Tracking exocytic vesicle movements reveals the spatial control of secretion in epithelial cells.

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Abstract

Understanding how specific secretory cargoes are targeted to distinct domains of the plasma membrane in epithelial cells requires analysing the trafficking of post-Golgi vesicles to their sites of secretion. We used the RUSH (retention using selective hooks) system to synchronously release an apical cargo, Cadherin 99C (Cad99C), and a basolateral cargo, the ECM protein Nidogen, from the endoplasmic reticulum and follow their movements to the plasma membrane. We also developed an interactive vesicle tracking framework, MSP-tracker and viewer, that exploits developments in computer vision and deep learning to determine vesicle trajectories in a noisy environment without the need for extensive training data. MSP-tracker outperformed other tracking software in detecting and tracking post-Golgi vesicles, revealing that Cad99c vesicles predominantly move apically with a mean speed of 1.1µm/sec. This is reduced to 0.85 µm/sec by a dominant slow dynein mutant, demonstrating that dynein transports Cad99C vesicles to the apical cortex. Furthermore, both the dynein mutant and microtubule depolymerisation cause lateral Cad99C secretion. Thus, microtubule organisation plays a central role in targeting apical secretion, suggesting that *Drosophila* does not have distinct apical versus basolateral vesicle fusion machinery. Nidogen vesicles undergo planar-polarised transport to the leading edge of follicle cells as they migrate over the ECM, whereas most Collagen is secreted at trailing edges. The follicle cells therefore bias secretion of different ECM components to opposite sides of the cell, revealing that the secretory pathway is more spatially organised than previously thought.

Introduction

Many tissues and organs are composed of tubes or sheets of polarised epithelial cells that act as barriers between internal compartments or between the inside and outside of the body. A key function of epithelia is to control the passage of ions, solutes and nutrients from one side of the epithelium to the other and this requires localisation of ion channels and

transporters to either the apical or basolateral domains of the plasma membrane and the formation of lateral occluding junctions that act as barriers to paracellular transport [1,2]. In addition, many epithelia secrete specific factors from their apical or basal surfaces. To perform these diverse functions, epithelial cells must target secreted and transmembrane proteins to either the apical or basolateral plasma membrane.

Many secreted and transmembrane proteins are localised to the correct domain by polarised exocytosis. In this direct pathway, newly synthesised proteins traffic through the endoplasmic reticulum (ER) and Golgi to the trans-Golgi network (TGN), where they are sorted into post-Golgi carriers that move to and then fuse with the appropriate membrane [3,4]. Other proteins are localised by different mechanisms, however, such as diffusion and trapping or secretion to the opposite membrane, followed by polarised endocytosis and recycling. The direct route can be distinguished from indirect localisation mechanisms by following newly-synthesized protein molecules on their first passage to the plasma membrane by blocking them in the ER or Golgi and then releasing them. This is possible for specific proteins, such as a temperature-sensitive form of VSV-G protein and *Drosophila* Rhodopsin [5–10]. An alternative, more generally applicable strategy exploits the observation that proteins are retained in the Golgi of mammalian cells in culture at 20˚C, allowing their release as a synchronous wave of secretion by raising the temperature [11]. Experiments using these approaches have identified multiple sorting signals for apical and basolateral cargoes and have revealed that there are a variety of trafficking routes to each membrane domain, some of which pass through intermediate endosomal compartments [2,12,13]. Nevertheless, it is still unclear how specific cargoes are targeted to the appropriate membrane domain and how this is controlled by the apical-basal polarity system [1].

Although these approaches have proved powerful, they involve non-physiological conditions or work only for specific cargoes. For example, the low temperature used to arrest secreted proteins in the Golgi can also affect other cellular processes, while the total block to the exit of proteins and lipids from the TGN might have knock-on effects on the localisation of essential trafficking factors. These limitations have been overcome by the development of the Retention using Selective Hooks (RUSH) technique [14]. In this approach, the labelled

cargo is fused to the Streptavidin-binding peptide (SBP), and the hook is provided by Streptavidin fused to KDEL, the ER retention signal (Figure 1A). The binding of SBP to Streptavidin-KDEL retains the cargo in the ER, until the addition of biotin triggers its release by binding with high affinity to Streptavidin and displacing the SBP-tagged cargo, which continues along the secretory pathway.

Most studies of secretion in epithelia have been performed in a small number of immortalised mammalian cell lines, such as Madin-Darby canine kidney (MDCK) cells, derived from the kidney tubule epithelium. We therefore set out to exploit RUSH to investigate how secretion is targeted to the apical and basolateral domains in a more typical secretory epithelium that is amenable to genetic analysis, using the *Drosophila* follicular epithelium as a model [15]. Our results highlight the essential role of microtubule-based transport in targeting secretion and demonstrate that epithelial cells target secretion to multiple specific destinations.[16][17–25][19,26–32]

Results

Using RUSH to visualise polarised secretion

To examine apical secretion using RUSH in the *Drosophila* follicle cells, we inserted SBP and the HaloTag self-labelling tag after the signal peptide of Cadherin 99c (Cad99c) under the control of the GAL4 system (Figure 1B). Cadherin 99c is the *Drosophila* orthologue of the human Usher cadherin PCDH15 and is expressed in the follicle cells throughout oogenesis, where it localises to the apical microvilli [33,34]. For the basolateral cargo, we chose SNAP-tag labelled Nidogen, as it is a monomeric extracellular matrix protein that localises to the basement membrane beneath the follicle cells and is presumably secreted from the bottom third of the lateral membrane like Collagen IV [35–38].

When SBP-Halo-Cad99C is expressed in the follicle cells under the control of traffic jam-Gal4 in the absence of the Streptavidin-KDEL hook and labelled with cell-permeable fluorescent HaloTag ligand, it localises to the apical domain, although some protein leaks into the lateral domain because of over-expression (Figure 1C). Similarly, SBP-SNAP-Nidogen localises to the basement membrane like the endogenous protein, demonstrating that both constructs

contain functional trafficking signals (data not shown). Expressing two copies of UAS-Streptavidin-KDEL is sufficient to retain all SBP-Halo-Cad99C in the ER without activating the ER stress response, as monitored by the levels of BiP [39] (Figure S1). On the addition of biotin, it is released and most Cad99c is localised in the Golgi after 15 minutes (Figure 1C), partially co-localising with the trans-Golgi marker, Golgin 245 [40], and by 30 minutes, almost all labelled protein is localised to the apical microvilli, indicating that it rapidly transits the secretory pathway. Cad99c co-localises with Rab11 in puncta immediately below the apical membrane before it reaches the apical surface, indicating that it passes through the Rab11-positive recycling endosomes en route to the cell surface (Figure 1D)

To characterise the time course of Cad99c and Nidogen secretion, we made time-lapse movies of each labelled cargo after their release from the ER with biotin (Figure 2A and B, Movies S1&2). As seen in the fixed samples, most Cad99c localised to the Golgi after 10 minutes and to apical recycling endosomes after 20 minutes. Cad99c was detected on the apical surface before 20 minutes and reached maximal levels after 30 minutes. The trafficking of Nidogen to the basal surface showed very similar dynamics. We quantified the dynamics of trafficking through the secretory pathway in more detail by marking ER exit sites, the *cis*-Golgi and the *trans*-Golgi and TGN with endogenously-tagged Sec16, GMAP-210 and Golgin 245 respectively [41–43]. We labelled the plasma membrane with CellMask™ and measured the change in co-localisation of Cad99c with each marker over time using the normalised Pearson correlation coefficient (Figure 2C, D, and G, FigS2, Movie S3). Peak levels of Cad99C colocalised with ER exit sites at 11 minutes and with the *trans*-Golgi after 22-23 minutes, indicating that it takes about 11-12 minutes to exit the ER and traverse the Golgi cisternae. Cad99c first appeared at the plasma membrane after 11 minutes and the amount of apical protein increased approximately linearly over the subsequent 20 minutes. Cad99c accumulates at a similar rate at the TGN and at the plasma membrane with a time lag of 6-8 minutes, indicating that this is how long it takes to traffic protein from the TGN to the cell surface. Cad99c reaches the plasma membrane at a similar rate to that of Serpentine in the fly tracheal epithelium but is significantly faster than cargoes that have been tracked in tissue culture cells using RUSH or VSVG-ts [6,14,44]. This may be because the follicle cells and trachea are intact secretory epithelia rather than transformed tissue culture cells and are therefore more efficient at polarised exocytosis.

We performed a similar analysis on the dynamics of Nidogen secretion, except that we used wheat germ agglutinin as a plasma membrane marker instead of Cell Mask, which does not efficiently label the basal plasma membrane (Figure 2E, F and H, Figure S2, Movie S4). This revealed that a significant proportion of Nidogen already localises to ER exit sites before release and that it reaches maximum levels in the *trans*-Golgi at 14 minutes, 8 minutes earlier than Cad99c. This may reflect the fact that Nidogen is a soluble cargo and can therefore concentrate in the ER lumen near the exit sites. Perhaps as a consequence, Nidogen starts to accumulate extracellularly slightly earlier than Cad99c.

The MSP-tracker and MSP-viewer programs

To examine the movement of post-Golgi carriers from the TGN to the plasma membrane, we performed fast time-lapse imaging of labelled cargoes 15-20 minutes after the addition of biotin, i.e. just after their peak accumulation in the TGN. This revealed that many Cad99c positive vesicles show processive movements towards the apical surface, whereas Nidogen-containing vesicles show more randomly-directed trajectories. However, tracking these movements was laborious and challenging due to the large number of small vesicles, their low signal to noise ratio compared to the much brighter Golgi and their dispersed origins from Golgi mini-stacks throughout the cytoplasm (Figure 3A). Many different tracking algorithms have been developed in recent years that use deep learning (DL) methods to enhance target detection and object tracking [17–27,30–32]. However, these machine learning methods usually require an extensive training dataset that must be updated for each new experimental system, which can be impractical and time consuming, some were developed for particular imaging techniques, while others perform poorly in a noisy environment. To address these drawbacks, we have developed an MSP (Multi-Scale Particle) framework to facilitate particle tracking in a noisy environment.

MSP-tracker is an extended version of our particle tracking software that uses a convolutional neural network (CNN) for candidate vesicle selection and a two-step process to identify tracklets and then link them into trajectories [45]. The particle detection module consists of background subtraction step followed by the identification of spots of different sizes and brightness using a multi scale spot enhancing filter (MSSEF) [46] (Figure 3B). This first step creates a pool of candidate vesicles, but is rather noisy, as MSSEF cannot

distinguish between post-Golgi vesicles and Golgi ministacks or other background signals. A light-weight convolutional neural network (CNN) classifier then selects vesicles from the pool. The decreased number of parameters in the CNN compared to other approaches reduces the number of annotations required for training to a few hundred regions of interest (ROIs) and provides a fast candidate pruning process that significantly improves detection efficiency. The MSP-framework comes with a few networks that have already been trained, but the CNN classifier can be easily trained on new data if required. In the last step, the locations of the selected candidates are refined to provide sub-pixel localisation. Due to its computational efficiency, this step uses the iterative centre of-mass refinement [25]. This pruned and refined set of detected particles is then handed to the linking algorithms.

The detected particles in different frames are joined into tracks with a two-step linking approach. First, short tracks (tracklets) are formed based on the vesicles' proximity in time and space. These short tracklets are then linked based on their characteristics, in a process called track-linking. Given the complex and changeable behaviour of the particles, this multi-step approach builds trajectories without assumptions about the type of movement. The first step takes into account the distance between detections and existing tracks, solving the assignment problem sequentially for each frame. The problem can be described as minimum-weight matching and is solved with the Hungarian algorithm [47]. Detections that are not matched with any of the tracks start their own new track. The length of the track is limited, as well as the number of the sequentially missed frames. This way, short reliable tracks are formed based on the spatial and temporal distances.

A Bayesian network (BN) then calculates a connectivity score for each pair of tracklets, based on their positions in time and space, speeds and directions of movement and their appearances. The connectivity score is only calculated for tracklets that are close in space and time, as this significantly reduces computation time and increases the efficiency of the method. When all the possible tracklet pairs have been evaluated, the tracklets are assembled into tracks using the Hungarian algorithm [47]. The minimum number of points in the trajectory can be set manually by the user and it can be useful to filter by length to eliminate very short trajectories, which can arise from false detections. MSP-tracker allows

users to repeat the track-linking process with different settings to link tracks into larger trajectories, as a second pass can be beneficial in a dense environment or when some particles move much faster than the majority. In these cases, the first pass connects slow-moving particles (using a small value for the distance parameter) and the second pass connects faster moving ones (using a larger value for the distance parameter). The software displays the intermediate results from the vesicle detection and track-linking algorithms, revealing how parameter selection affects the output. The optimal parameters can then be saved in an external file for future reference. Finally, the extracted tracks are stored in a format compatible with MSP-viewer where the trajectories can be examined (Figure 3C).

MSP-viewer is the tool for exploring extracted tracks and allows the user to view the tracks, correct them if required, plot them and analyse their characteristics. The main window displays the image sequence overlaid with the plotted trajectories and a table of the individual trajectories (Figure 3C). The set of trajectories can then be filtered based on their duration (length), net or total distances travelled, mean curvilinear speed or trajectory orientation. The results can also be displayed as a circular plot of trajectory orientations based on track count or net distance travelled. Clicking on an individual track in the table opens a new window that shows the trajectory, a graph of the speed of vesicle movement over time and a table listing the net distance travelled, the mean curvilinear and straight line speeds over the whole trajectory and when the particle is moving, as well as the maximum curvilinear speed (Figure 3D-F). The code for both MSP-tracker and MSP-viewer and a detailed manual with step-by-step instructions for installation and parameter settings are available on github (https://github.com/MariiaVision/msp_tracker).

Evaluation of MSP-tracker

To evaluate the performance of MSP-tracker under controlled conditions, we used twelve image sequences of 100 frames of computer-generated data from the particle tracking challenge [20]. The scenario mimics real data of moving vesicles at three density levels (low, middle and high), and at four different signal-to-noise ratios (SNR) of Poisson noise (1, 2, 4 and 7) (Figure 4A). We compared the performance of MSP-tracker to the tracking methods that participated in the particle tracking challenge using the five performance measures used in the original challenge paper (Figure 4B) [20]. Linking

performance is quantified by the degree of matching between the ground truth and estimated tracks, expressed by the α and β measures, and by two Jaccard similarity coefficients, one for entire tracks, JSC_θ, and one for track points, JSC. The α measure does not take into account spurious tracks and ranges from 0 (worst) to 1 (best), while the β measure has a penalty for spurious tracks, and therefore ranges from 0 to α. Both Jaccard similarity coefficients range from 0 to 1. The particle localisation accuracy is evaluated by the root mean square error (RMSE) of matching points in the paired tracks. The lines in Figure 4A represent the values for MSP-tracker and the coloured areas show the range of scores reported in the original challenge paper [20].

The performance of MSP-tracker falls within the upper range of the published results of the particle tracking challenge for the α and β measures but gives lower results for high density samples in the Jaccard similarity coefficient for track points, JSC. This may reflect the fact that the convolutional neural network was not trained on this type of artificial data and was developed to detect the vesicles of interest against a background of other fluorescent objects in the cell, such as the Golgi. However, MSP-tracker performs extremely well for the Jaccard similarity coefficient for the entire tracks, JSC_θ. This highlights its ability to link detections into the correct tracks, even if the detections inside the tracks are not complete.

A more relevant test for MSP-tracker is to evaluate its performance on real data that has been manually tracked to determine the ground truth. We therefore analysed whether MSP-tracker correctly identified tracks in 12 video sequences containing a total of 391 annotated tracks. To examine the performance of the tracker under different conditions, we included movies taken using both Airyscan and spinning disk microscopes, four different RUSH cargoes and imaged along cross-sectional and planar views of the egg chamber. The ground truth data was manually annotated using the ImageJ multi point tool [48] and MSP-viewer, which provides a faster and more convenient platform for tracing vesicle trajectories. The manually tracked data do not provide subpixel particle localisations unlike automated methods, and we therefore did not use the RMSE measure for the evaluation.

The results from MSP-tracker were compared with the performances of the most recent version of Trackmate [23,32], which is widely used as an ImageJ plugin and

SpotTracking [49], which scored in the top 3 in most categories of the particle tracking challenge[20]. In addition to the linking measures that have already been mentioned, α, β, JSC and JSC_θ, we used a novel measure ɣ, which represents the proportion of manually annotated tracks that were detected by the software. MSP-tracker produced significantly better results than the two other tracking programs in all metrics (Figure 4C). This is mainly due to the detection component of the tracker, where the candidate pruning improves detection and therefore boosts tracker performance. Nevertheless, all three programs detect some spurious tracks, which is reflected in the low β and JSC_θ scores. This is largely due to the nature of the data itself. The dispersed mini-Golgi stacks can resemble vesicles, while some vesicles, located away from the focal plane appear as blurry and noisy areas that are challenging to detect. Given the uncertainty about how many of these "false positive" tracks are genuine, ɣ represents a more meaningful performance metric, since it measures the proportion of verified tracks that each software detected. MSP-tracker performed much better than the other programs on this metric, as it detected nearly 90% of the annotated tracks compared to 40% for Trackmate and SpotTracking (Figure 4C).

A key part of our analysis pipeline is to use MSP-viewer to manually curate the tracks after the automated track detection by MSP-tracker. We also manually add in any tracks that have been missed by MSP-tracker, but are visible to the human eye. MSP-viewer provides a straightforward and efficient platform to do this, resulting in a final track list that is as close to the ground truth as possible.

Tracking Cadherin 99c and Nidogen vesicles

We used automated tracking with manual curation to analyse the direction of Cad99c-positive vesicle movements at different stages of oogenesis and at different time points after the addition of biotin. At stage 8 when the follicle cells are still cuboidal, Cad99c-positive vesicles move in all directions. To quantify this, we counted the number of apically-directed tracks (defined as tracks whose orientation fell within 45˚of apical), compared to lateral (left and right 90˚ quadrants) and basal. 31% of vesicles moved apically, 52% moved laterally and 17% basally (Figure 5A, Table S1, Movie S5). The apical tracks are on average longer than the basal ones, however, and this results in a mean apical displacement per track of 0.49 μm ±0.26 (s.e.m), which is sufficient to deliver Cad99c to the apical plasma membrane (Figure 5D). The columnar follicle cells at stage 9 show a much stronger

directional bias towards the apical surface, with 73% of the Cad99c-positive vesicles moving apically compared to 17% basally, giving a mean apical displacement per track of 1.32 μm ± 0.18 (s.e.m) (Figure 5B,D, Table S1, Movie S5). This increase in directionality correlates with the more highly polarised organisation of the microtubules along the apical-basal axis of columnar follicle cells compared to the cuboidal cells at stage 8 (Fig S8G and K) [50–52]. The strong directional bias in the movement of Cad99c vesicles starts to diminish after 30 minutes post biotin addition, when most Cad99c has already reached the apical membrane, presumably because secretion is now balanced by endocytosis in the opposite direction (Figure 5C, Movie S5), indicating that MSP-tracker can also map the trajectories of endocytic vesicles. We saw the same trend when tracking vesicle movements imaged on a spinning disc microscope, with a strong apical bias in Cad99c tracks (Figure S5A) that is lost when imaging later after biotin addition (Figure S5B). A similar analysis of the trajectories of Nidogen vesicles revealed that they move in more random directions with a slight bias towards the basal side (Figure 6A, Table S1, Movie S6). 33% of vesicles move towards the basal quadrant, compared to 25% apically and 41% laterally (Table S1). Nevertheless, the weak directional bias leads to a mean basal displacement per track of 0.17 μm ± 0.09 (s.e.m) (Figure 6B).

Studies on the secretion of another ECM protein, Collagen IV, in the follicle cells indicate that it is secreted in a planar-polarised manner from the basal third of the lateral membrane and that it is transported there in Rab10^{+ve} vesicles by Kinesin and Khc73 [37,38]. During stages 3-8 of oogenesis, the follicle cells collectively migrate over the underlying basement membrane, causing the egg chamber to rotate around the anterior-posterior axis, in a process that aligns the collagen fibrils so that they form a molecular corset around the dorsal ventral axis [38,53]. Imaging of Rab10^{+ve} vesicles revealed that these move towards the trailing edges of the migrating cells, where Collagen IV is thought to be secreted [37,54]. We therefore examined whether Nidogen carriers also show planar-polarised movements basally by imaging horizontal planes of the epithelium either in the middle of the cell or just above the basal surface (Figure 6C and D, Movie S6). The vesicle tracks showed little planar-polarised bias in their directions of movement in the middle of the cell, but showed a pronounced planar polarisation basally, with 45% moving towards the leading edge, compared to 30% towards the trailing edge (Table S1). Surprisingly, this is the opposite bias

to that reported for Rab10^{+ve} vesicles, which are presumed to contain Viking (Collagen IV) [37]. Movies collected on the spinning disc microscope showed the same bias (Figure S6).

To test whether Nidogen is transported to the leading edge in Rab10^{+ve} vesicles, we co-expressed UAS-Rab10-YFP with the Nidogen RUSH construct. Rab10 co-localises with Nidogen in large vesicular compartments at the base of the cells and remains associated with Nidogen vesicles after they leave this structure (Figure 7A and B, Movie S7). Fast imaging of the basal side of the cells 15 minutes after the release of Nidogen with biotin revealed that lots of moving Nidogen-containing vesicles were also Rab10^{+ve} (Figure 7C; Movie S7). As Rab10 signal bleached easily, and not all Rab10 tracks survived filtering, we loaded the Nidogen track coordinates onto the Rab10 movies in MSP-viewer and manually determined whether we could see Rab10 signal along the trajectory and moving with the trajectory. Over 75% of Nidogen tracks had Rab10 associated with them for at least part of their length. Tracking Rab10^{+ve} vesicles in these movies revealed a similar directional bias to Nidogen vesicles, with 45% moving towards the leading edge (Figure 7D-E, Table S1, Movie S7). This is the opposite bias to that observed by Zajac and Horne-Badovinac (2022) in cells that did not over-express Nidogen. We therefore tracked Rab10^{+ve} vesicles in cells without UAS-Nidogen and observed a bias towards the trailing edge (Figure 7F, Movie S7). Thus, releasing a pulse of Nidogen through the secretory pathway shifts the direction of Rab10^{+ve} vesicle movement towards the leading edge.

Since these results suggest that follicle cells secrete Nidogen and Collagen IV from opposite sides of the basal region of the lateral surface, we set out to confirm that Collagen IV is secreted from the trailing edges of the cells. Since Collagen IV is a dimer, it was not possible to use RUSH to examine its movement within the cell. However, it accumulates laterally between cells, before being drawn into alignment in the basal ECM by attachment to the existing ECM and follicle cell rotation[38]. We therefore imaged egg chambers containing clones of cells lacking GFP-Collagen IV surrounded by cells expressing GFP-Collagen IV from the *viking* protein trap line. By labelling the extracellular Col IV in living egg chambers using a fluorescently-labelled anti-GFP nanobody, we could examine from which side of the cell Col IV is secreted while observing the direction of rotation. As expected, more lateral GFP-Col IV could be detected at trailing edges that faced GFP-Col IV non-

expressing cells than at leading edges (Figure 7G). Thus, follicle cells transport Nidogen and Col IV in opposite directions along the basal array of microtubules to bias their secretion at the leading and trailing edges respectively.

The role of microtubules in Cadherin 99c and Nidogen transport

We examined the effects on Cad99c trafficking of disrupting microtubule-based transport. In most epithelial cells, microtubules are nucleated from noncentrosomal microtubule organising centres at the apical cortex and form apical-basal arrays with their minus ends apical [51,55–57]. Cad99c carriers are thought to be transported along these microtubules by the minus end directed motor protein, Dynein [52]. We therefore used the MSP package to analyse Cad99c carrier movements in stage 8 and 9 follicle cells heterozygous for a dominant mutation in the Dynein heavy chain, *Dhc*$^{8-1}$, which reduces the speed of the dynein-dependent transport [58] (Figure 8A, Figure S8A, Movie S8). The net speed of apical movements at stage 9 was significantly reduced in the slow dynein mutant: in wildtype egg chambers, vesicles moved apically with a mean net speed of 1.1 µm/sec ± 0.34 (sd), whereas basally-directed vesicles moved more slowly at 0.85 µm/sec ± 0.19 (sd). In the *Dhc*$^{8-1}$/+ mutant, however, the apically-directed vesicles moved at the same speed as the basally-directed vesicles (0.85 µm/sec ±0.34 (sd) and 0.83 µm/sec ±0.22 (sd) respectively), which were unaffected by the mutation (Figure 8D). This observation confirms that Dynein is the major motor that transports Cad99C vesicles to the apical surface, whereas another, slower, motor mediates the basal movements. *Dhc*$^{8-1}$/+ follicle cells also showed a loss of the apical bias in the direction of vesicle movement, with equal numbers of tracks extending basally and apically in stage 9 egg chambers (Table S1). This correlates with a 40% reduction in the frequency of Cad99C vesicle movements, suggesting that the mutant Dynein is less active than the wild-type motor. As a consequence, the mean displacement per track is altered to 0.1 µm ±0.23 (s.e.m.) basally in stage 8 egg chambers, and 0.07 µm ± 0.3 (s.e.m) basally in stage 9 egg chambers, indicating that Cad99c is not translocated apically (Figure 8E). Consistent with this, Cad99C was secreted exclusively laterally at stage 8 in 15.8% of movies of *Dhc*$^{8-1}$/+ mutant cells and a further 26.3% showed secretion at the lateral membrane before the apical membrane (Figure 8F).

Colcemid treatment leads to the depolymerisation of almost all microtubules in the follicle cells, but a few stable microtubules persist in the apical region of the cells (Figure S8). This leads to a dramatic reduction in the number of Cad99c vesicle movements, indicating that almost all Cad99c trafficking is microtubule-dependent (Figure 8B, Figure S8B, Movies S8). Depolymerising microtubules with colcemid had an even more dramatic effect on where Cad99C is secreted, with 71% of movies showing exclusively lateral secretion or secretion laterally before apically (Figure 8G). This indicates that dynein-dependent apical transport along microtubules is the primary determinant of where Cad99c is secreted and implies that Cad99c-containing vesicles are competent to fuse with the lateral membrane. During the final stages of its secretion, Cad99C normally accumulates in Rab11^{+ve} recycling endosomes beneath the apical membrane (Figure 1D) and is then transported to the membrane by Myosin V [52]. Consistent with this, colcemid treatment leads to the partial relocalisation of the Rab11^{+ve} endosomes to the lateral side of the cell (Figure S8D).

In contrast to Cad99c, the movements of Nidogen-containing vesicles were unaffected by the *Dhc*$^{8-1}$ mutant, suggesting that their trafficking does not require Dynein (Figure 8D and I-J, Movie S9). Apically-directed Nidogen-containing vesicles move at the same speed as basally-directed ones in both wild-type cells and *Dhc*$^{8-1}$ mutant cells (Figure 8D). Furthermore, both move much faster than Cad99C vesicles, with an average speed of 1.5 μm/sec ± 0.4 (sd) apically and 1.4 μm/sec ±0.3 (sd) basally. This is significantly faster than kinesin or dynein, suggesting that the basal transport is mediated by Khc73, a member of the KIF3 family of super-processive, fast plus end-directed motors that transports Col IV basally [37,59,60]. Since almost all microtubules grow from the apical surface with their plus ends extending basally, Khc73 is unlikely to be responsible for the fast apical movements of Ndg vesicles.

The planar polarised movements of Nidogen vesicles along the basal surface are significantly slower, with a mean speed of 0.7 μm/sec, in agreement with the proposal by Zajac et al. (2022) that Kinesin is the predominant motor that transports vesicles along basal microtubules. Consistent with the requirement for Kinesin and Khc73 for Nidogen vesicle transport, depolymerising microtubules with colcemid blocked Nidogen secretion (Figure

8H). Indeed, most Nidogen appeared to remain in the Golgi, as shown by its co-localisation with Golgin245, suggesting that the exit of Nidogen from the TGN requires the pulling forces exerted by microtubule motor proteins. Finally, the movements of Nidogen vesicles along the basal side of the cell in both directions were significantly faster in *Dhc*$^{8-1}$ mutant cells, although the bias in the direction of Ndg tracks was not affected (Figure 8D and K-L, Movie S9). This suggests that transport towards both the leading and trailing edges is mediated by plus end directed motors that are antagonised by dynein. Furthermore, vesicle movements towards the leading edge are significantly faster than those towards the trailing edge in *Dhc*$^{8-1}$/+, suggesting that different motors move the vesicles in each direction.

Discussion

[20]The trafficking of Cad99C to the apical surface of the follicle cells has been shown to depend on dynein and microtubules in studies in fixed samples [52]. Consistent with these results, we observe that Cad99c vesicles predominantly move towards the apical surface, particularly at stage 9, and that these movements are microtubule-dependent, as they largely cease after treatment with colcemid. Analysis of the vesicle trajectories reveals that they move faster and further towards the apical surface than they do basally, suggesting that different motors mediate their movements in each direction. Furthermore, a slow dynein mutant reduces the speed of apical movements without affecting the basal ones. These results demonstrate that dynein directly transports Cad99c vesicles towards the apical surface, whereas an unidentified, slower motor mediates the basal movements.

Both the slow Dynein mutant and colcemid treatment lead to the secretion of Cad99c at the lateral membrane of the follicle cells, indicating that the apical-basal organisation of the microtubule cytoskeleton is the main determinant of where different cargoes are secreted. This observation is surprising since the fusion of vesicles containing apical cargoes with the apical membrane requires a specific t-SNARE protein in mammalian cells, Syntaxin 3, whereas the fusion of basolateral cargo vesicles requires Syntaxin 4 [61,62]. *Drosophila* lacks

clear homologues of Syntaxin 3 and 4, and it is therefore possible that the apical and basolateral membranes contain the same fusion machinery, so that vesicles with apical cargoes are competent to fuse with the basolateral membrane and vice versa. In this case, the site of Cad99c secretion would be solely determined by its Dynein-dependent transport to microtubule minus ends at the apical membrane, explaining why it is mistargeted laterally when Dynein and microtubules are disrupted. Similarly, the secretion of Collagen IV at the basal part of the lateral membrane depends on its basal transport by Khc73 and Kinesin, with knockdown of both motors leading to its secretion at the apical region of the lateral domain [37]. However, it is also possible that the mis-targeting of secretory cargoes is an indirect effect of disrupting microtubule-based transport, as t-SNARES also need to be delivered to the correct membrane. Microtubule disruption causes the mis-localisation of Syntaxin 3 to the basolateral domain of MDCK cells, inducing the secretion of apical cargoes basolaterally [63]. Thus, there may be specific apical fusion factors in *Drosophila* whose localisation also depends on Dynein and microtubules, and which lead to the lateral secretion of Cad99c when mislocalised, but the nature of these factors is unknown.

Knockdown studies have indicated that Khc73 is the main motor that transports ECM cargoes vesicles basally, while Kinesin may play a larger role in the planar polarised transport along the basal side of the cell [37]. Our results support this idea, since we observe that the Nidogen-containing vesicles move towards the basal side at 1.4 μm/sec, consistent with transport by the fast Kinesin 3 family member, Khc73, whereas the planar polarised movements across the basal surface occur at half this speed, as expected for Kinesin 1. Nidogen vesicles show a clear bias in their planar-polarised basal movements towards the leading edge of the migrating follicle cells, suggesting that Nidogen is mainly secreted on this side of the cell. This is the opposite side from where most Collagen IV is secreted, as inferred from the trafficking of Rab10^{+ve} vesicles and the accumulation of Viking-GFP at the trailing edge of cells that abut non-expressing cells [37,54]. The membrane proteins that guide the follicle cell migration are also planar-polarised, with Semaphorin 5c and Lar localising to the leading edges of cells, and Fat2 and Plexin A to the trailing edges [64–66]. Thus, the follicle cells have mechanisms to target the secretion of specific proteins to opposite sides of the basal surface. Epithelial cells are generally thought to traffic

secreted proteins to either the apical or basolateral plasma membrane, but the behaviour of Nidogen and Collagen IV demonstrates that they can target cargoes much more precisely.

Understanding how secretory cargoes are secreted to the correct domain of the plasma membrane requires being able to track and analyse vesicle movements at scale in a reliable and reproducible manner and MSP-tracker provides a powerful tool to do this. The CNN only requires small training sets and the same trained model can be used to analyse multiple experiments if the vesicles are similar in size and appearance. It is particularly good at tracking vesicle movements in noisy environments where other organelles are also visible in the same channel, such as the mini-Golgi stacks in our RUSH experiments. The user-friendly interface of MSP-tracker also allows the results to be monitored at each step of the algorithm, facilitating the selection of the optimal parameters for vesicle selection and track linking. Testing the performance of MSP-tracker showed that it performs particularly well with real data, correctly classifying 90% of the ground truth vesicle trajectories identified by manual annotation. Two other leading tracking algorithms identified fewer than half of these ground truth trajectories, demonstrating that MSP-tracker is significantly better at analysing vesicle movements in a noisy environment. The companion program, MSP-viewer allows users to review and correct the tracking results before analysing the extracted trajectories. This feature significantly improves the resulting data, as it allows users to delete spurious trajectories, which are usually short and can be removed simply by filtering by length. One can also add any trajectories that were missed by MSP-tracker, which are most often tracks that were not linked correctly. Once this straightforward manual curation is complete, MSP-viewer can then be used to analyse the speeds and directions of the resulting trajectories, providing detailed information on the dynamics of vesicle movement. Both MSP-programs are available at https://github.com/MariiaVision/msp_tracker and the software can be run on Windows, MacOS or Unix machines. One can also import tracks from other algorithms into MSP-viewer and use the simple, but powerful, interface to curate the tracks.

Our results demonstrate that MSP-tracker in combination with the RUSH system provide a efficient approach to visualise the complex trafficking of secretory cargoes in epithelial cells.

Combining these approaches with mutations and drugs that disrupt motors and trafficking factors may reveal the underlying logic of polarised secretion in epithelia.

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Materials & Methods

Imaging systems

Two microscopes were used to collect the RUSH tracking data for this study, a Zeiss 880 Airyscan microscope using the Airyscan "Fast" mode, using the 63x Water objective 1.2 NA, and a custom built spinning disk microscope (Sirinakis et al., 2022).

The UAS-Rab10-YFP tracking data were collected on the Zeiss 880 Airyscan using Airyscan "Fast" mode with the 63x oil immersion objective 1.4 NA.

All other images were obtained using a Leica SP8 with white light laser, using the 63x oil objective, 1.4 NA.

For information on acquisition speeds for the tracking movies, microscope system used, Halo/SNAP dye used, and other parameters see figure legends.

Fly Husbandry and lines

Fly lines used in this study are described in **Table 2**.
For RUSH experiments flies were raised at 25˚C until pupation, at which point they were moved to 18˚C to reduce leakiness of the RUSH system.

Flies heterozygous for *Dhc64c*$^{8-1}$ show reduced trafficking speeds of *bicoid* mRNA particles [58], and we therefore used this mutant to test if the speeds of Cad99c and Ndg vesicles movements were affected by this allele.
UAST-SBP-Halo-Cad99c was created by cloning the signal peptide of the Cad99c followed by a GSGSGSG linker, the sequence for streptavidin binding peptide (SBP), PAG, then the HaloTag sequence, another GSGSGSG linker, and the rest of Cad99c into pUAST(AttB) using NEB HiFi assembly. UAST-SBP-SNAP-Ndg was created by cloning the signal peptide of cg25c followed by the SBP sequence, then a Pro-Ala-Gly linker, then the SNAP sequence, a GSHMRSRPTS linker and the Ndg sequence into pUAST(AttB) using NEB HiFi assembly. The cargoes were designed to ensure that the SBP was facing the lumen of the ER and therefore could access the streptavidin-KDEL hook. Transgenic flies made by injecting into the listed landing sites.

Sec16 was endogenously tagged using CRISPR/Cas-mediated homology-directed repair [67,68]. A pCFD3-gRNA-Sec16 plasmid and a donor construct were injected together into TH_attP2 embryos at a concentration of 75ng/µl and 100ng/µl, respectively. To make the donor construct, EGFP along with a GSGSGSG linker and ∼1000bp long homology arms were cloned into pBluescriptSK+ using NEB HiFi Assembly. The endogenous Sec16 ATG was removed, and to prevent Cas9 from re-cutting the repaired DNA ,the PAM site was mutated. Injected flies and their progeny were screened for the presence of the GFP sequence and multiple independent stable lines were recovered.

Table 2 – Fly lines used in this study

Line		Location	Reference	Notes

1	2xUAST-SA-KDEL/CyO	attP40 and attP51C1	[44]	
2	UAST-SBP-Halo-Cad99c	attP2	This Study	
3	UAST-SBP-SNAP-Ndg	attP2	This Study	
4	TrafficJam-Gal4/CyO (P{GawB}NP1624)	2L	[69]	
5	GFP-Sec16	Endogenous locus (X)	This Study	
6	GFP-Golgin-245	Endogenous locus (2R)	[70]	
7	GFP-GMAP (Gmap^{CC00492})	Protein trap (X)	[71]	
8	w;; Dhc64c$^{8-1}$/TM6B	3L	[72]	
8	w; 2xUAST-SA-KDEL/CyO; UAST-SBP-Halo-Cad99c/TM6B			Combination of 1 & 2
9	w; 2xUAST-SA-KDEL/CyO; UAST-SBP-SNAP-Ndg/TM6B			Combination of 1 & 3
10	GFP-Sec16; TrafficJam-Gal4/CyO			Combination of 4 & 5
11	w; TrafficJamGal4, GFP-Golgin245/CyO			Recombination of 4 & 6
12	GFP-GMAP; TrafficJam-Gal4/CyO			Combination of 4 & 7
13	w; TrafficJam-Gal4/CyO; Dhc64c[8-1]/TM6B			Combination of 4 & 8
14	y, w; P{UASp-YFP-Rab10}21	3L	RRID:BDSC_9789	
15	y1 w*; P{PTT-un}vkgG00454, FRT40A / CyO	2L	RRID:BDSC_98343 recombined with FRT 40A	Recombination of Vgk-GFP trap line and FRT40A
16	hsFlp; P{w[+mC]=Ubi-mRFP.nls}2L P{ry[+t7.2]=neoFRT}40A/CyO	2L	hsFlp added to RRID:BDSC_34500	

Sample preparation for live imaging

Flies were fattened on yeast for 2 days at 18˚ C. Ovarioles were dissected out of ovaries and the muscle sheath in Schneider's insect medium supplemented with insulin (7.5 µg/ml, Merck I9278). For SNAP-Ndg flies, ovarioles were initially labelled in Schneider's plus 0.5 µM SNAP-SurfaceBlock for 5-10 min to block non-egg-chamber-autonomous Ndg labelling of the ECM. Ovarioles were labelled in Schneider's plus 1 µM SNAP or HaloTag ligands for 15-20 minutes at room temperature before being transferred to an 8-well poly-lysine coated µ-slide (Ibidi) in 100 µl Schneider's medium. To induce release of the cargo from the ER, 100 µl Schneider's containing 800 µM D-Biotin (Merck) was added to the medium, to give a final concentration of 400 µM D-Biotin.

For colcemid treated ovarioles the abdomens of flies were injected with 0.4 mg/ml colcemid in Schneider's medium 1 hour before dissection. Labelling and cargo release were carried out as above, but 0.4 mg/ml colcemid was present in all the solutions. Colcemid stocks were dissolved in DMSO, as the usual carrier ethanol caused independent effects on the egg chambers, while DMSO did not.

Correlation analysis for cargoes with CellMask, GFP-Sec16, and GFP-Golgin245

Image analysis was carried out using Fiji [48]. i[73]Pearson's correlation coefficient was calculated for the cargo and marker (e.g. Cell Mask, GFP-Sec16, GFP-GMAP, GFP-G245) signal in each frame of the time series using a custom script (https://github.com/gurdon-institute/Egg_Chamber_Analysis/blob/main/Pearson_frames.py).

For Cell Mask, GFP-GMAP, GFP-G245, and UAS-Rab10-YFP, the zero timepoint was set to a value of zero, and the difference between other timepoints and the zero timepoint was calculated for each movie. For GFP-Sec16, as there is significant overlap at the zero timepoint, we set the endpoint of the movie to a value of zero, and calculated the difference between the other timepoints and the endpoint for each movie.

The changes in correlation were then entered into GraphPad Prism, and each dataset transformed to be on a scale of 0-1 for graphing purposes. The transformation was Y=Y/K, where K=the mean maximum change in correlation. We were not able to use the raw correlation values, as the brightness of SNAP/Halo and CellMask labelling varies from egg chamber to egg chamber, whereas the change in correlation is consistent between samples.

Quantifying Nidogen secretion with WGA (Wheat Germ Agglutinin)

We were unable to use the Pearson's correlation for WGA as the staining changed both in brightness and localisation throughout the movie (i.e. in the beginning of the movie the basal surface of the egg chamber was labelled, but by the end it was brighter, and the lateral and apical membranes were also labelled)., We therefore quantified basal signal of Nidogen by using the basal WGA signal to define a basal region.
Egg chamber basal signal was quantified using a custom script (https://github.com/gurdon-institute/Egg_Chamber_Analysis/blob/main/Jenny_Basal_MeasureROICheck1_5basalR.py) to identify egg chambers and measure a 1.5 µm inner band containing the basal membranes of follicle cells. In order to robustly map egg chamber ROIs, the script first finds the largest WGA signal region in each frame, and then uses the convex hull if its area is greater than twice that of the original ROI. Egg chambers in each frame were then filtered to use the mapping from the previous frame if there is an overlap of less than 0.75. This gives reliable, roughly ellipsoid ROIs in frames where signal intensity varies, and a continuous boundary is not detected.

The output of the script includes a measurement of the mean brightness value in the Basal ROI. This was used to calculate the change in brightness over time, with time zero set to a value of zero. The values were then entered into GraphPad Prism, and each dataset was again transformed to be on a scale of 0-1 for graphing purposes. The transformation was Y=Y/K, where K=the mean maximum change in brightness.

Table 3 Number of movies used for cargo marker correlation analysis

Cargo →	**Cad99c**	**Ndg**
Marker ↓		

Sec16	10	11
GMAP	8	15
G245	11	12
CellMask	9	N/A
WGA	N/A	9
Rab10	N/A	16

Antibody stainings of RUSH egg chambers

For antibody stainings of egg chambers, ovaries of fattened flies were dissected into Schneider's medium supplemented with insulin. Ovarioles were incubated in Schneider's medium plus 400 µM biotin for 0, 15, or 30 minutes before being transferred to 4% formaldehyde in PBS and fixed for 20 minutes with rotation. Ovarioles were washed 3x5min in PBS, then incubated with Halo-TMR ligand (Promega) for 1 hour at 37˚C with shaking at 500rpm. Samples were washed 3x5 min in PBS then blocked in PBT (0.2% Tween20) with 10% BSA overnight at 4˚C. Ovarioles were then incubated for 48 hours at 4˚C in PBT 1% BSA with 1:2000 Rabbit anti-Rab11 [74] and 1:1000 Goat anti-Rabbit. Ovarioles were then washed 4x30min in PBT 1% BSA, then stained overnight at 4˚C with 1:500 Donkey anti-Rabbit Alexa488 and Donkey anti-Goat Alexa647 secondary antibodies in PBT 0.1% BSA. Samples were washed 3x10 min in PBT and mounted in Vectashield plus DAPI (Vectorlabs). Images were taken on the Leica SP8 WLL with the 63x oil objective. 1.4 NA.

Microtubule stainings of egg chambers.

For microtubule stainings of egg chambers, fattened flies were either mock injected with Schneider's medium, or with 0.4 mg/ml colcemid in Schneider's medium, 1 hour before dissection. Ovaries were dissected into either Schneider's medium supplemented with insulin, or also containing 0.4 mg/ml colcemid. Ovarioles were incubated for 30 minutes before being transferred to 10% formaldehyde in PBT (2% Tween20) and fixed for 10

minutes. Ovarioles were washed 3x5min in PBT (2% Tween20), then blocked in PBT (0.2% Tween20) with 10% BSA for 2 hours. Ovarioles were then incubated overnight at 4˚C in PBT (0.2% Tween20) 1% BSA and a 1:200 of FITC-conjugated anti-α-tubulin antibody (F2168, Merck, clone DM1A). Ovarioles were then washed 6x10min in PBT (0.2% Tween20) and mounted in Vectashield plus DAPI (Vectorlabs). Images were taken on the Leica SP8 WLL with the 63x oil objective. 1.4 NA.

Staining and quantification of extracellular Collagen IV at leading and trailing cell edges

hsFlp / + ; P{PTT-un}vkgG00454, FRT40A / nls-RFP, FRT40A females were collected and fattened on yeast (with males present), and subjected to 2-3 x 2hour 37˚C heatshock at 12 hour intervals. Alternatively pupae were heatshocked for 3 days with 2hour 37˚C heatshock at 12 hour intervals, and then adults fattened for 24-48 hours at 25˚C with yeast.

Ovaries were dissected out in Schneider's supplemented with insulin, and the ovarioles removed from the muscle sheath. Ovarioles were transferred to a drop of Schneider's with 1:50 dilution of Atto647n GFP-nanobody (Proteintech) to label the extracellular Collagen for 20 minutes. Ovarioles were transferred to fresh Schneider's and imaged as previously on an 8-well poly-lysine coated μ-slide (Ibidi). Timelapse Z-stacks were taken to allow the direction of rotation to be determined. The amount of extracellular collagen at the leading and trailing edges of the cells was then measured as follows. In FIJI [48] a segmented line was drawn along the leading or trailing edges of a patch of cells. This was converted to an area, the area expanded to a band of ~0.2um wide and the integrated density was measured. The integrated density was divided by the number of cells in the patch and used to compare Collagen levels at the leading and trailing edges of the cells. Non boundary cells (i.e. those that contacted Vkg-GFP exprssing cells on both sides) were measured as a control.

Using MSP-tracker and MSP-viewer to track Cad99c, Ndg and Rab10 particles.

MSP-tracker was used to find particle tracks by following the method described in the user manual. MSP-viewer was used to assess the tracks found by MSP-tracker. Tracks were

filtered by length to remove short (<700 nm) tracks. Remaining tracks were assessed on a track by track basis, and tracks were added manually, extended, edited, or deleted as necessary. Once tracks had been curated they were filtered to remove anything of less than 1000 nm long, and data were exported for further analysis. Excel was used to filter data into tracks going in different orientations, and Graphpad Prism was used to represent the data.

Western blotting

For each sample, three ovaries were homogenised in 25 µl Schneider's medium and 25 µl 2x Laemmli sample buffer and boiled at 95˚C. 10 µl of sample was loaded per lane on a 4-12% Bis-Tris polyacrylamide gel. The membrane was probed for BiP and Tubulin using rabbit anti-GRP78 (BiP) (StressMarq biosciences SPC-180S) at 1:1000, and mouse anti-tubulin (DM1A) at 1:1000. Secondaries used were goat anti-mouse Alexa 680 and goat anti-rabbit Alexa790 at a dilution of 1:10,000. The blot was imaged using a LiCor Odyssey system.

The tracking algorithm

The particle tracking algorithm of the MSP-framework consists of particle detection, and frame-to-frame object association (linking), Figure 2B. A pre-processing step enhances the appearance of the particles and improves the detection results. The background intensity is estimated from m consecutive frames (parameter can be set manually) and subtracted from the original frame. Finally, Contrast Limited Adaptive Histogram Equalization is applied to each frame to enhance the contrast.

The particle detection exploits an extended multiscale version of the spot enhancing filter (SEF). The SEF highlights spots by convolving the original image g(x, y) with a Laplacian-of-Gaussian kernel LoG(x, y, σ). In the multi scale spot enhancing filter (MSSEF) [46], a set of SEF is applied sequentially with an intensity-based threshold after each iteration. This way, each iteration can be represented by Equation 1.

$$f(x, y, \sigma^k) = LoG(x, y, \sigma^k) * \left(b(x, y, \sigma^{k-1}) g(x, y)\right), \qquad (1)$$

where σ^k is a standard deviation of the LoG, and $b(x, y, \sigma^{k-1})$ is a binary mask extracted from the previous iteration. The threshold for each iteration T^k is defined by the mean intensity of the image μ^k and its standard deviation ς^k weighted by a user defined value c, Equation 2.

$$T^k = \mu^k + c\,\varsigma^k, \quad (2)$$

The MSSEF exposes spots of different sizes and intensities and provides a reduction of the false detection rate related to the noise levels. The particle candidates are extracted based on the local maxima of the final MSSEF image. The coordinates of the candidates are used to define a region of interest (ROI) for each candidate. The default ROI size is 16x16 pixels, but the value can be adjusted for larger particle sizes. The CNN network classifies the ROIs to provide the final detection results. Training on small ROIs instead of an entire image allows one to use a light-weight CNN and apply the same learned model for imaging modalities provided that the appearance and size of the particles are similar.

The light-weight CNN architecture consists of two convolutional layers followed by max pooling and two fully-connected layers. The softmax activation function is used for the output layer, and Relu activation for the rest. The network is trained with binary cross-entropy loss function and root mean square propagation optimiser. The training data is augmented with rotation and flipping.

FInally, the locations of the selected candidates are refined to provide sub-pixel localisation using the iterative centre-of-mass refinement for the task [25].

The data association of the detected particles is solved with a two-step linking approach. The first step of the data association considers the distance between detections and existing tracks, solving the assignment problem sequentially for each frame with the Hungarian algorithm [47]. The weight is represented by the Euclidean distance between the detection and already existing tracks. The formed tracklets are used to build longer trajectories.

A Bayesian network (BN) is used to compute the connectivity score for each pair of the tracklets. The score expresses the probability of these tracklets being linked based on their characteristics. Each variable (node) of the BN represents a single characteristic of the tracklet pair. These characteristics can be divided into four main groups: particle appearance, motion, location, and temporal position.

The particle appearance is represented by the intensity node. It indicates the similarity in average intensity between the ROIs in the last frame of the first tracklet and the first frame of the second tracklet. We expect the intensity of the particle to change along the trajectory but have similar values in succeeding frames. The motion is defined by two nodes: orientation and speed, where the similarity in orientation and speed of particle movement is defined. The location of the tracklets is defined based on the Euclidean distance between the coordinates of the last detection in the first tracklet and the first detection in the second tracklet. The temporal position is defined by sequence and gap variables. The sequence node characterises the successiveness of the first and second tracklets. This node is defined by two parent nodes: an order and an overlap. The gap node represents a temporal gap between the last detection in the first tracklet and the first detection in the second tracklet. The conditional probability table defines the contribution of each parent node. The introduced connectivity score is a binary variable with True meaning that the pair of tracklets are connected and belong to the same trajectory. The inference over the BN provides the probability of the connectivity score (CS) to be True, P_c:

$$Pc = P\left(CS = True \middle| I, O_r, S_p, O_d, O_v, G\right), \quad (3)$$

where I is a difference in intensities, O_r – in orientation, S_p- in speed, O_d – order of the tracklets, O_v – presence of the overlap and G is a temporal gap between the tracklets. When all the possible tracklet pairs are evaluated, the assignment problem is addressed with the Hungarian algorithm [47], and the tracklets are assembled into the tracks.

Parameters for detection and linking can be set in MSP-tracker GUI before running the tracking algorithm for the entire timelapse sequence.

Trajectory evaluation in track-viewer

The individual trajectory window of the MSP-viewer provides several quantitative trajectory measures, Table 3. Travelled distance is described by the total, net and maximum distances. The total distance is the entire trajectory length. In contrast, net distance represents the length between the first and the last points, and the maximum distance is defined by the largest interval between any two points of the trajectory. The total trajectory time is the number of frames scaled by the frame rate. The net orientation is defined by the

first and last trajectory's points. The curvilinear speed is calculated from the total distance and can be defined as the mean of the instantaneous speeds, while the net distance defines the straight-line speed.

Table 3. Quantitative trajectory measures provided by the MSP-viewer

Measures	Formulation
Total distance travelled	$d_{total} = \Sigma_{i=1}^{N-1} d(p_i + p_{p+1})$
Net distance travelled	$d_{net} = d(p_i + p_N)$
Maximum distance travelled	$d_{max} = \max_i (d(p_1 + p_i))$
Total trajectory time	$t_{traj} = (N-1)t$
Net orientation	α
Mean brightness (normalised)	$b = \frac{1}{b_{max}N} \Sigma_{p=1}^{M} b_p$
Mean curvilinear speed	$v = \frac{1}{N-1} \Sigma_{i=1}^{N-1} \frac{d(p_i + p_{i+1})}{t}$
Mean straight-line speed	$v_{line} = \frac{d_{net}}{t_{traj}}$
Max curvilinear speed over a segment	$\max(v_1, v_2, \dots v_w)$
Number of moving segments	k
Average moving segment time	$t_{avg} = \frac{\Sigma_{j=1}^{k} t_j}{k}$
p_i - position of the ith particle; N – number of detections in the trajectory; t – trajectory time; M- number of pixels in the region of interest; b_p- pixel brightness; b_{max}- value of the brightest pixel in the image sequence	

The speed is evaluated for the entire trajectory (average speed), for the trajectory segments where the particle is moving (moving speed), and the maximum curvilinear speed is calculated for a given time interval by a sliding window over the moving segments. To allow segmentation of short trajectories, where the traditional Mean Square Displacement (MSD) methods are not applicable, we exploit a 1D U-Net segmentation [75]. The method provides segmentation of the directed motion of the particle from the remaining trajectory. The software provides both options, MSD and 1D U-Net based approaches.

Registration of the image sequence

Some image sequences have an undesirable movement of the egg chamber in the frame. We have registered the sequences before using the tracking software to prevent the movement's influence on the trajectory evaluation. We have used our custom Python code and the SimpleITK library [76], using mutual information between two neighbouring frames to register image sequences.

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Figure Legends

Figure 1. The RUSH system

A) Diagram of the RUSH system. The Halo-tagged cargo is retained in the ER by the binding of the Streptavidin binding peptide (SBP) to Streptavidin (StrepA), which is fused to the ER retention signal, KDEL. The cargo is synchronously released upon the addition of biotin, which outcompetes SBP for binding to Streptavidin.

B) Diagram of the tagged cargo constructs used in this study. A signal sequence is followed by the SBP, then the tag, and the cargo. Cadherin 99C (Cad99c) was used as an apical cargo and Nidogen (Ndg) as a basal cargo.

C) Time course of SBP-Halo-Cadherin99C trafficking in fixed samples. At 0 minutes, Cad99c localises to the ER throughout the cytoplasm. 15 minutes after release from the ER with

biotin, Cad99c (green) has accumulated in the Golgi, marked by Golgin245 (magenta). By 30 minutes, almost all Cad99c has reached the apical membrane. Scale bar 10 µm.
D) 25 minutes after release from the ER, Cad99c (green) localises to sub-apical puncta that are labelled by Rab11 (magenta). Scale bar 10 µm.
In all figures with a cross-section of the follicle cells, apical is toward the top of the image. And basal toward the bottom.

Figure 2. Time course of Cad99c and Nidogen secretion

A-B) Stills from RUSH movies of SBP-Halo-Cadherin 99c (A) and SBP-SNAP-Nidogen (B) trafficking in the follicle cells after release from the ER. Cad99c is shown in an early stage 9 egg chamber, and the Ndg in a stage 8 egg chamber. Scale bars 10 µm. Time indicates minutes since the addition of biotin. Cad99c is labelled with Halo-OregonGreen, and Ndg with SNAP-SiR. The large cytoplasmic puncta visible 10 minutes after cargo release are Golgi ministacks.
C) Stills from a RUSH movie of SBP-Halo-Cadherin 99c (labelled with Halo-JF646; magenta) in cells expressing GFP-Sec16 (green), which marks ER exit sites. Scale bars 5 µm. Cad99c partially co-localises with Sec16 at ER exit sites after 10 minutes, and by 20 minutes is predominantly localised to the Golgi, which lie adjacent to the ER exit sites (See supplementary Figure 2).
D) Stills from a RUSH movie of SBP-Halo-Cadherin 99c (magenta) in cells in which the plasma membrane is labelled with CellMask™. Some Cad99c has already reached the apical membrane by 20 minutes and much more is localised apically at 30 minutes.
E) Stills from a movie of SBP-Halo-Ndg RUSH in cells expressing GFP-Sec16. Some colocalisation of Ndg with Sec16 can be seen at time 0, presumably as Ndg is a soluble protein present in the ER, and is able to partly accumulate at the ER exit sites. Most Nidogen localises at ER exit sites after ten minutes and in the adjacent Golgi mini-stacks after 20 minutes.
F) Stills from a movie of SBP-Halo-Ndg RUSH (SNAP-SiR; magenta) in cells labelled with alexa488-wheatgerm agglutinin (WGA; green), which labels the basal membrane. Scale bars 5 µm. Colocalisation of Ndg with WGA can be seen from 20 minutes, and is stronger at 30 minutes as Ndg has been secreted and bound to the ECM.

G) Graph showing the change in Cad99c colocalisation with GFP-Sec16 (ER exit sites), GFP-GMAP (*cis*-Golgi), GFP-G245 (*trans*-Golgi and TGN) and CellMask™ (plasma membrane) over time. Peak colocalisation has been normalised to 1 to allow easier comparison between markers. Cad99c colocalisation with Sec16 peaks at 11 minutes after biotin addition, its colocalisation with GMAP peaks between 12-14 minutes and with G245 is at 22-23 minutes. Cad99c starts to co-localise with CellMask™ at 11 minutes and increases over time. The number of movies used for each marker is detailed in the materials and methods.
H) Graph showing the change in Nidogen colocalisation with GFP-Sec16, GFP-GMAP, GFP-Golgin245 and with WGA, which marks the basal membrane. Peak colocalisation has been normalised to 1 to allow easier comparison between markers. Ndg colocalisation with Sec16 is already high before biotin addition, and peaks at 12 minutes after biotin addition, which is also when the peak colocalisation with GMAP occurs, indicating rapid transit of Ndg from the ER exit sites to the *cis*-Golgi. Peak Ndg colocalisation with G245 is at 14 minutes, again indicating rapid transit through the Golgi. Basal Ndg signal is observable from 6 minutes, and increases over time. It is important to note that as a soluble protein, the Ndg present in the basement membrane is not necessarily from the egg chamber being observed. The number of movies used for each marker is detailed in the materials and methods.

Figure 3. The MSP-tracker and viewer analysis pipeline

A) Examples of frames from a Cad99c RUSH movie, highlighting both particles of interest (magenta circles), background/ER signal (blue circle), and Golgi mini-stacks (green circle). Scale bar 2 µm.
B) Workflow for MSP-Tracker showing the steps taken from .tiff file to trajectories.
C) An example of the output of MSP-viewer showing the trajectories of Cad99c vesicles overlaid onto a movie still. The image was obtained using the trajectories:save image command in the mspviewer software.
D) Example of a Cad99c track as seen in the “trackviewer” window of MSP-viewer.
E) Example of the trajectory segmentation section of the “trackviewer” window in MSP-viewer. The marked speed is the fastest segment of the track over a 1 second time interval.

F) Trajectory information as viewed in the “trackviewer” window. These data are output into a .csv file using the trajectories:save info to csv function.

Figure 4. Evaluation of the MSP-tracker

A) Performance of the proposed MSP-tracker evaluated on the particle tracking challenge data [**Chenouard2014].** The performance is evaluated by α and β measures, the Jaccard similarity coefficient for track points JSC, the Jaccard similarity coefficient for the entire tracks JSC_θ and root mean square error (RMSE) of matching points in the paired tracks. The lines represent the MSP-tracker results and coloured areas refer to the range of the results presented in the original challenge paper [**Chenouard2014]**.

B) Frame examples of the particle tracking challenge data.

C) Performance of the proposed MSP-tracker, Trackmate and SpotTracking evaluated on the original data. Linking performance is evaluated by α and β measures, the Jaccard similarity coefficient for track points JSC, as well as for the entire tracks JSC_θ and a fraction of paired up tracks ɣ.

Figure 5. Cad99c tracks are apically biased.

Arrows superimposed on movie stills indicate the vectors of the vesicle tracks from the movie. Cyan arrows are apically directed, red arrows basally directed and yellow arrows laterally directed.

A) Tracks from representative movie of Cad99c trafficking in a stage 8 egg chamber 21 minutes after biotin addition. The polar histogram on the right shows the orientation of Cad99c tracks from 3 Airyscan movies. This and the following polar histograms represent the number of tracks that travelled a net distance of at least 1 µm in each 10˚ direction, with apical (A) at the top and basal (B) at the bottom. The plots are normalised to show tracks per minute and speeds are colour coded according to the key. All movies were collected at 4.5 fps. Cad99c was labelled with Halo-JF549. Scale bars are 5 µm.

B) Tracks from representative movie of Cad99c trafficking in a stage 9 egg chamber, 29 minutes after biotin addition. The polar plot represents data from 5 Airyscan movies and shows a strong apical bias in the direction of Cad99c vesicle movements.

C) Tracks from representative movie of Cad99c trafficking in a stage 9 egg chamber 39 minutes after biotin addition. The polar plot represents data from 3 airyscan movies. By this timepoint, the strong apical bias is lost, as the exocytosis of C99c is balanced by endocytosis.
D) Graph showing the net displacement of Cad99c vesicle tracks along the apical-basal axis, with positive displacement towards apical (A), and negative towards basal (B). The bar shows the mean, and the error bars the s.e.m. Individual track displacements are indicated by the small circles. The mean displacement for stage 8 is 0.49 µm ±0.26 (s.e.m), and for stage 9 is 1.32 µm ±0.18 (s.e.m). Significance was calculated using a Wilcoxon Signed-Rank test for comparing the displacement to zero.

Figure 6. Analysis of Ndg tracks reveals planar polarised transport basally.

A) Tracks from a representative movie of Ndg trafficking in a stage 8 egg chamber 23 minutes after biotin addition. The polar plot on the right represents data from 3 Airyscan movies and shows a weak bias towards the basal side of the cell. Speeds are colour-coded according to the key. Movies were taken at 3.7 fps. Arrows superimposed on movie stills indicate the vectors of the vesicle tracks from the movie. Cyan arrows are apically directed, red arrows basally directed and yellow arrows laterally directed.
B) Graph showing the net displacement of Ndg vesicle tracks along the apical-basal axis (A, B; lefthand column) and along the leading edge to trailing edge axis (L,T) in the middle of the cell (middle column) and on the basal side of the cell (righthand column). The bars and error bars show the means and s.e.m. Individual tracks are indicated by the small circles. P indicates planar. Mean displacement towards basal is 0.17 µm ±0.09 (s.e.m). The mean displacement for planar polarised tracks in the middle of the cell was 0.11 µm ±0.07 (s.e.m) towards the leading edge, and for the basal tracks, 0.44 µm ±0.13 (s.e.m) towards the leading edge. Significance was calculated using a Wilcoxon Signed-Rank test comparing the displacement to zero .
C) Ndg tracks in a transverse section through the middle of the follicle cell epithelium (see diagram). The large white arrow indicates the direction of egg chamber rotation and the coloured arrows indicate the vectors of vesicle tracks, with tracks towards the leading edge in cyan, towards the trailing edge in red and tracks perpendicular to the axis of rotation in

yellow. The polar plot on the right represents data from 3 Airyscan movies taken at 2.3 fps. The Leading and trailing (L and T) directions of the egg chamber's rotation are indicated.
D) Ndg tracks in a transverse section through the basal section of the follicle cell epithelium. The direction of rotation and the tracks are labelled as in (C). The polar plot represents data from 3 Airyscan movies taken at 2.3 fps.

Figure 7. Ndg is transported in Rab10 positive vesicles.
A) Stills from a RUSH movie of SBP-SNAP-Nigogen (labelled with SNAP-SiR; magenta) in cells expressing UAS-Rab10-YFP (green). Scale bar 5 µm. Significant colocalisation of Ndg with Rab10 in large vesicular structures is seen 15 minutes after biotin addition, but this colocalisation has largely disappeared by 30 minutes.
B) Graph showing the change in Nidogen colocalization with GFP-Sec16, GFP-GMAP, GFP-Golgin245, UAS-Rab10-YFP and basal WGA over time. Peak colocalisation is set to 1 to enable comparison between markers. Ndg colocalisation with Rab10 peaks at 19 minutes, after the peak with G245, indicating Ndg leaves the Golgi and transits through a Rab10 positive compartment on its way out of the cell. The graph is the same as Figure 2H, but with the addition of UAS-Rab10-YFP to show its position in the pathway.
C) Still from an Airyscan movie of the basal side of cells expressing UAS-Rab10-YFP and Ndg. The yellow arrowheads indicate examples of Rab10 vesicles; cyan arrowheads indicate examples of Rab10 vesicles that contain Ndg.
D) A polar plot of Rab10 tracks from movies at the basal side of cells also expressing SNAP-Ndg. Rab10 vesicle movements show a bias towards the leading edge. The polar plot represents tracks from two Airyscan movies.
E) A polar plot of Ndg tracks from the same movies as D), also showing a leading edge bias.
F) A polar plot of Rab10 tracks from movies of cells not expressing SNAP-Ndg, showing a bias towards the trailing edge. The plot represents tracks from two Airyscan movies. Speeds are colour coded according to the key.
G) Stills from movies looking at Collagen IV secretion from Vkg-GFP clones. Double RFP (Vkg-GFP negative) cells that do not express Collagen IV are marked in blue, and the clone boundary is marked by a blue line. The direction of migration in each movie is indicated by a

white arrow. Extracellular Collagen IV was labelled with GFP-booster conjugated to Atto647n. Intra- and extracellular Collagen IV is marked with GFP.
H) Quantification of extracellular Collagen IV levels at the leading and trailing edges of cells, from movies such as those represented in G). Cell whose leading edges lie at the boundary with non-expressing double RFP cells were used to measure the amount of Collagen IV secreted at the leading edge, cells with trailing edges at the boundary were used to measure the amount of Collagen IV secreted at the trailing edge, and non-boundary cells were used to measure the total amount of secreted Collagen IV. The majority of the Collagen IV is secreted from the trailing edge.

Figure 8. The effects of a slow dynein mutant and microtubule depolymerisation on Cad99c and Ndg trafficking.

A) Tracks from a representative movie of Cad99c trafficking in a *Dhc*$^{8-1}$ heterozygous stage 9 egg chamber, 13 minutes after biotin addition. The polar plot on the right represents data from 3 Airyscan movies and shows the loss of the apical bias in Cad99c vesicle movements seen in WT egg chambers. Scale bar 5 µm.
B) Tracks from a representative movie of Cad99c trafficking in a stage 9 egg chamber, 26 minutes after biotin addition that has been treated with colcemid to depolymerise microtubules. The polar plot on the right represents data from 4 movies. Scale bar 5 µm.
C) A polar plot of Cad99c tracks in wild-type stage 9 egg chambers for comparison with (A) and (B). The plot represents data from 5 movies, and is the same as in Figure 5B.
The scales in A) B) and C) are the same to allow for easy comparison.
D) Graph showing the maximum curvilinear speeds (over a segment) of Cad99c and Ndg vesicles travelling apically or basally in wild-type and *Dhc*$^{8-1}$*/+* cells and for planar polarised Ndg tracks along the basal surface. A stands for Apical, B for Basal, L for Leading Edge, and T for Trailing Edge. Cad99c vesicles move significantly faster apically than basally, and significantly faster than apical tracks in the *Dhc*$^{8-1}$/+ mutant. The *Dhc*$^{8-1}$ mutation has no effect on the speeds of Ndg vesicle movements along the apical-basal axis, but increases the the speed of planar-polarised vesicle movements at the basal side of the cell. The bars and error bars represent the means and standard deviations and the circles represent individual tracks. Statistical significance was determined with a Kruskal-Wallis test using Dunn's

multiple comparisons test, with significant differences between conditions indicated: * = $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$.

E) Graph showing the mean displacement of Cad99c and Ndg tracks in wild-type and *Dhc*$^{8-1}$/+. Positive displacement is towards the apical side (A) of the cell, with negative values indicating displacement towards the basal side (B). The last two columns show the displacement of Ndg vesicles on the basal surface towards the leading edge of the cell (L). Bars and error bars represent the means and standard errors of the means (s.e.m), and circles represent individual tracks. The y axis is compressed at large displacement values to fit all the data in. Statistical significance for the C99c dataset was determined by a Kruskal-Wallis test using Dunn's multiple comparisons test, with significant differences between conditions indicated. Statistical significance for the Ndg pairs determined by Mann-Whitney test. * = $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$.

F) An image of Cad99c RUSH in a *Dhc*$^{8-1}$/+ background, taken 43 min after biotin addition. Cad99c has been secreted to the lateral membrane, but not the apical membrane. Scale bar 10 µm.

G) A projection of a Z-stack from a movie of Cad99c RUSH in a colcemid treated egg chamber 54 minutes after biotin addition. Cad99c has been delivered to the lateral membrane, but not to the apical membrane. Scale bar 10 µm.

H) Image from a movie of Ndg trafficking in a colcemid-treated stage 8 egg chamber expressing GFP-G245. The image was taken 30 min after biotin addition and shows that most Ndg is still trapped in the Golgi ministacks. Scale bar 10 µm.

I) Tracks from representative movie of Ndg trafficking in a stage 8 *Dhc*$^{8-1}$/+ egg chamber taken 24 minutes after biotin addition. Scale bar 5 µm. The polar plot on the right represents 3 movies and shows the number of tracks per minute.

J) A polar plot showing the number of Ndg tracks per minute in WT stage 8 egg chambers for comparison with (I).

K) Tracks from representative movie of Ndg trafficking at the basal side of a stage 8 *Dhc*$^{8-1}$/+ egg chamber taken 22 minutes after biotin addition. Scale bar 5 µm. The polar plot represents 2 movies and shows the number of tracks per minute.

L) A polar plot showing the number of Ndg tracks per minute in wild-type stage 8 egg chambers for comparison with (K)

Supplementary Figures

Supplement for Figure 1 – RUSH does not cause ER stress.

BiP expression in egg chambers expressing the Cad99c and Ndg RUSH constructs, with or without the 2x Streptavidin-KDEL insertions that retain the cargoes in the ER. Tubulin was used as a loading control. BiP levels do not change when the 2x Streptavidin-KDEL hook was expressed to retain the cargoes in the ER, indicating that trapping the cargoes does not cause ER stress.

Supplement for Figure 2 – Time course of Cad99c and Nidogen secretion.

A) Still images taken at different time points are biotin addition from a RUSH movie of SBP-Halo-Cadherin 99c (magenta) trafficking in cells expressing GFP-GMAP (*cis*-Golgi), GFP-Golgin245 (*trans*-Golgi and TGN) as secretory pathway markers. Scale bars 5 µm.

B) Stills from a RUSH movie of SBP-SNAP-Nidogen trafficking in cells expressing GFP-GMAP, or GFP-Golgin245. Scale bars 5 µm.

Supplement for Figure 5 – Spinning disc imaging of Cad99c trafficking

A) Tracks from representative movie taken on a spinning disc microscope of Cad99c trafficking in a stage 9 egg chamber, 30 minutes after biotin addition. The polar plot on the right shows the strong apical bias in the direction of the tracks. Movies were taken at 2 fps using a custom-built spinning disk system.

B) Tracks from representative movie taken on a spinning disc microscope of Cad99c trafficking in a stage 9 egg chamber, 35 minutes after biotin addition. The polar plot on the right represents data from 2 spinning disk movies and shows that the strong apical bias has disappeared, as the exocytosis of C99c is now balanced by endocytosis.

Supplement for Figure 6 – Spinning disc imaging of Nidogen trafficking

A) Tracks from representative movie taken on a spinning disc microscope of Ndg vesicle movements along the apical-basal axis of a stage 8 egg chamber. The polar plot on the right represents data from 2 spinning disk movies and shows a slight basal bias in Ndg movements.

B) Tracks from representative movie taken on a spinning disc microscope of Ndg vesicle movements Ndg tracks in a transverse section through the middle section of the follicle cells (see diagram). The polar plot represents data from 3 spinning disk movies and shows no clear bias in track direction.

C) Tracks from representative movie taken on a spinning disc microscope of Ndg vesicle movements Ndg tracks in a transverse section at the basal side of the follicle cells (see diagram). The leading and trailing (L and T) directions of follicle cell migration are indicated in the polar plot on the right, which shows that most tracks are directed towards the leading edge.

Supplement for Figure 8 – *Dhc*$^{8-1}$*/+* and colcemid treatments.

A) Tracks from a representative movie of Cad99c trafficking in a *Dhc*$^{8-1}$ heterozygous stage 8 egg chamber 33 minutes after biotin addition. Scale bar 5 µm. The polar plot on the right represents data from 3 movies.

B) Tracks from a representative movie of Cad99c trafficking 24 minutes after biotin addition in a stage 8 egg chamber that has been treated with colcemid to depolymerise microtubules. Scale bar 5 µm. The polar plot on the right represents data from 4 movies

C) Polar plot representing data from 2 movies showing the number of Cad99c tracks per minute in wild-type stage 8 egg chambers.

Graph scales in A) B) and C) are the same to allow for easy comparison.

D) Rab11 and Cad99c localisation in colcemid-treated egg chambers 30 minutes after the addition of biotin. Rab11 positive recycling endosomes are no longer enriched apically and can be seen laterally and basally with Cad99c. Scale bar 5 µm.

E), G), I), K) β-tubulin staining in mock injected flies. Scale bar 10 µm.

F), H), J), L) β-tubulin staining in flies injected with colcemid. These images were collected with the same microscope settings and laser power colour scale as the mock injected images. Scale bar 10 µm. F'), H'), J'), L') have all been adjusted to show the details of the remaining microtubules.

G) and H) show microtubules in cuboidal follicle cells.

K) and L) show microtubules in columnar follicle cells.

Table S1

The number of vesicles travelling in each direction for all tracked RUSH movies of Cad99c and Ndg.

Chi^2 tests were performed to determine if the tracks were directionally biased. The null hypothesis was that equal numbers of tracks would travel apically/basally, or leading/trailing.

Movies

Name of Movie	Movie Legend	Associated Figure
Movie1	Cadherin99c RUSH – Images taken every 5min. SBP-Halo-C99c labelled with Halo-OregonGreen. Time represents time since biotin addition. Cadherin99c can be seen trafficking through the secretory pathway in an early stage 9 egg chamber. The large puncta that can be seen at 10 minutes after cargo release are the Golgi ministacks. The small puncta seen subapically in the cells from 15 minutes onward correspond to Rab11 positive recycling endosomes, through which the Cad99c travels.	Figure 2A
Movie2	Nidogen RUSH – Images taken every 5min. SBP-SNAP-Ndg labelled with SNAP-SiR. Time represents time since biotin addition. Nidogen can be seen trafficking through the secretory pathway in a stage 8 egg chamber. The large puncta seen at 10 minutes after cargo release are the Golgi ministacks.	Figure 2B
Movie3	Cadherin99c RUSH with secretory pathway markers. Images taken every 1 min. SBP-Halo-C99c labelled with Halo-JF646, except when combined with CellMask DeepRed (then labelled with Halo-OregonGreen). Time represents time since biotin addition. The movies are ordered to follow the route through the secretory pathway. The first section shows Cad99c partially colocalising with Sec16 (ER exit sites) en route to the Golgi, the second shows colocalisation with GMAP (*cis*-Golgi), and the third with Golgin245 (*trans*-Golgi). The final section shows the accumulation of Cad99c at the apical membrane, labelled by CellMask.	Figure 2 and Figure S2
Movie4	Nidogen RUSH with secretory pathway markers. Images taken every 1 min. SBP-SNAP-Ndg labelled with SNAP-SiR. Time represents time since biotin addition. The movies are ordered to follow the route through the secretory pathway. The first section shows Nidogen partially colocalising with Sec16 (ER exit sites) en route to the Golgi, the second shows colocalisation with GMAP (*cis*-Golgi), and the third with Golgin245 (*trans*-Golgi). The final section shows the accumulation of Nidogen in the basement membrane, labelled by Alexa488-Wheatgerm agglutinin.	Figure 2 and Figure S2
Movie5	Cadherin99c RUSH – All movies were taken on the Zeiss Airyscan using Airyscan fast mode at 4.5 frames per second.	Figure 5

	SBP-Halo-Cad99c was labelled with Halo-JF549. Time represents time since biotin addition. Vesicle tracks >1 µm are shown overlaid on the righthand side of the movie. Part 1 Stage8 - 21min after addition of biotin; vesicles move in a generally apical direction. Part 2 Stage9 – 29 min after addition of biotin; almost all Cad99c move apically. Part 3 Stage9 – 39 min after addition of biotin; vesicles can be seen moving basally as well as apically, as Cad99c that has reached the apical membrane is being endocytosed at this point.	
Movie6	Nidogen RUSH – All movies taken on the Zeiss Airyscan using Airyscan fast mode. SBP-SNAP-Nidogen labelled with SNAP-SiR. Time represents time since biotin addition. Vesicle tracks >1 µm are shown overlaid on the righthand side of the movie. Part1 Stage8, 23min post-biotin. Taken at 3.8fps on the Airyscan. Cross section through follicle cells. Vesicles move rapidly both apically and basally. Part 2 Stage8, 40min after biotin addition. Taken at 2.3 frames per second. Section through the middle of the follicle cells. Vesicle tracks are not planar polarised. Part 3 Stage8, 33min after biotin addition. Take at 2.3 frames per second. Section through the basal region of the follicle cells. Tracks are mostly planar polarised along the axis of egg chamber rotation.	Figure 6
Movie7	Nidogen RUSH with UAS-Rab10-YFP – All sections through the base of the follicle cells. SBP-SNAP-Nidogen labelled with SNAP-SiR. Part 1 Stage8 – taken on Leica SP8 at 1 frame per minute. Time represents time since biotin addition. Around 11 minutes after the addition of biotin, some Nidogen starts to colocalise with the Rab10 puncta, with peak colocalisation around 20 minutes after the addition of biotin. Part 2-4 all Stage8 and taken on the Zeiss Airyscan with Airyscan fast mode at 1.59 frames per second. Part 2 – showing Rab10 (Green) and Nidogen (Magenta). Yellow arrowheads indicate examples of Rab10 positive vesicles; cyan arrowheads indicate examples of Ndg and Rab10 positive vesicles. There are plenty of Rab10 vesicles that do not contain Nidogen, but for most Nidogen vesicles are labelled with Rab10 for at least part of the track. Part 3 – Rab10 channel only. Part 4 – Nidogen channel only. Part 5 – UAS-Rab10-YFP expressed in the absence of UAS-SBP-SNAP-Nidogen. Stage 8 and taken on the Zeiss Airyscan with Airyscan fast mode at 1.56 frames per second.	Figure 7

Movie8	Cadherin99c RUSH in *Dhc*$^{8-1}$/+ and after colcemid treatment – All movies were taken on the Zeiss Airyscan using Airyscan fast mode at 4.5 frames per second. SBP-Halo-C99c was labelled with Halo-JF549. Time represents time since biotin addition. Tracks > 1 µm are shown overlaid on the right hand side of the movie. Part 1 Cadherin99c RUSH in *Dhc*$^{8-1}$/+ at stage 9. There are many fewer tracks than in wildtype cells, and the apical bias is lost. Part 2 Cadherin99c *Dhc*$^{8-1}$/+ RUSH – Stage 8. Part 3 Cadherin99c RUSH Colcemid treated – Stage 9. There are far fewer tracks than in non-treated egg chambers, and those that are present are less polarised. Part 4 Cadherin 99c RUSH Colcemid treated – Stage8. There are far fewer tracks than in non-treated egg chambers.	Figure 8 and Figure S8
Movie9	Nidogen RUSH with slow Dynein - All movies taken on the Zeiss Airyscan using Airyscan fast mode. SBP-SNAP-Ndg was labelled with SNAP-SiR. Tracks > 1 µm are shown overlaid on the righthand side of the movie. Part 1 Nidogen in *Dhc*$^{8-1}$/+ at stage 8. Taken at 3.8 frames per second. Cross section through the follicle cells. Nidogen trafficking is similar to wild-type, with lots of tracks moving apically and basally. Part 2 Nidogen in *Dhc*$^{8-1}$/+ at stage 8. Taken at 2.3 frames per second. Section through the base of the follicle cells. Tracks are still planar-polarised along the rotational axis.	Figure 8

All movies show the movements of RUSH cargoes after release with biotin in *Drosophila* follicle cells at stages 8-9.

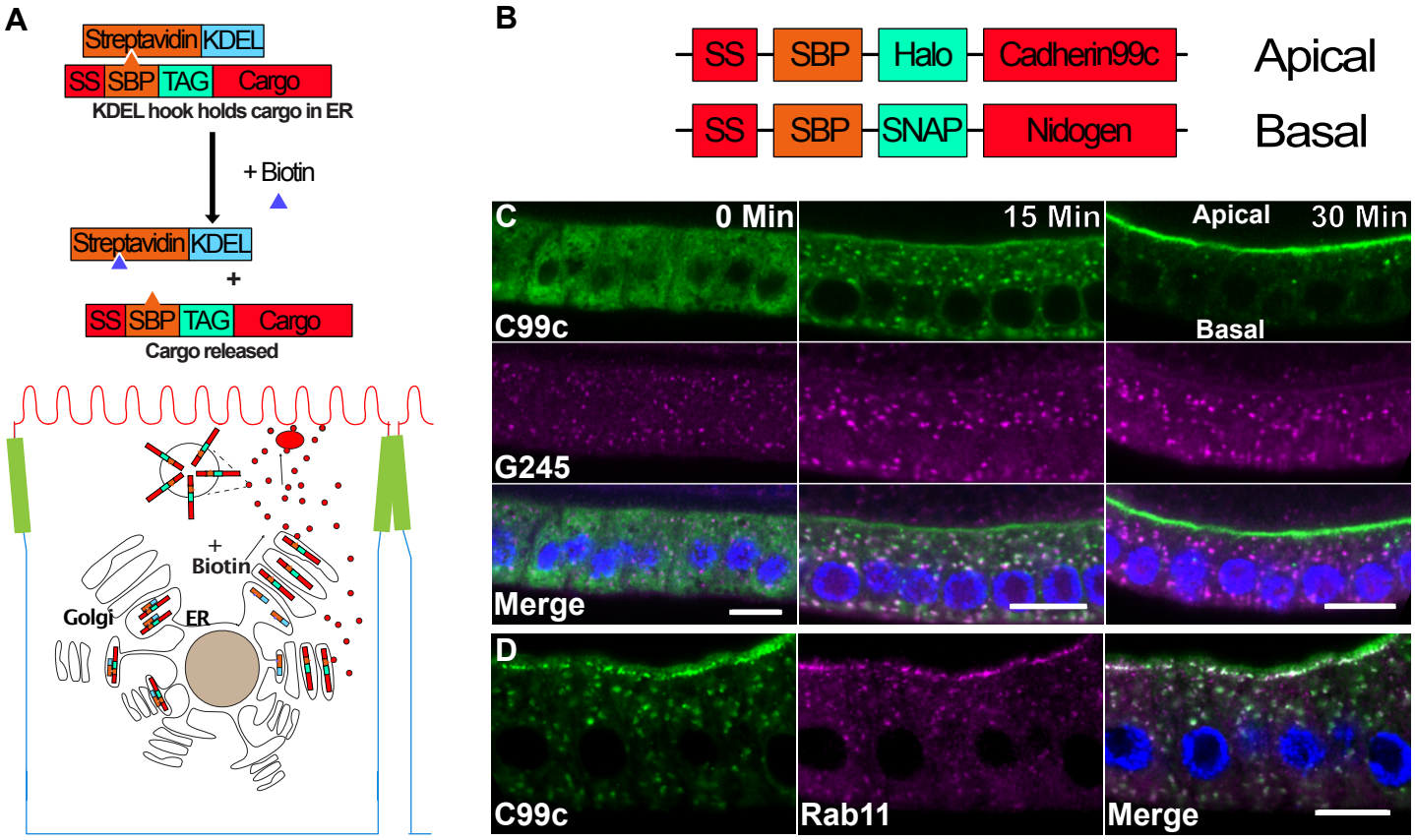

Figure 1. The RUSH system

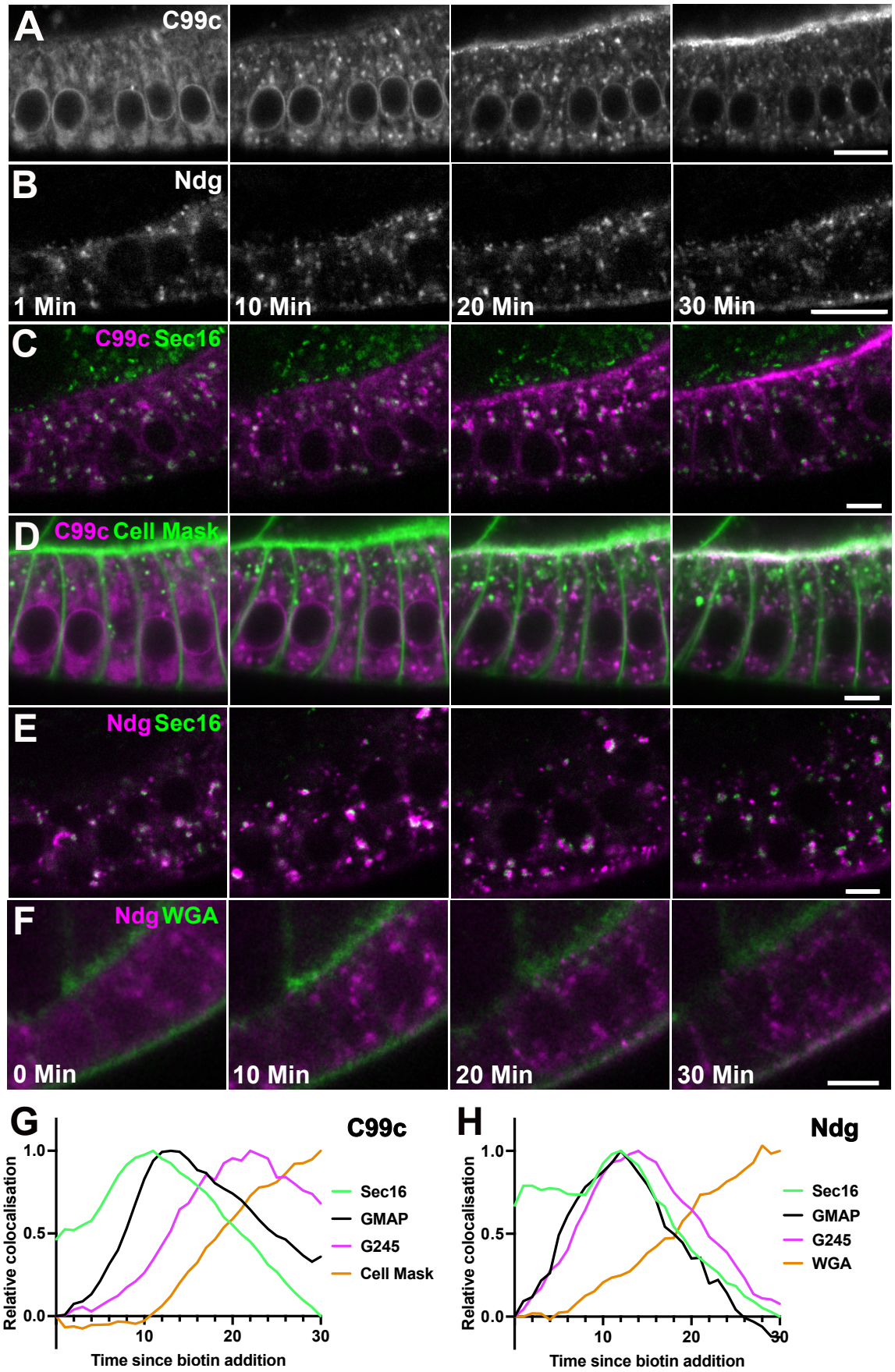

Figure 2. Time course of Cad99c and Nidogen secretion

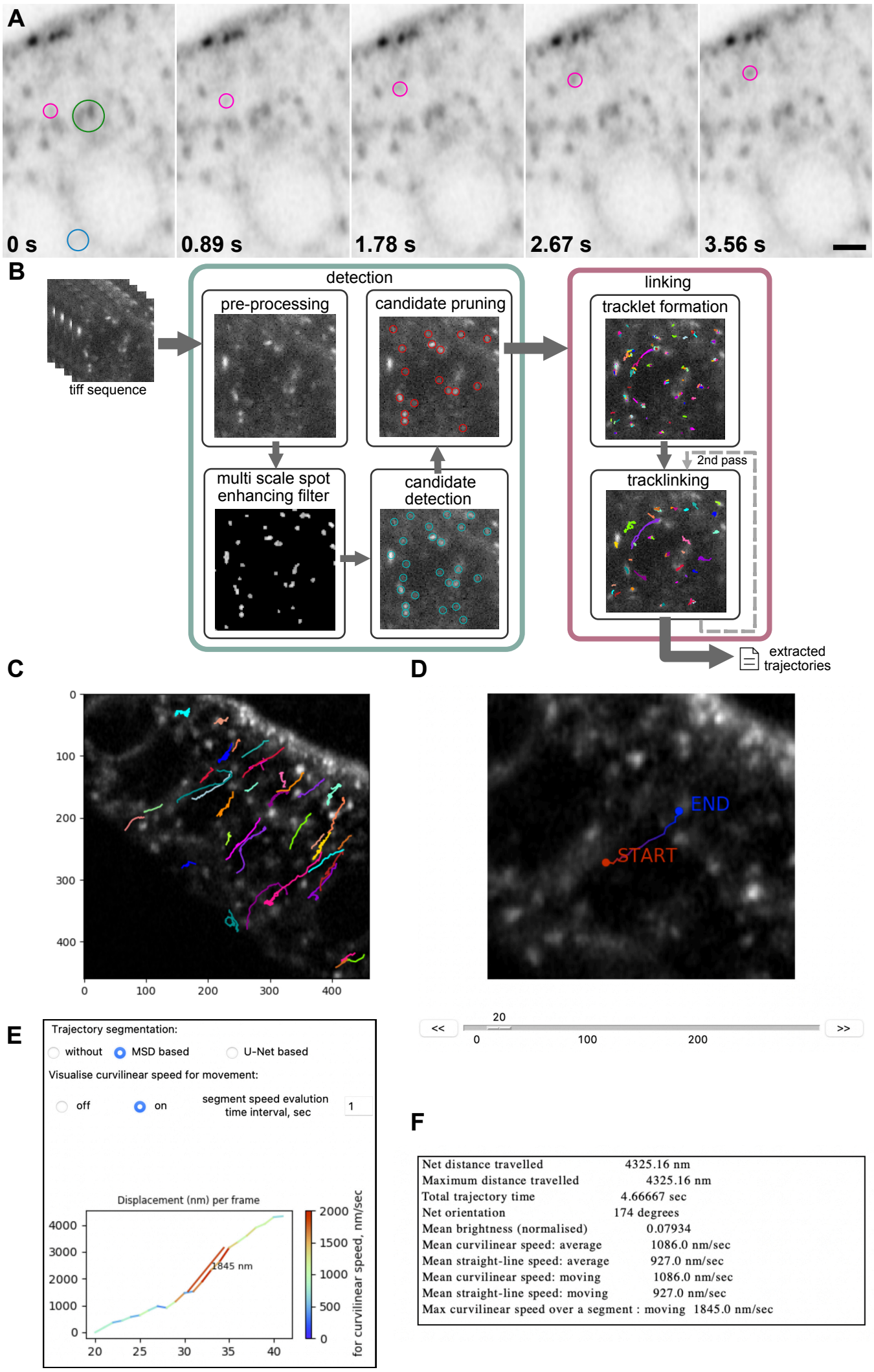

Figure 3. The MSP-tracker and viewer analysis pipeline

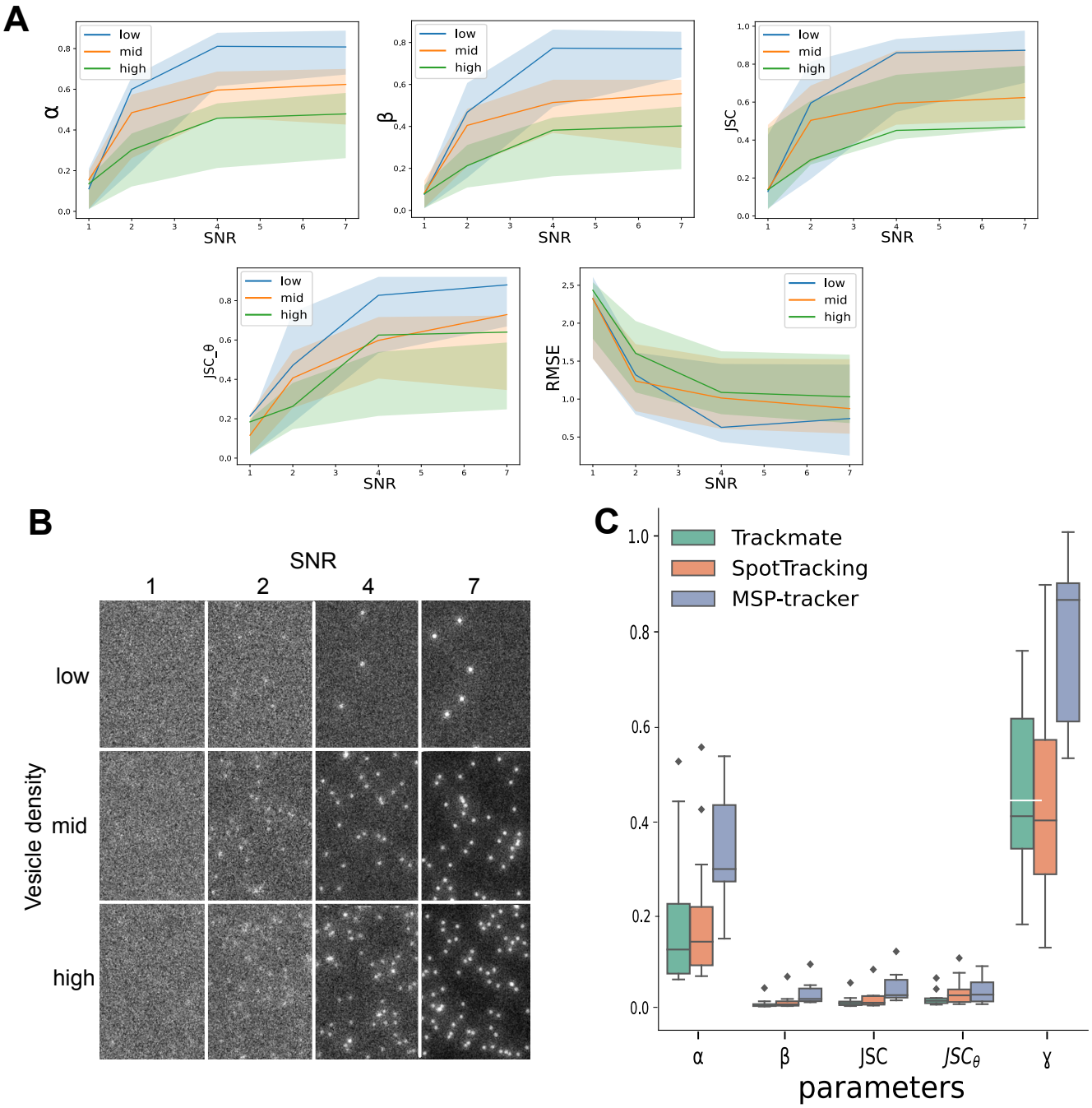

Figure 4. Evaluation of the MSP-tracker

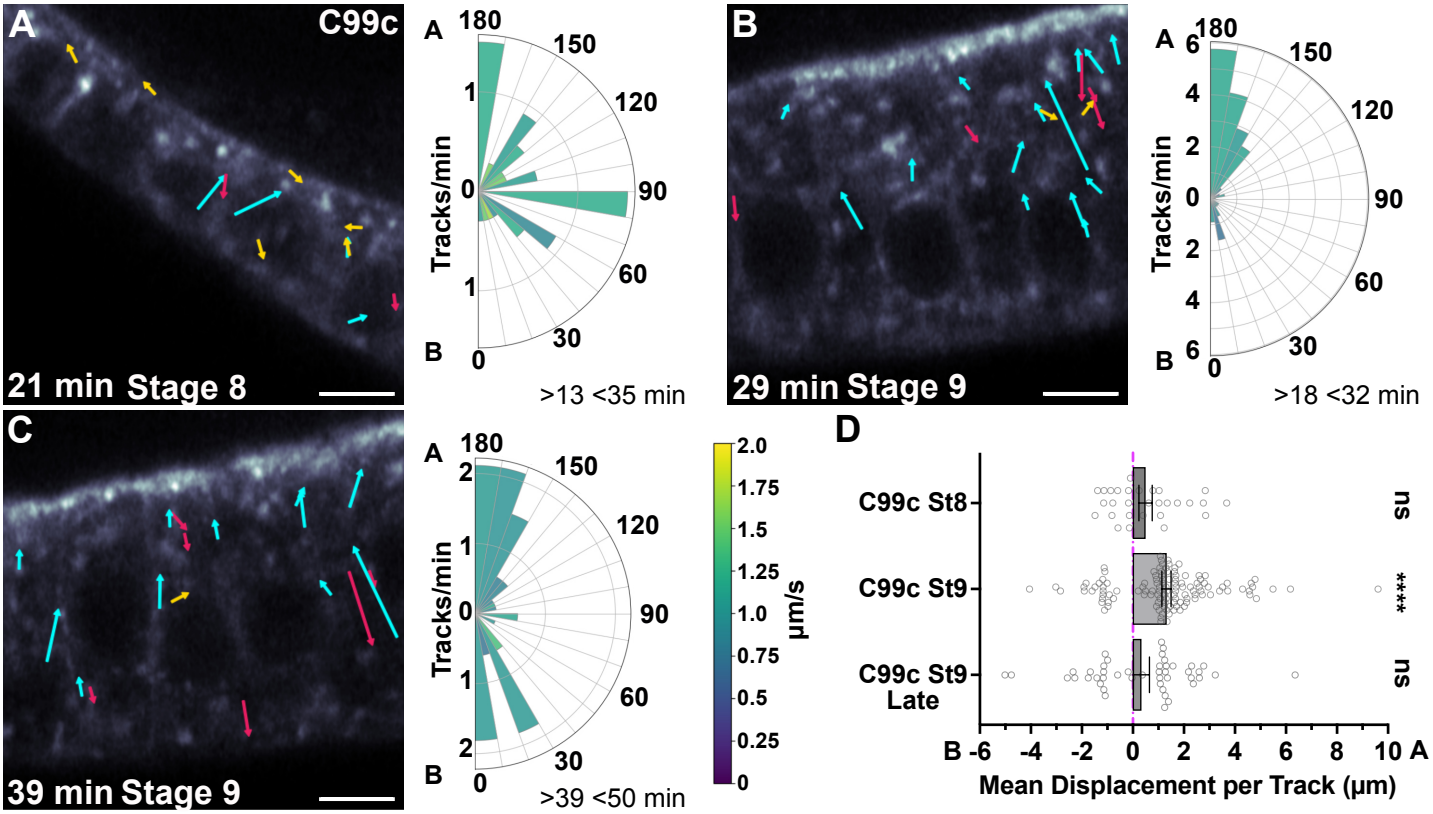

Figure 5. Cad99c tracks are apically biased.

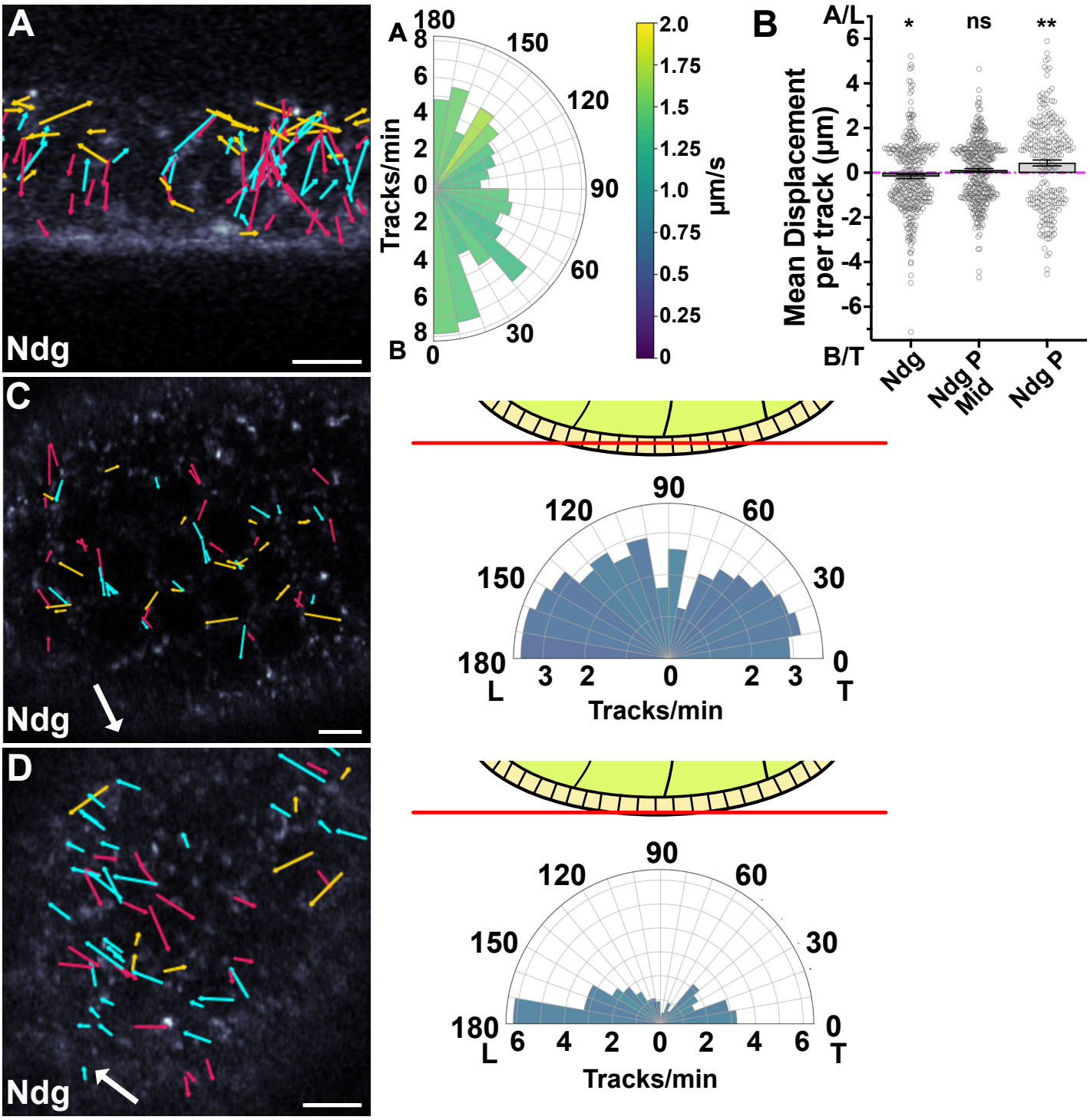

Figure 6. Analysis of Ndg tracks reveals planar polarised transport basally.

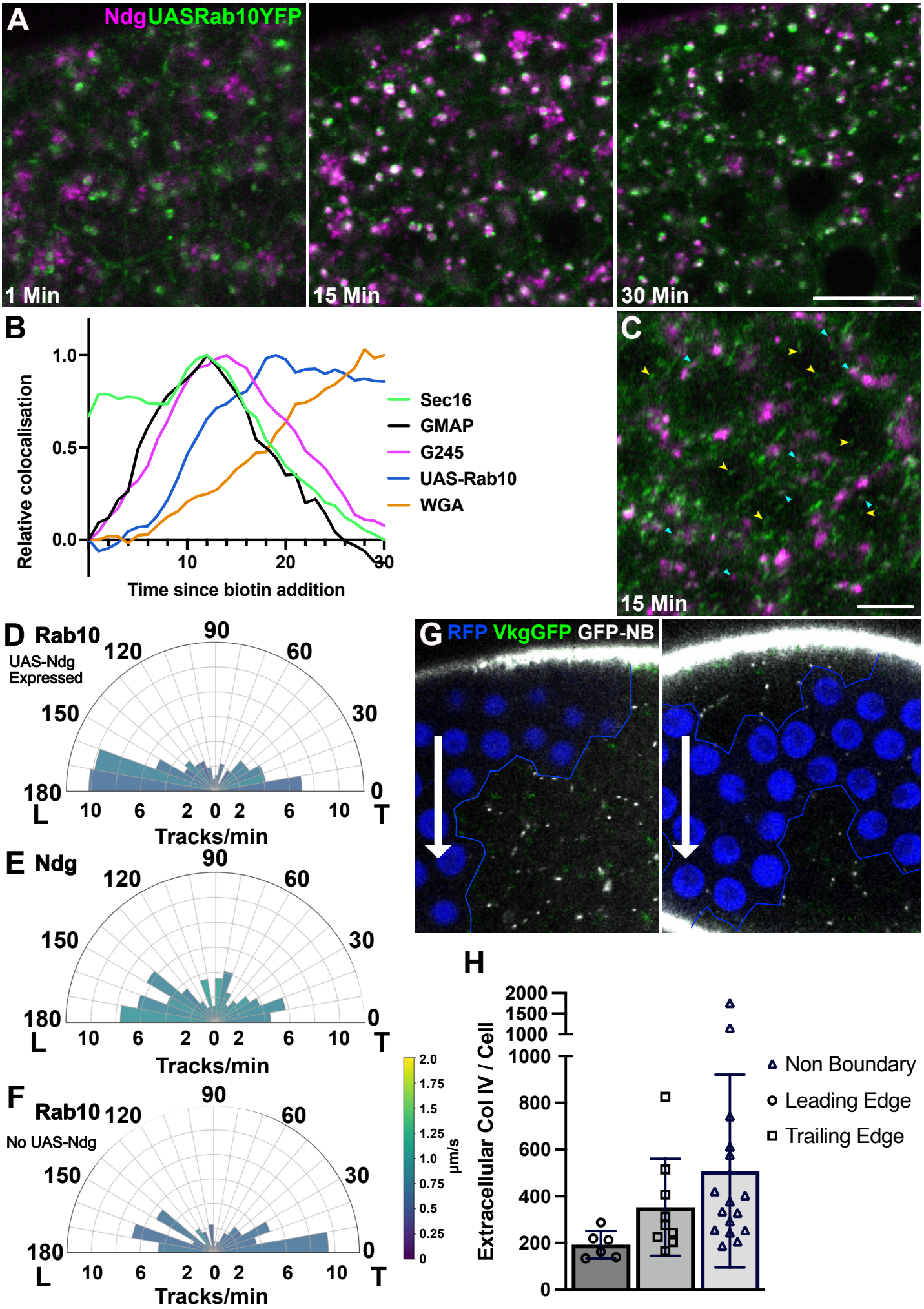

Figure 7. Ndg is transported in Rab10 positive vesicles.

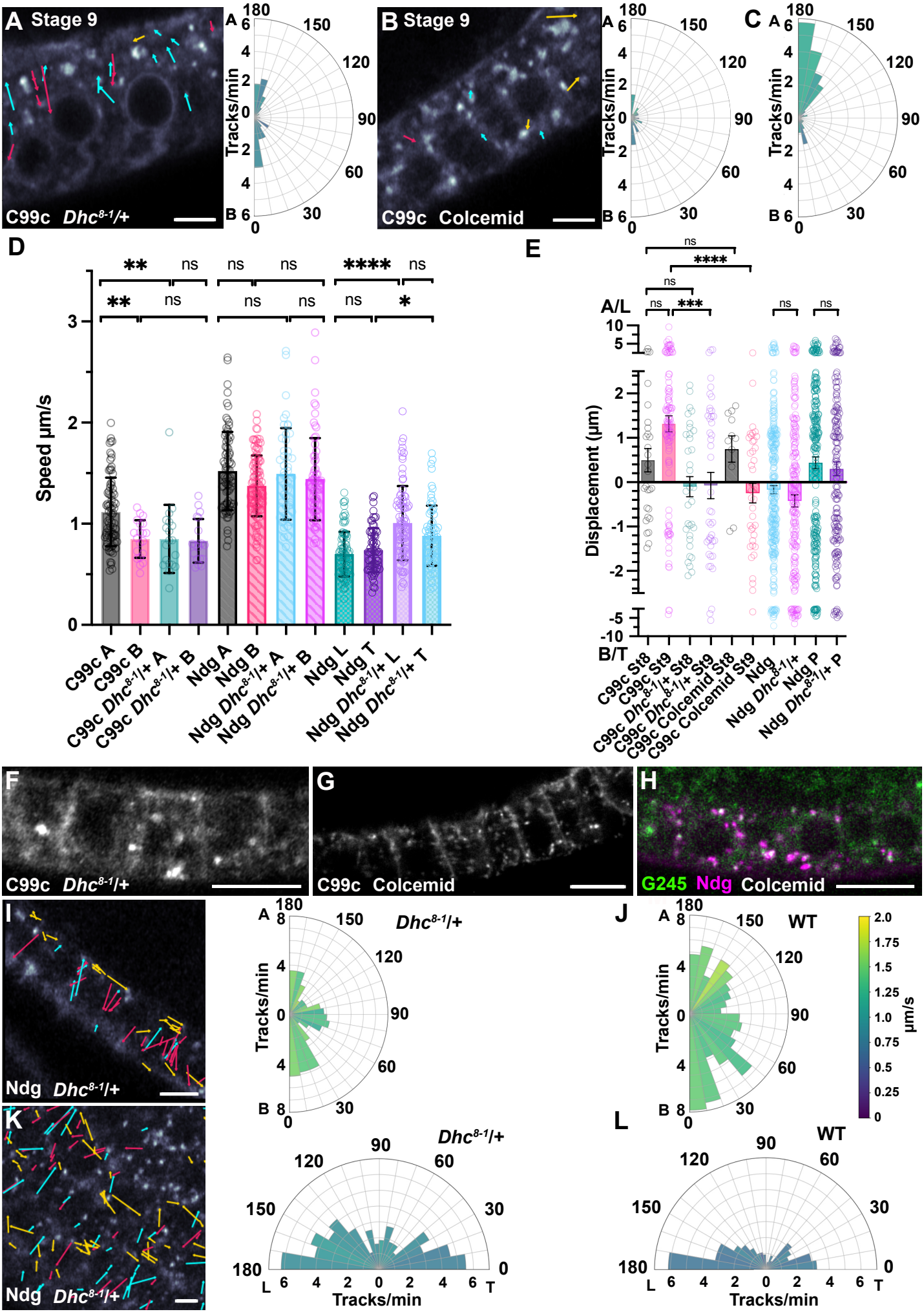

Figure 8. The effects of a slow dynein mutant and microtubule depolymerisation on Cad99c and Ndg trafficking.

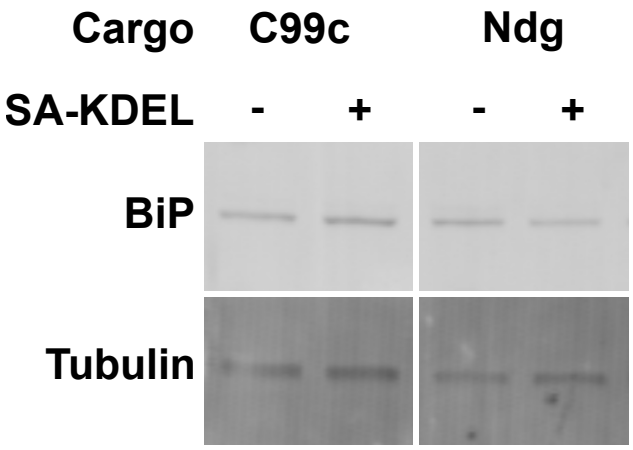

Figure S1 – RUSH does not cause ER stress.

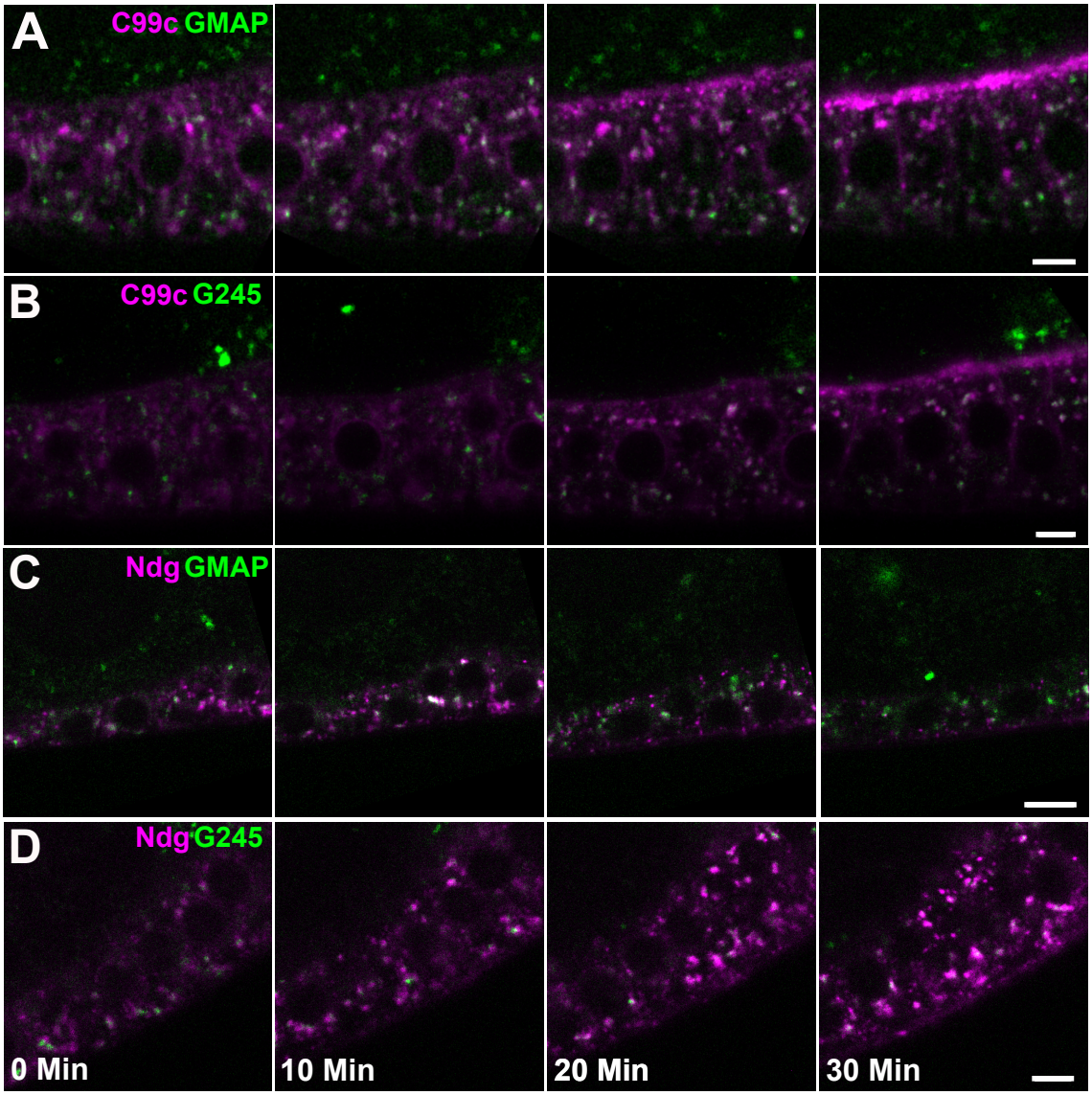

Figure S2 – Time course of Cad99c and Nidogen secretion.

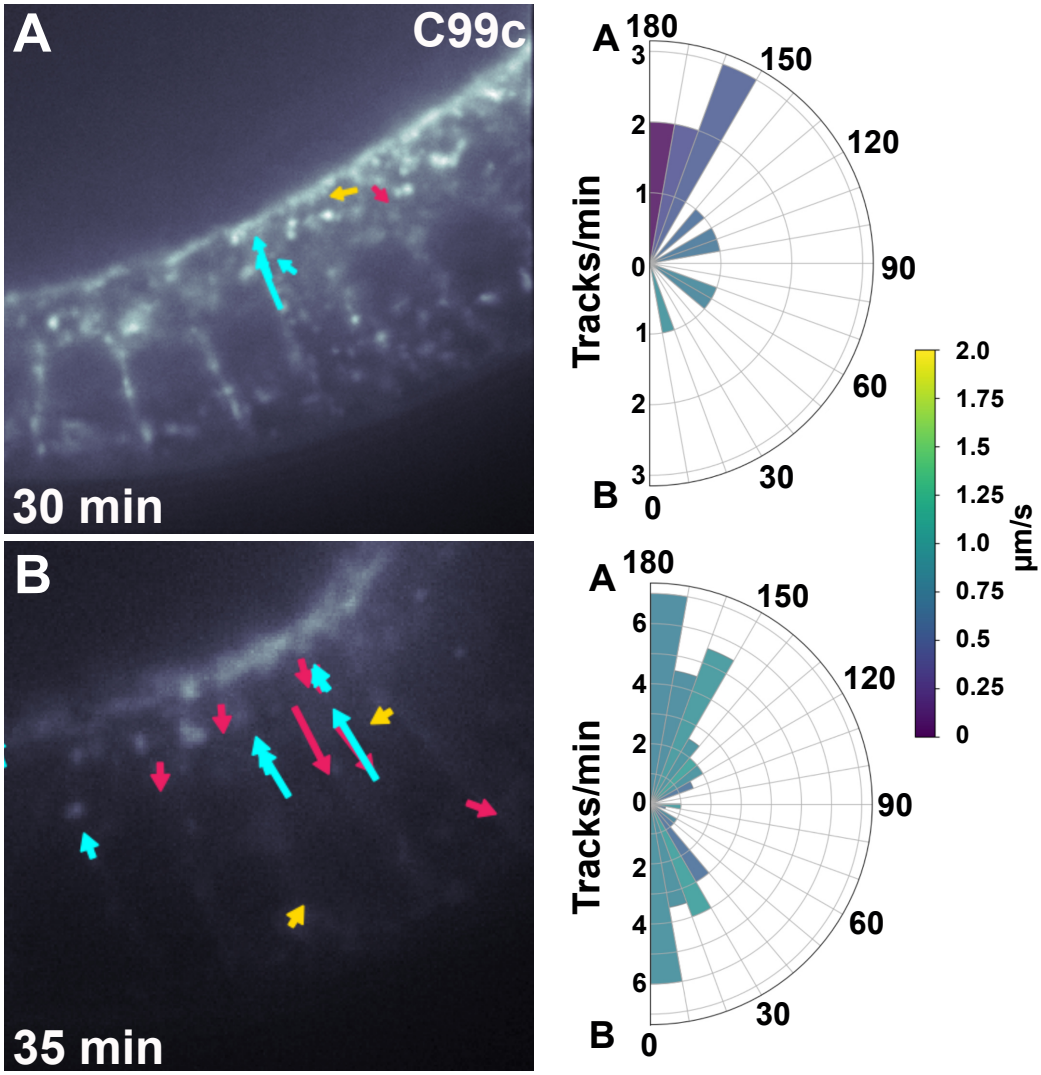

Figure S5 – Spinning disc imaging of Cad99c trafficking

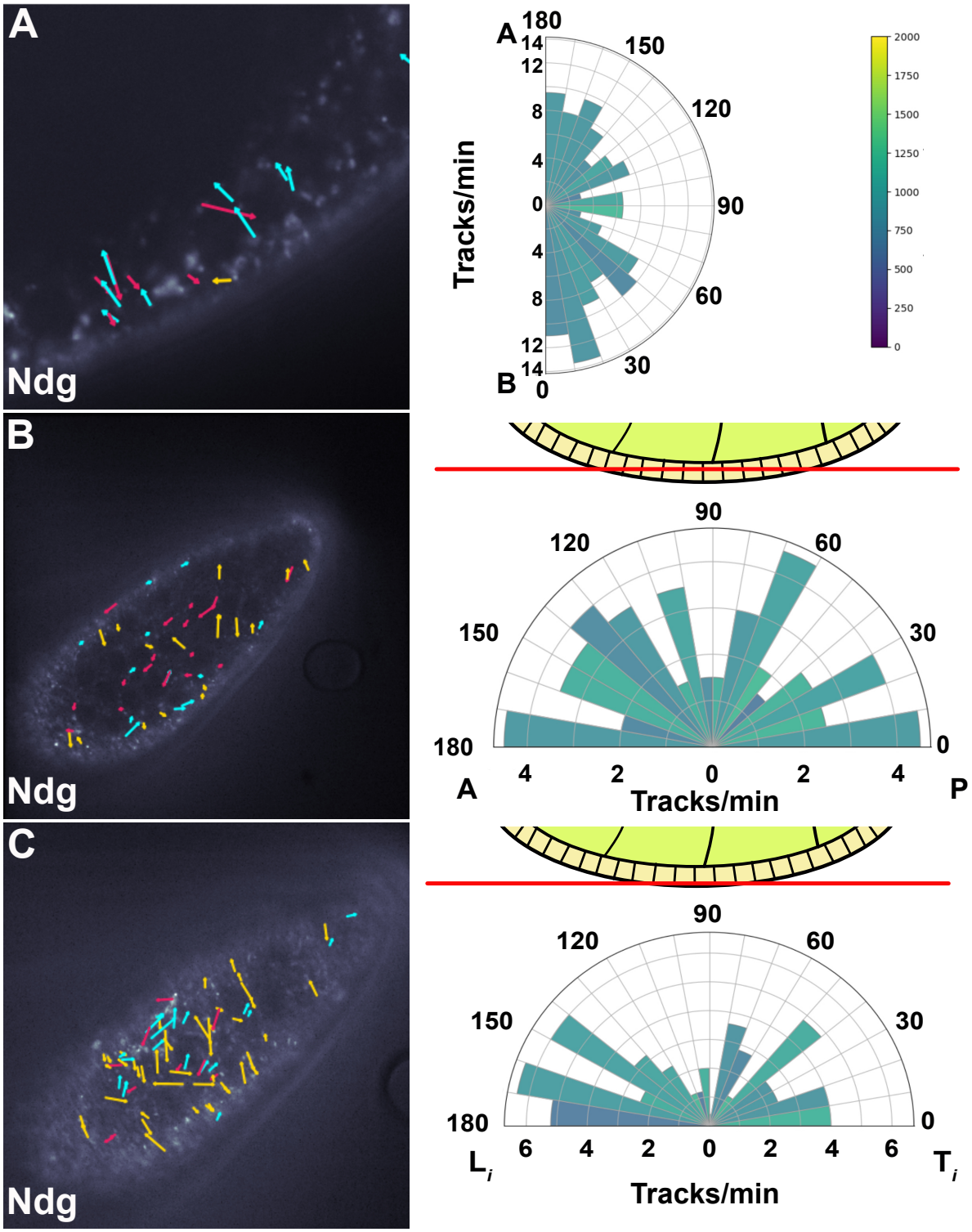

Figure S6 – Spinning disc imaging of Nidogen trafficking

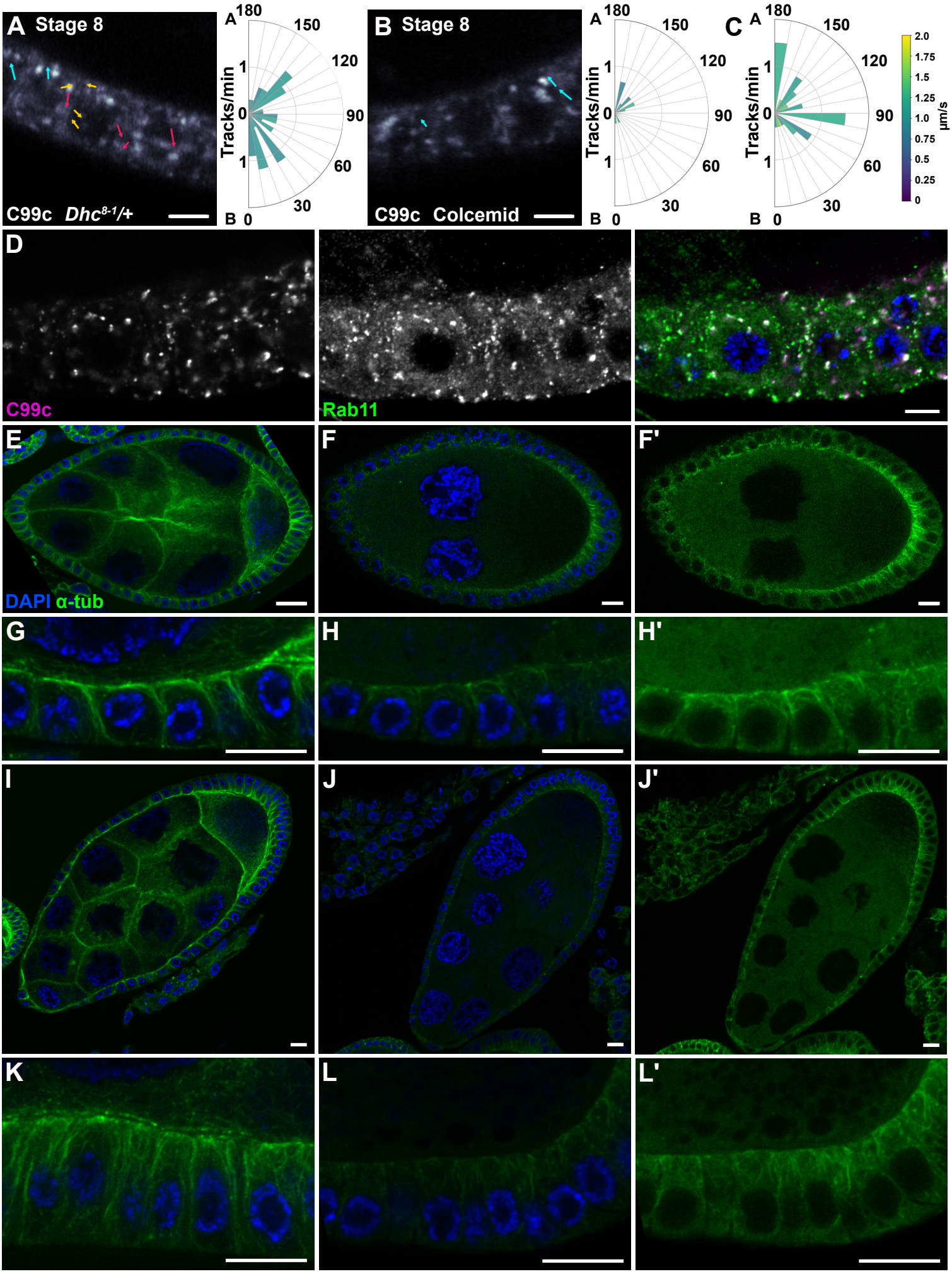

Figure S8 – *Dhc*$^{8\text{-}1}$/+ and colcemid treatments.

Cargo	Stage	No of Movies	Apical	Basal	Lateral	Total	% Apical	%Basal	%Lateral	Significance
C99c	8	3	9	5	15	29	31.03	17.24	51.72	ns
C99c	9	5	86	20	12	118	72.88	16.95	10.17	****
C99c Late	9	3	23	16	6	45	51.11	35.56	13.33	ns
C99c dhc8−1/+	8	3	7	12	15	34	20.59	35.29	44.12	ns
C99c dhc8−1/+	9	3	19	19	5	43	44.19	44.19	11.63	ns
C99c colcemid	8	4	6	2	3	11	54.55	18.18	27.27	ns
C99c colcemid	9	4	11	14	13	38	28.95	36.84	34.21	ns
Ndg	8	3	83	109	136	328	25.30	33.23	41.46	ns
Ndg dhc8−1/+	8	3	47	70	80	197	23.86	35.53	40.61	ns
			Leading	Trailing	Sideways	Total	% Leading	% Trailing	% Sideways	
Ndg basal	8	3	103	69	58	230	44.78	30.00	25.22	*
Ndg mid	8	3	110	88	134	332	33.13	26.51	40.36	ns
Ndg basal dhc8−1	8	2	90	76	84	250	36.00	30.40	33.60	ns
Rab10 alone	8	2	43	49	27	119	36.13	41.18	22.69	ns
Rab10 with Ndg	8	2	96	62	52	210	45.71	29.52	24.76	*
	Stage	No of Movies	Rab10 +ve	Rab10 −ve or unclear	Total	% Rab10 +ve				
Ndg	8	2	164	47	211	77.73				

Table S1