vesicles. Green and red labels small vesicles derived from the apical side of the cell body; green ones move toward the lamellipodium while the red ones move inside the cell body. Blue labels small vesicles produced from the lamellipodium. The small vesicles (green, red and blue) appear more dynamic than large ones (yellow). Top: top view; bottom: lateral view. Time unit (minute : second). Relating to Figure 2A.

Movie S4: Vesicles move along MTs. Vesicles and MTs are labeled with Membrane-scarlet (red) and 2G4-GFP (green), respectively. One large vesicle in the posterior side of the cell are relatively stationary and are not included for colocalization analysis between vesicles and MTs. Time unit (minute : second). Relating to Figure 3A.

Movie S5: Vesicle motion in cell body (left) and front (right). Vesicles and MTs are labeled with Membrane-scarlet (red) and 2G4-GFP (green), respectively. An arrow pointing to the network structure of MTs in the cell body.

Movie S6: The dynamics of MTs. The plus end of MTs is labeled with EB3-scarlet (red) and the brightest spot stands for the microtubule organizing center. Time unit (minute : second). Relating to Figure 3D.

Movie S7: Infrequent Phagocytosis. Live imaging in a large field of view to cover more than twenty cells (Membrane-YFP) as they move from the dorsal neural tube and the notochord. Only one cell engulfs the debris of a dead cell in the time duration from 06:00 to 06:20 (arrows). Time unit (hour : minute). Relating to Figure 4A.

Movie S8: The cellular process of non-canonical macropinocytosis (Membrane-YFP), during which the cell extends its protrusion and then retracts and degrades into vesicles (arrows). Time unit (minute : second). Relating to Figure 4B.

Movie S9: Dextran (red) is engulfed by the cell (Membrane-YFP, green), rapped by a vesicle (spot segmented) and transported to the front. The trajectory of this vesicle is color encoded relative to time. Relating to Figure S3A.

Movie S10: Type I Vesicles Transport F-actin. The retracting tail (Membrane-YFP, green) breaks into small vesicles, which wrap F-actin patches (Utrophin-scarlet, red) and deliver them to the lamellipodium. Left: 3D view; right: slicer view. In the slicer view, the new formed vesicles are spot segmented with

dragon tails, and their migration is normalized to cell movement. Time unit (minute : second). Right and left movies are related to Figure 4C and the insert, respectively.

Movie S11: As the lipid portion integrates into the membrane (Membrane-YFP, green), F-actin (Utrophin-scarlet, red) released from the same vesicle (arrows) merges with the actin branches in the lamellipodium. Time unit (minute : second). Relating to Figure 4D.

Movie S12: F-actin (Utrophin-scarlet, red) is enriched in the vesicle (Membrane-YFP, green) (left movie); in contrast, total actin (Actin-scarlet, red) (middle movie) and cortactin (Cortactin-scarlet, red) (right movie) is expressed in a low level in the vesicles, relating to Figure S3D, S3E, and S3F, respectively. Time unit (minute : second).

Movie S13: During canonical macropinocytosis, the folding membrane (Membrane-YFP, green) is transiently bound by F-actin (Utrophin-scarlet, red). The whole process is pointed by arrows. top: top view; bottom: segmented and lateral view. Time unit (minute : second). Relating to Figure 5A and 5B.

Movie S14: Photo-conversion on the nocodazole treated cells expressing Farnesylated-Dendra2 shows opposite directions of membrane flow in basal (left) and apical (right) region. The principle is the same to Movie S2. Time unit (minute : second).

Movie S15: A migrating cell (Membrane-YFP) in the presence of nocodazole. This cell does not generate the fan-shaped lamellipodium, but instead forms thin protrusion. Time unit (minute : second). Relating to Figure 6E.

Movie S16: A segmented view of vesicle movement inside the nocodazole treated cell. Note the vesicles (yellow) do not move close to the cell periphery. Top: top view; bottom: lateral view. Time unit (minute : second). Relating to the segmented snapshot in Figure 6E.

Movie S17: Bleb expansion (actin absence) and retraction (actin presence) in the Nocodazole treated cells (pointed by arrows). Cell membrane and F-actin is labeled with Membrane-YFP (green) and Utrophin-scarlet (red), respectively. Time unit (minute : second). Relating to Figure 6I.

Movie S18: A migrating cell (Membrane-YFP, green) expressing DN-Rab11-2A-mCherry (Red). The lamellipodium forms but they are not sustainable in one particular direction. Time unit (minute : second). Relating to Figure 7E.