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Novel transport function of adherens junction revealed by live imaging in *Drosophila*

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ABSTRACT

Adherens junctions are known for their role in mediating cell–cell adhesion. DE-cadherin and Echinoid are the principle adhesion molecules of adherens junctions in *Drosophila* epithelia. Here, using live imaging to trace the movement of endocytosed Echinoid vesicles in the epithelial cells of *Drosophila* embryos, we demonstrate that Echinoid vesicles co-localize and move with Rab5-or Rab11-positive endosomes. Surprisingly, these Echinoid-containing endosomes undergo directional cell-to-cell movement, through adherens junctions. Consistent with this, cell-to-cell movement of Echinoid vesicles requires the presence of DE-cadherin at adherens junctions. Live imaging further revealed that Echinoid vesicles move along adherens junction-associated microtubules into adjacent cells, a process requiring a kinesin motor. Importantly, DE-cadherin- and EGFR-containing vesicles also exhibit intercellular movement. Together, our results unveil a transport function of adherens junctions. Furthermore, this adherens junctions-based intercellular transport provides a platform for the exchange of junctional proteins and signaling receptors between neighboring cells.

1. Introduction

Adherens junctions (AJs) are specialized structures at the interface of two neighboring epithelial cells. In electron micrographs, AJs appear as two thick bands, 20 nm apart and bridged by rodlike structures. At the molecular level, AJs contain the E-cadherin-catenin complex and nectin-Afadin complex that promote actin polymerization and AJ assembly in mammalian cells [1]. DE-cadherin (DE-cad) and Echinoid (Ed) are the counterparts of E-cadherin and nectin in *Drosophila* epithelia [2]. AJs are known for their roles in cell adhesion, cell recognition and morphogenesis. As AJs link cells into a sheet-like structure, information exchange between these cells is crucial for determining cell fate during animal development. Diverse mechanisms have been documented to mediate long range and local intercellular communication. Cytonemes and tunneling nanotubes use actin-based cellular process to mediate transfer of signaling molecules and membrane vesicles between unconnected cells [3,4]. Argosomes are exogenously derived lipoprotein particles (released from fat body) that facilitate intercellular transfer of lipid-linked proteins such as Hedgehog and Wingless [5]. Exosomes are endosomal-derived exovesicles and can travel over several cell diameters [6]. To date, only a few mechanisms including gap junctions and somatic ring canals have been shown to mediate direct cell-to-cell communication of small molecules and proteins between adjacent cells [7,8].

Here, using live imaging to trace the movement of endocytosed Ed vesicles in the epithelial cells of *Drosophila* embryos, we uncovered a new mechanism for endosomes shuttling between neighboring epithelial cells. We showed that these intercellular endosomes move along the microtubules (MTs) within a cell and, through the AJ-associated MTs, undergo cell-to-cell transport. Thus, AJs play a transport role in addition to cell–cell adhesion.

Abbreviations: AJs, adherens junctions; SJs, septate junctions; DE-cad, DE-cadherin; Ed, Echinoid; *shg*, *shotgun*; MTs, microtubules; *khc*, *kinesin heavy chain*; *en*, *engrailed*; *da*, *daughterless*; dEGFR, *Drosophila* EGF receptor.

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2. Materials and methods

Details are in supplementary material.

3. Results

3.1. Live imaging reveals cell-to-cell movement of Ed-GFP vesicles through AJs

We used the *Drosophila* embryo as a model system to study the function of AJs. We focused on stage 14/15 of embryogenesis, a late stage of dorsal closure during which two opposing and elongated epithelial cell layers extend dorsally towards the dorsal midline to cover the underlying amnioserosa (Fig. 1A). We and others reported previously that Ed is expressed in lateral epithelial cells but not in the amnioserosa, and Ed is required for cell shape change and coordinated migration/zippering of opposing epithelial layers [9–11]. As shown in Fig. 1A, the lateral epithelial cells (about three cells away from the leading edge) have an elongated prism shape. Immunostaining revealed the lengths of the AJs and septate junctions (SJs) to be 1.2 and 2.5 μm, respectively. Further, the angle between the junctions is approximately 180° along the apical–basal axis, with the AJs followed by the SJs. We ectopically expressed Ed-GFP using *en-Gal4* or *da-Gal4* in the epithelial cells. Interestingly, we observed apically localized Ed-GFP particles with particle sizes up to 0.5 μm (Fig. 1B). To determine whether this is an artifact caused by Ed overexpression, we performed immunostaining with Ed antibody to wild-type embryos of similar stages. We confirmed the presence of endogenous Ed particles (Fig. 1C) localized mainly to AJs (78%, n = 332 particles) with a few at the SJs (22%).

Next, we performed live imaging to trace the movement of Ed-GFP vesicles in the epithelia. We fixed the focal plane (0.7 μm thick) of the confocal microscope at Ed-GFP-enriched AJs and then observed the movement of Ed-GFP vesicles in lateral epithelial cells about three cells away from the leading edge (Fig. 1A). Live imaging of *en > Ed-GFP* embryos revealed that Ed-GFP vesicles might be stationary, move in the cytosol or move out of the cell membrane. Besides the intracellular movement of Ed-GFP vesicles, we unexpectedly observed cell-to-cell movement of Ed-GFP vesicles as they moved across the plasma membrane of one cell (referred to as the donor cell) into the adjacent cell (referred to as the recipient cell) (Fig. 1D and Video S1A). The frequency of cell-to-cell movement of Ed-GFP vesicles was 0.42 vesicle/cell/minute and the average speed was 0.176 μm/s. On average they traveled 3.08 ± 1.75 μm (n = 36), or up to 5 μm, after entering the recipient cell (Fig. 1E and Video S1B). Because the dimensions of this facet of lateral epithelial cells are approximately 12 × 4 μm (Fig. 1A), this would also suggest that Ed-GFP vesicles continue moving further from the AJs and deep into the recipient cells. Interestingly, intercellular movement of Ed-GFP vesicles can occur at multiple positions along the AJs of the recipient cell. As shown in Fig. 1D, one recipient cell received two Ed-GFP vesicles at different locations on the AJ from different donor cells. After crossing the plasma membrane, intercellular Ed-GFP vesicles can fuse with another Ed-GFP vesicle (Fig. 1E), become stationary or move out of focus (data not shown). Moreover, we found that 70% of intercellular Ed-GFP vesicles were transported

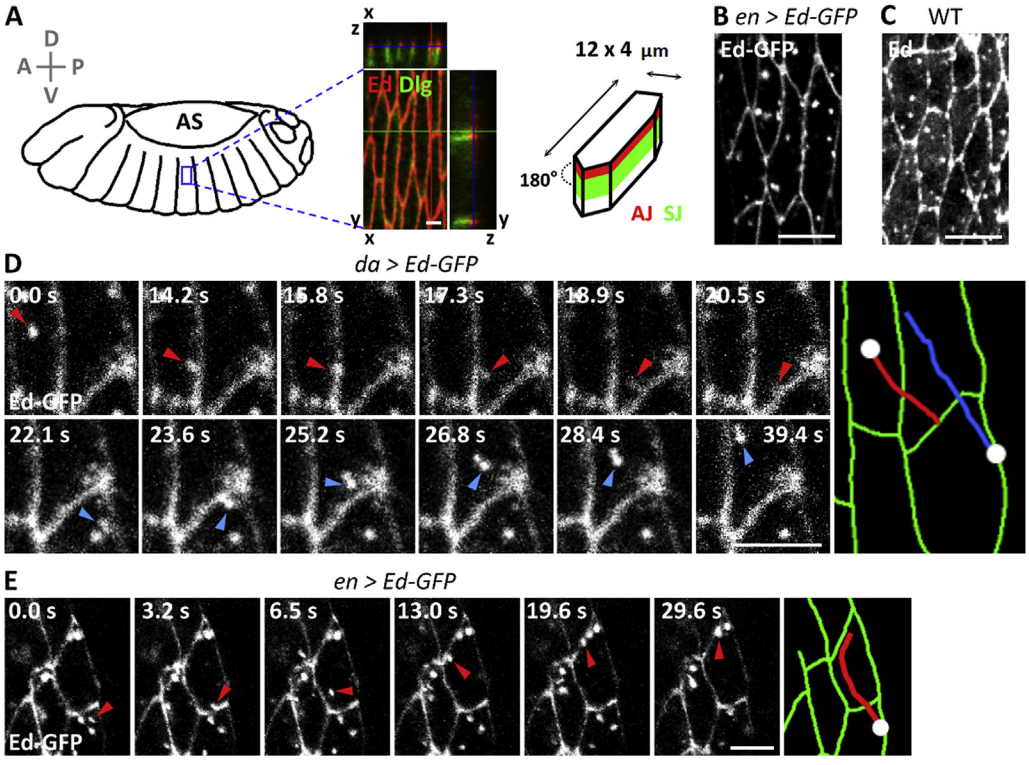

Fig. 1. Live imaging reveals cell-to-cell movement of Ed-GFP vesicles through AJs. (A) (Left) Schematic of a stage 14 embryo undergoing dorsal closure. AS indicates amnioserosa. (Middle) Lateral epithelial cells (about three cells away from the leading edge) possess apical AJs (Ed, red) followed by SJs (Discs large, green), shown in xy, xz and yz planes. (Right) Schematic showing a lateral epithelia cell. The shape resembles a prism, with polygonal apical and basal faces, and the angles between AJs (red) and SJs (green) were approximately 180° along the apical–basal axis. (B) The presence of apically localized Ed-GFP particles in live *en > Ed-GFP* embryo. (C) The presence of endogenous Ed particles, revealed by immunostaining and Z-stack projection, in wild-type epithelial cells. (D) Movie sequences showing cell-to-cell movement of Ed-GFP vesicles in *da > Ed-GFP* embryos. One cell received two Ed-GFP vesicles (red and blue arrowheads) at different positions along the AJ. (E) Movie sequences showing an Ed-GFP vesicle (arrowhead) crossing the plasma membrane and fusing with another Ed-GFP vesicle in *en > Ed-GFP* embryo. Summaries of Ed-GFP vesicle trajectories are shown, with a white dot indicating the starting position of each vesicle (D and E). Bars, 2 μm (A) and 5 μm (B,C,D,E).

into recipient cells via the dorso-ventral–directed AJs (n = 93) as opposed to other anterior-posterior–directed AJs. Importantly, intercellular movement of Ed-GFP vesicles can also be detected in an *ed* protein trap line (Video S1C), suggesting that this phenomenon is not an artifact of Ed overexpression.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.125.

One possibility for the observed movement of Ed vesicles through AJs is that a slightly out-of-focus Ed vesicle may be moving basal to the AJs but still within the donor cell (Fig. S1B). To eliminate this possibility we fixed the 0.7 μm-thick focal plane at a depth containing the Ed-GFP-labeled AJs, which have an average length of 1.2 μm, while the adjacent SJs are 2.5 μm in length. The average movement of Ed vesicles was 3.08 ± 1.75 μm after entering the recipient cell (Fig. 1D and E). Thus, if the vesicle was in fact out of focus, we would expect that the angles between the AJ and SJ to be significantly less than 180° (compare Fig. S1B and S1A). However, as shown in Fig. 1A, both the apical and basal faces of the lateral epithelial cells are polygonal in shape, and therefore the angles between the AJs and SJs are by necessity approximately 180°. Another possibility is that Ed vesicle may be moving apical to the epithelial cells in the perivitelline space (Fig. S1C). To exclude this possibility we analyzed serial confocal z sections of live *en > Ed-GFP* embryos. We found that Ed-GFP particles were first detected apically at the level of Ed-GFP-labeled AJs (Fig. S2A). Consistent with this, Ed-GFP particles were never detected above Ed-GFP-labeled AJs when we imaged *en > Ed-GFP* embryos with lattice light sheet microscopy (Fig. S2B). Thus it is unlikely that Ed vesicles move extracellularly into the perivitelline space. The third possibility is that Ed vesicles move through filopodia extending from the adjacent lateral epithelial cell (Fig. S1D). Because the particle size of some intercellular Ed-GFP vesicles can be as large as 0.5 μm, the filopodia containing these vesicles should be readily detectable. Using live imaging in *en > actin-GFP* embryos, filopodia were clearly visible in the dorsal-most epithelial cells (as demonstrated in Lin et al., 2007), but no filopodia-like protrusions were detected along the AJs of lateral epithelial cells (data not shown). Similarly, no filopodial protrusions were detected in a TEM section of wild-type lateral epithelial cells (data not shown). To confirm the presence of cell-to-cell transport of Ed vesicles that occur at the level of AJs (as shown in Fig. S1A), we performed live imaging with thin optical sections in *en > Ed-mCherry + DE-cad-GFP* (or *α-catenin-GFP*) embryos. Three-dimensional reconstruction of the trajectory of Ed-mCherry vesicles revealed that Ed-mCherry vesicles indeed crossed, via DE-cad-GFP-labeled AJs, from the donor cell into the recipient cell (Fig. 2A and Video S2). Moreover, in rare cases, we observed a tubular-shaped Ed-mCherry vesicle penetrating the α-catenin -GFP-labeled AJs, with one end of vesicle in the recipient cell while the other end remained in the donor cell (Fig. 2B). Consistent with this, TEM analyses revealed that AJs between epithelial cells were interrupted by vesicles apparently moving into an adjacent cell (Fig. 2C).

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The interpretation that Ed-GFP vesicles move through AJs could be due to Ed-GFP vesicle merging with the plasma membrane of a donor cell that, in turn, causes the release of a distinct Ed-GFP vesicle from the recipient cell. To test this possibility, we photobleached a segment of Ed-GFP-enriched AJs and then observed the movement of an Ed-GFP vesicle across the photobleached AJs (Fig. 2D and Video S3). We reasoned that if this possibility is correct, we would see the merging of Ed-GFP vesicles with photobleached AJs while being unable to detect new Ed-GFP vesicles released from the photobleached membrane of the recipient cell. Instead, we observed the same Ed-GFP vesicle moving across the photobleached AJ into the recipient cell (Fig. 2D). Altogether, our results suggest that this is a true cell-to-cell movement.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.125.

As intercellular movement of Ed-GFP vesicles occurs at the level of AJs, we next examined whether intercellular movement of Ed-GFP vesicles requires the presence of DE-cad at the AJs. Embryos with complete loss of *shotgun (shg)* function exhibit loss of epithelial integrity early in development [12,13]. shg^{g317} is a dominant negative allele, and zygotic shg^{g317} mutant embryos contain very low levels of DE-cad in the epidermal cells [12,14]. We therefore examined the intercellular transport of Ed-GFP vesicles in zygotic shg^{g317} mutant embryos. Interestingly, the distribution of Ed at the AJ was largely maintained in this background, and Ed-GFP vesicles formed normally. However, the frequency of intercellular movement of Ed-GFP vesicles was significantly reduced (Fig. 2E). This result suggests that the presence of DE-cad at AJs is required for intercellular movement of Ed-GFP vesicles.

3.2. Cell-to-cell movement of Ed-containing endosomes

We previously showed that in the interommatidial cells of the eye disc, Ed is endocytosed via an AP-2-, dynamin- and Rab5-dependent pathway [15], and we confirmed this in the lateral epithelial cells (Fig. S3 B-D). We further found that Rab11-mediated recycling of endosomes is critical in recycling Ed vesicles back to the plasma membrane as increased accumulation of cytosolic Ed-GFP was detected when a dominant negative form of Rab11, but not Rab4, was co-expressed (Fig. S3 E and F). To gain insight into the origin of Ed vesicles undergoing intercellular movement, we examined whether these intercellular Ed-GFP vesicles represent endosomes. Towards this, we performed live imaging in embryos co-expressing Ed-GFP and mCherry-Rab5 or mCherry-Rab11. Strikingly, 43.5% (n = 154) of Ed-GFP vesicles co-localized and moved with mCherry-Rab5 during cell-to-cell movement (Fig. S4A and Video S4A) while 41.9% (n = 191) of Ed-GFP vesicles co-localized and moved with mCherry-Rab11 during cell-to-cell movement (Fig. S4B and Video S4B). Together, our results suggest that intercellular Ed-GFP vesicles include at least early endosomes and recycling endosomes.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.125.

3.3. Cell-to-cell movement of Ed vesicles depends on MTs

The movement of endocytosed vesicles is associated with MTs [16]. It has been shown that apical MTs align along the dorso–ventral axis during the zippering stage of dorsal closure [17]. We further found that MTs were positioned adjacent to AJs of the prism-shaped epithelial cells (Fig. S5 A and B). Moreover, live imaging in embryos double-labeled with Ed-mCherry and Jupiter-GFP revealed that Ed-mCherry vesicles exhibited directional (dorso-ventral) movement along the Jupiter-GFP labeled MT arrays (Fig. 3A and Video S5). Jupiter associates with MTs [18]. Next, we asked whether Ed vesicles might move along the MT track and then cross the AJ membrane into the recipient cell. To test this, we labeled the cell membrane with CellMask Deep Red stain in embryos double-labeled with Ed-mCherry and Jupiter-GFP. Importantly, we observed that Ed-mCherry vesicles moved along the MT arrays and, through MT arrays accumulating on both sides of AJs, they directly cross the CellMask Deep Red-labeled cell membrane into the recipient cell (Fig. 3B and Video S6). Interestingly, after moving across the AJ, an Ed vesicle sometimes changed direction if it fell off the MT (Fig. S5C). Thus, Ed vesicles move along MTs within a cell and then, through the AJs-associated MTs, they undergo cell-to-cell

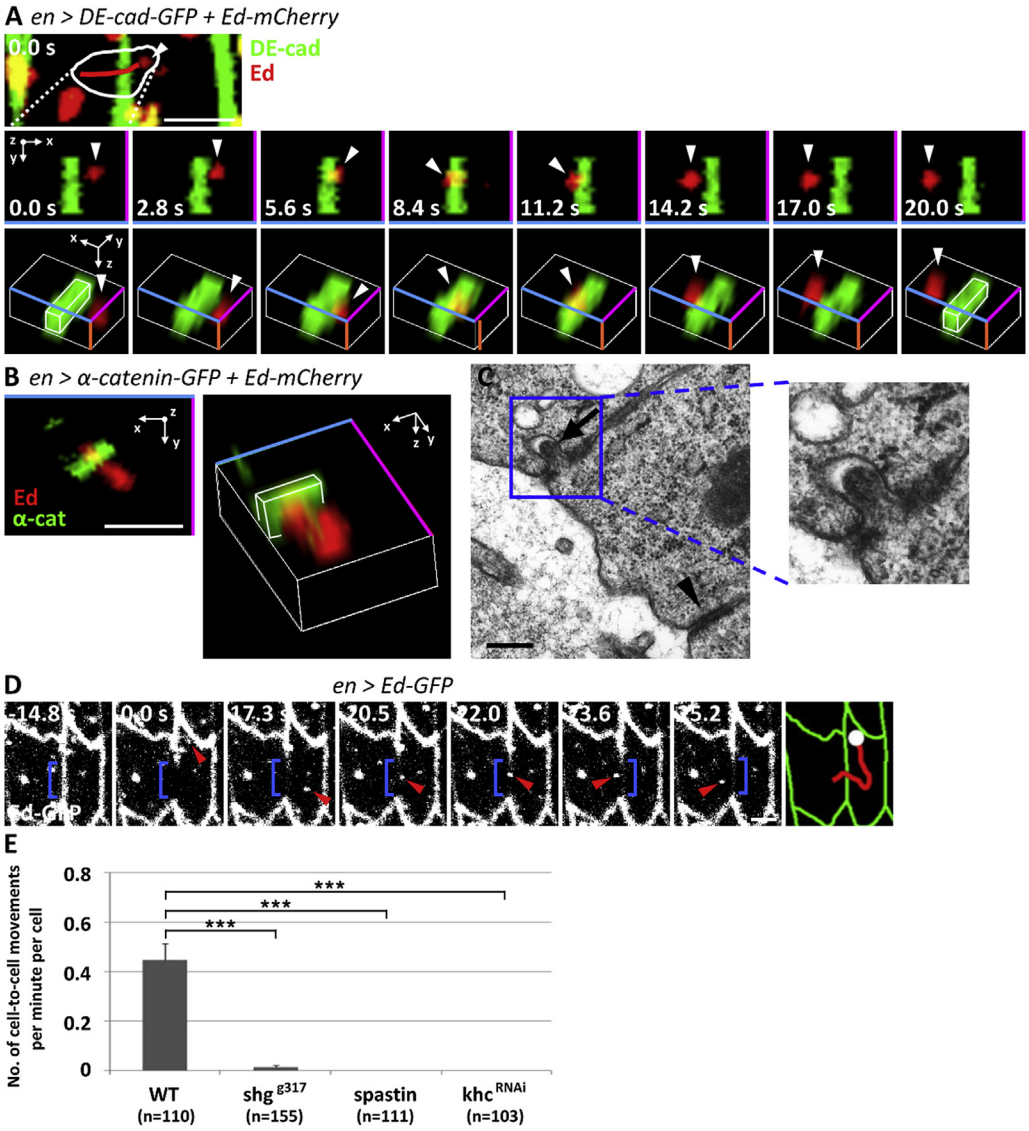

Fig. 2. Demonstration of cell-to-cell movement of Ed-GFP vesicles. (A) 3D reconstruction of Ed-mCherry vesicle trajectory in *en > DE-cad-GFP + Ed-mCherry* embryo. (Upper) Summary of the trajectory of an Ed-mCherry vesicle (arrowhead) crossing the DE-cad-GFP–labeled AJs into recipient cell. (Lower) Movie sequences showing the trajectory in xy and xyz planes of an Ed-mCherry vesicle crossing the DE-cad-GFP–labeled AJs. (B) Stills in xy and xyz planes showing a tubular-shaped Ed-mCherry vesicle (red) spanning the α-catenin-GFP-labeled AJs (green) in *en> α-catenin-GFP + Ed-mCherry* embryo. (C) TEM image revealed that AJs between epithelial cells were interrupted by a vesicle (arrow) apparently moving into the upper cell. Arrowhead indicates the wild type AJs. Of note, there is an invagination of the membrane of the upper cell around the vesicles. (D) Movie sequences showing an Ed-GFP vesicle (arrowhead) moving across the photobleached AJ (bracket) into a recipient cell in *en > Ed-GFP* embryo. Summary of Ed-GFP vesicle trajectory is shown, with a white dot indicating the starting position of the vesicle. (E) The frequency of cell-to-cell movement of Ed-GFP vesicles was scored in wild-type, shg^{g317}, *en > spastin-EGFP* and *en > khc RNAi* embryos. Bar diagram shows means ± s.e.m. Asterisks indicate $p < 0.001$ with Student's *t* test. n indicates the cell number. Bars, 0.2 μm (C) and 2 μm (A,B,D).

transmission. We next proceeded to determine whether MT arrays are required for the intercellular movement of Ed vesicles by overexpressing Spastin-EGFP to sever MTs [17]. Live imaging revealed that when MT arrays were lost, Ed-GFP vesicles became largely stationary (Video S7B) and intercellular movement of Ed-GFP vesicles was completely blocked (Fig. 2E). To further test whether MT motors, which move organelles along MTs, are involved in this process, we performed live imaging in embryos co-expressing Ed-GFP and *kinesin RNAi*. At stage 14/15, when *kinesin heavy chain (khc)* was depleted, Ed-GFP vesicles moved slowly but they still can approach AJ's membranes (Video S7C). However, intercellular movement of Ed-GFP vesicles was still blocked (Fig. 2E). Altogether, our results suggest that intercellular movement of Ed vesicles depend on MTs.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.125.

3.4. Cell-to-cell transport of DE-cad- and EGFR-containing endosomes

Ed is an AJ molecule and exhibits intercellular movement. We wondered whether other AJ or septate junction (SJ) molecules are also endocytosed and exhibit intercellular movement. To explore this possibility, we performed live imaging in embryos with *en-Gal4*-driven *DE-cad-GFP*, *Armadillo-GFP* (encoding an AJ-associated protein), *Discs large-GFP* (encoding a SJ protein) and *Lethal giant larvae-mCherry* (encoding a SJ protein). No Armadillo-GFP vesicles were detected whereas there were few Discs large-GFP and Lethal

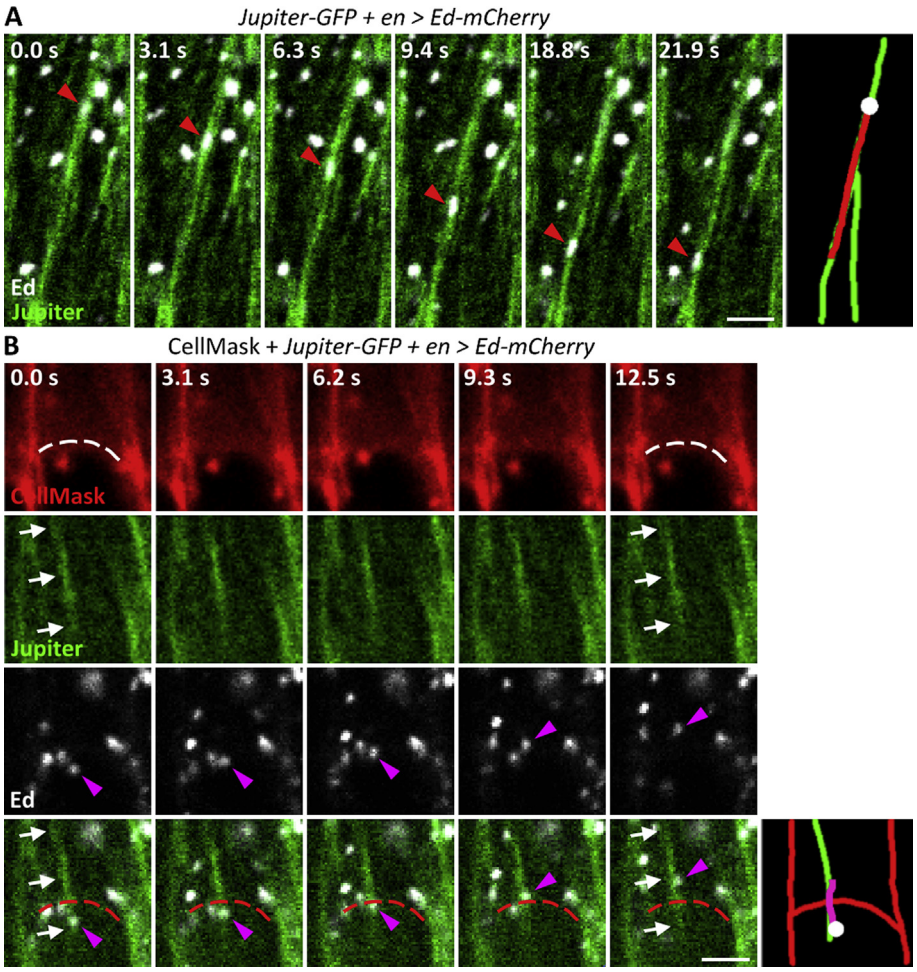

Fig. 3. Cell-to-cell movement of Ed vesicles depends on MTs. (A) Movie sequences from a live imaging of *Jupiter-GFP + en > ed-mCherry* embryo showing an Ed-mCherry vesicle (white, arrowhead) moving along the Jupiter-GFP labeled MT arrays (green). (B) Movie sequences from a live imaging of *Jupiter-GFP + en > ed-mCherry* embryo labeled with CellMask Deep Red showing an Ed-mCherry vesicle (white) moving along MT arrays (green) and, through MT arrays at the AJ, crossing the CellMask Deep Red-labeled cell membrane (red) into the upper cell. Arrowheads indicate the locations of the Ed-GFP vesicle and white arrows indicate MT arrays; dashed line indicates cell–cell boundary. Summary of Ed-GFP vesicles trajectories are shown, with the white dot indicating the starting position of each trajectory. Bars, 2 μm.

giant larvae-mCherry vesicles present at the SJ level, but they were stationary (Video S8). Conversely, we observed intercellular movement of DE-cad-GFP (Fig. 4A). Thus Ed and DE-cad are two cell adhesion molecules localized at the AJs that exhibit intercellular movement. Interestingly, live imaging of embryos co-expressing Ed-GFP and DE-cad-mCherry revealed that many DE-cad-mCherry vesicles co-localized and moved with Ed-GFP and a subpopulation of these co-localized vesicles underwent further intercellular movement (Fig. 4B and Video S9A). We next asked whether signaling receptors such as *Drosophila* EGF receptor (dEGFR), localized at AJs, might also exhibit intercellular movement. Live imaging of embryos co-expressing dEGFR-GFP and Ed-mcherry revealed that 38.2% (n = 283) of dEGFR-GFP vesicles co-localized and moved with Ed-mcherry and a subpopulation of these co-localized vesicles underwent intercellular movement (Fig. 4D and Video S9B). Thus, adhesion molecules and signaling receptors localized at AJs, once endocytosed, can be sorted to the same endosomes that, in turn, undergo cell-to-cell movement. As shown in Fig. 2E, cell-to-cell transport of Ed-containing vesicles required DE-cad. Similarly, cell-to-cell transport of dEGFR-containing endosomes also required DE-cad (Fig. 4E). We next determined whether Ed is required for the intercellular movement of endosomes containing DE-Cad. Towards this, we detected the intercellular movement of DE-cad-GFP in embryos overexpressing *ed RNAi*. Importantly, DE-cad-GFP vesicles formed normally, however, the frequency of intercellular movement of DE-cad-GFP vesicles was significantly reduced (Fig. 4C). Thus, cell-to-cell transport of DE-cad-GFP vesicles requires Ed.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.125.

4. Discussion

Our live imaging studies have revealed a new mechanism for endosomes shuttling between neighboring epithelial cells. These intercellular endosomes contain junctional proteins and signaling receptors. This AJ-based transport mechanism is different from those of (1) actin-based cytonemes/tunneling nanotubes, (2) secreted exosomes and (3) exogenously derived argosomes (lipoproteins) that mediate intercellular transfer of lipid-linked proteins. It is also distinct from the somatic ring canal that is located basal to AJs [19,20]. Because the crossing of the AJs by Ed vesicles can take about 3 s (from 14.2 to 17.3 s and from 23.6 to 25.2 s of Fig. 1D) to complete and they often did not change their direction during the crossing (Fig. 1D and E), one possibility is that Ed vesicles move through a pre-existing pore filled with MTs. Live imaging and

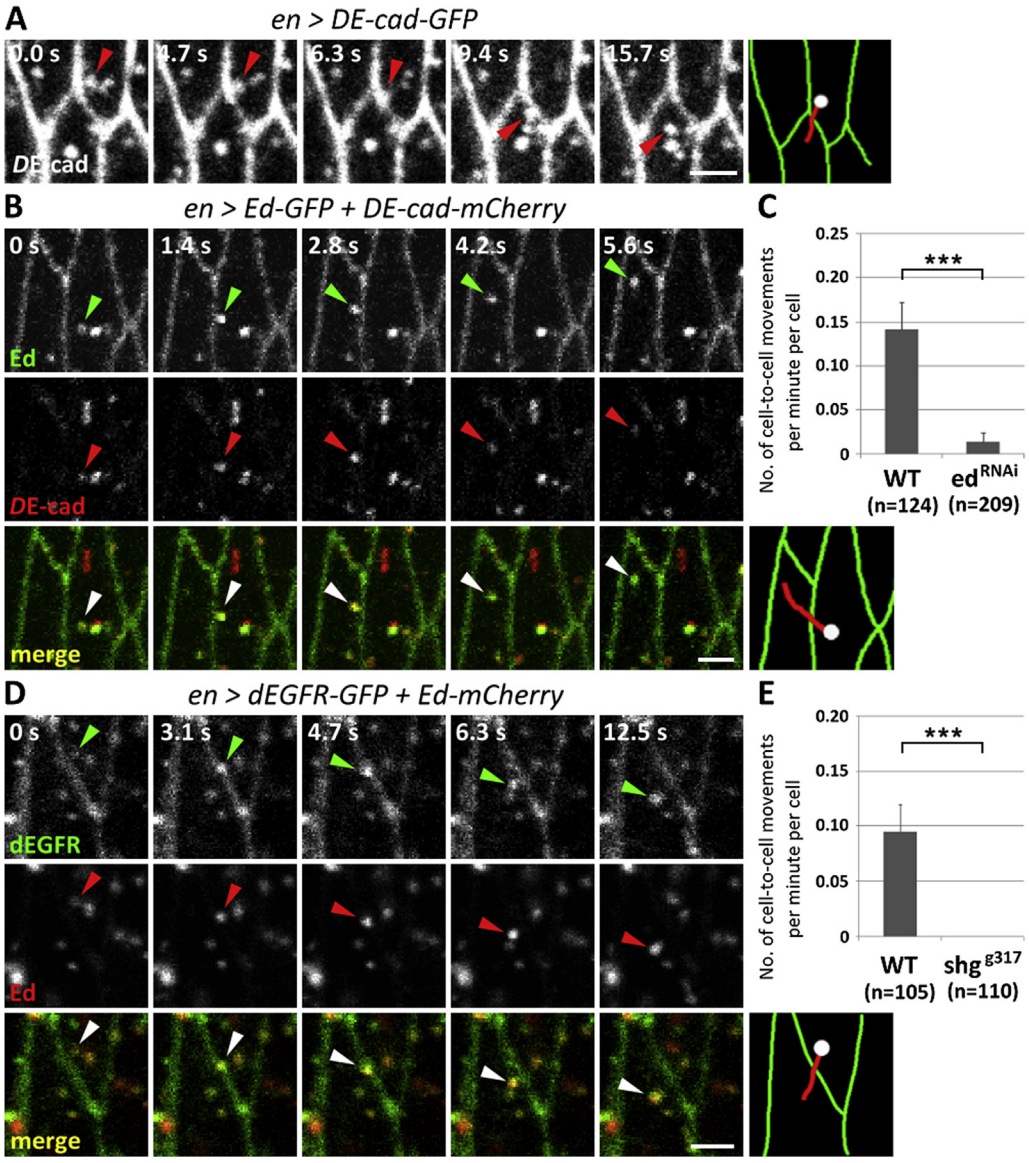

Fig. 4. Cell-to-cell transport of DE-cad- and EGFR-containing endosomes. (A) Movie sequences showing intercellular movement of DE-cad-GFP vesicles (arrowhead) in *en > DE-cad-GFP* embryo. (B,D) Movie sequences of (B) *en > Ed-GFP + DE-cad-mCherry* and (D) *en > dEGFR-GFP + Ed-mCherry* embryos. (B) DE-cad-mCherry (red) co-localized and moved with Ed-GFP (green) during intercellular movement. (D) dEGFR-GFP (green) co-localized and moved with Ed-mCherry (red) during intercellular movement. Arrowhead indicates the location of co-localized vesicles. Summary of DE-cad-GFP (A), DE-cad-mCherry (B) and dEGFR-GFP (D) vesicles trajectories are shown, with white dot indicating the starting position of each trajectory (A,B,D). Bars, 2 μm. (C) The frequency of cell-to-cell movement of DE-cad-GFP vesicles was scored in wild-type and *ed-RNAi* embryos. (E) The frequency of cell-to-cell movement of dEGFR-GFP vesicles was scored in wild-type and shg^{g317} embryos. Bar diagram shows means ± s.e.m. Asterisks indicate $p < 0.001$ with Student's *t* test. n indicates the cell number.

electron microscopy of stage 14/15 embryos, however, reveals no pre-existing cytoplasmic channel at the AJs of lateral epithelial cells (unpublished data). We therefore favor an alternative possibility that Ed vesicles might move along MTs followed by an endocytic transfer of endosomes across the AJ membrane to allow intercellular transport. In support of this interpretation, TEM analyses have revealed an invagination of the membrane of the upper cell around the vesicles at AJs (Fig. 2C). Further study is required to confirm this mechanism.

As only a subpopulation of endosomes exhibits cell-to-cell transport, it is possible that intercellular transport is a regulated process. Alternatively, intercellular transport may be a stochastic event depending on the attachment of endosomes to MT arrays that happen to associate with AJs. We showed that Ed is a component of intercellular endosomes. Thus, Ed might engage in non-cell-autonomous processes. Consistent with this, we previously demonstrated that *ed* acts non-autonomously to generate extra R7 photoreceptor cells and to promote ommatidial rotation during eye development [15,21]. Finally, many signaling receptors remain active in the endosomes following receptor-mediated endocytosis [22]. Thus, through cell-to-cell transport of EGFR-containing endosomes, activated receptors might be spread to neighboring cells. It is currently unclear whether this will affect the spatial range/extent of receptor activation or simply create signal noise.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.125.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.125.

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