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Adherens Junctions on the Move—Membrane Trafficking of E-Cadherin

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Cadherin-based adherens junctions are conserved structures that mediate epithelial cell–cell adhesion in invertebrates and vertebrates. Despite their pivotal function in epithelial integrity, adherens junctions show a remarkable plasticity that is a prerequisite for tissue architecture and morphogenesis. Epithelial cadherin (E-cadherin) is continuously turned over and undergoes cycles of endocytosis, sorting and recycling back to the plasma membrane. Mammalian cell culture and genetically tractable model systems such as *Drosophila* have revealed conserved, but also distinct, mechanisms in the regulation of E-cadherin membrane trafficking. Here, we discuss our current knowledge about molecules and mechanisms controlling endocytosis, sorting and recycling of E-cadherin during junctional remodeling.

The ability of epithelial cells to organize into monolayered sheets is a prerequisite for multicellularity, thereby providing tissue integrity, barrier function, and tissue polarity in metazoan organisms. Adherens junctions (AJs) are conserved key structures that mediate cell–cell adhesion in invertebrates and vertebrates. In many polarized epithelial sheets, AJs form a continuous adhesive belt at the apical–lateral interfaces of cell–cell contacts, the zonula adherens. The structural and functional core components of epithelial AJs are clusters of dimeric E-cadherin, a calcium-dependent, homophilic cell–cell adhesion receptor (Fig. 1). High-resolution microscopy analyses, however, recently revealed that E-cadherin clusters also accumulate throughout the lateral junctions below the zonula adherens (Wu et al. 2014; Yap et al. 2015).

Classical cadherins such as E-cadherin are single-pass membrane proteins with characteristic extracellular cadherin (EC) repeat domains that mediate *trans*-homophilic interactions between neighboring cells. While the numbers of ECs vary between different species, their intracellular domains are highly conserved from flies to humans and form a complex with catenins that link AJs to the actin cytoskeleton. The juxtamembrane domain of the cadherin intracellular tail interacts with p120 catenin, whereas the carboxy-terminal part directly binds β-catenin, which, in turn, binds α-catenin mediating a dynamic linkage to the actin cytoskeleton (Fig. 1) (Drees et al. 2005; Gates and Peifer 2005; Yamada et al. 2005). This dynamic interaction between AJs and the actin cytoskeleton is tightly linked to junctional maintenance, dynamics, and plastic-

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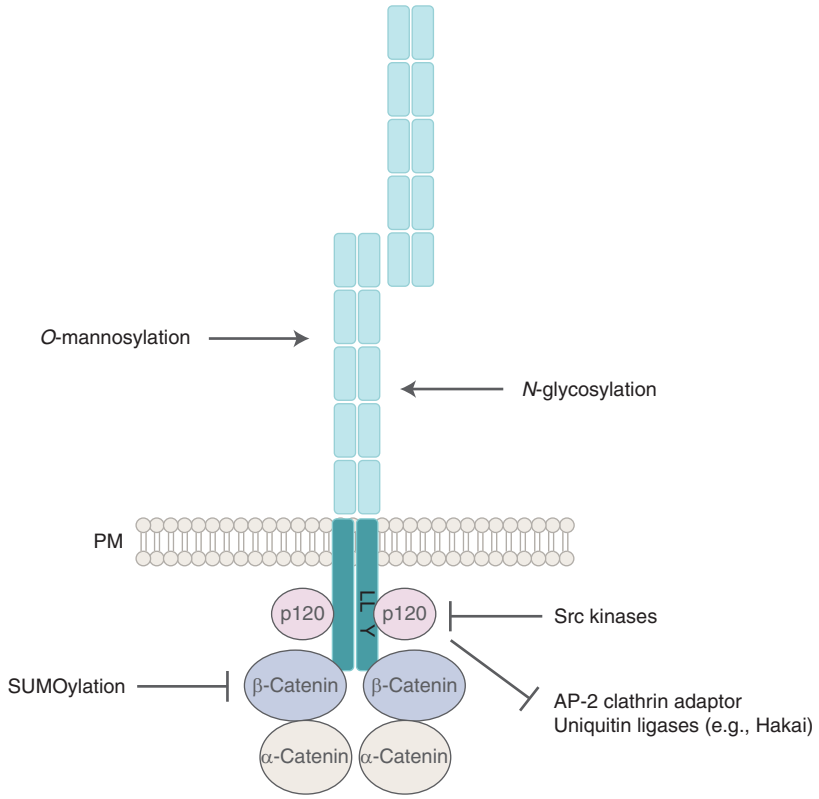

Figure 1. The core E-cadherin/catenin complex at adherens junctions (AJs). The stability and turnover of the core E-cadherin/catenin complex is regulated by different molecules and posttranslational modifications, for further details see main text.

ity in developing and differentiated tissues (Michael and Yap 2013).

The dual properties of stability and plasticity of AJs were first observed in calcium chelation experiments (Kartenbeck et al. 1982). Depletion of extracellular calcium results in a rapid disruption of cell–cell adhesion in cultured epithelial monolayers because of the endocytic internalization of cadherins from AJs (Kartenbeck et al. 1982, 1991). The significance of cadherin internalization under physiological conditions is now well established. Over the last three decades, numerous studies showed that junctional proteins such as E-cadherin are dynamically turned over at the cell surface and this is fundamental for tissue remodeling during morphogenesis and tissue homeostasis (Kowalczyk and Nanes 2012; Takeichi 2014). Mammalian cell culture studies combined with in vivo models like *Drosophila* have led to the identification of conserved molecules and the underlying regulatory mechanisms driving cadherin trafficking in a large variety of morphogenetic and developmental processes. Here, we review our current knowledge about the proteins and the mechanisms controlling endocytosis, sorting and recycling of E-cadherin.

E-CADHERIN IS CONSTANTLY INTERNALIZED FROM THE CELL SURFACE

Dynamic changes in cell shape within tissues require a constant remodeling of cell junctions. Initial metabolic labeling experiments in cultured Madin–Darby canine kidney (MDCK) epithelial cells showed a half-life of endogenous E-cadherin at the cell surface of ~5–10 h (McCrea and Gumbiner 1991; Troxell et al.

1999). Recent fluorescence recovery after photobleaching (FRAP) and photoconversion experiments in living epithelial layers of the *Drosophila* embryo confirmed a relatively slow biosynthetic turnover of E-cadherin clusters of about 1 h in vivo (Cavey et al. 2008). Thus, the comparatively slow transcriptional regulation of E-cadherin cannot account for all rapid changes in cell adhesion strength during fast cellular movements and tissue remodeling. Instead, cadherins are constantly removed from the plasma membrane through endocytosis and recycled back by exocytosis. Depending on the cellular context, E-cadherin can be internalized through different endocytic pathways. Most studies analyzed clathrin-mediated endocytosis of E-cadherin (Le et al. 1999; Palacios et al. 2002; Paterson et al. 2003). However, growth-factor-induced non-clathrin-mediated pathways of E-cadherin, including Rac1-dependent macropinocytosis, have been reported (Braga et al. 1997, 1999; Akhtar and Hotchin 2001; Lu et al. 2003; Bryant et al. 2007).

LOCAL REMOVAL OF E-CADHERIN FROM THE PLASMA MEMBRANE BY CLATHRIN-MEDIATED ENDOCYTOSIS

Unlike macropinocytosis, clathrin-mediated endocytosis allows a spatially controlled internalization. Since clathrin does not bind directly to cargo receptors, selection of cargo relies on adaptor proteins that recognize internalization motifs within the cytoplasmic region of transmembrane receptors (Kelly and Owen 2011). E-cadherin associates with several endocytic adaptors including AP-2, Dab-2, and Numb (Ling et al. 2007; Miyashita and Ozawa 2007b; Yang et al. 2007; Sato et al. 2011). A central adaptor in clathrin-mediated endocytosis is AP-2, which forms a tetrameric complex that directly binds clathrin and recruits several classes of receptors bearing an acidic dileucine internalization signal in their cytoplasmic tail (Fig. 2) (Traub 2003, 2009; Kelly and Owen 2011). Vertebrate E-cadherin contains an AP-2 binding motif and mutations in this dileucine motif affect the localization of E-cadherin by preventing its clathrin-mediated endocytosis (Miranda et al. 2001; Miyashita and Ozawa 2007a,b).

The budding of clathrin-coated vesicles requires the core endocytic machinery including the GTPases Dynamin and Rab5. Reduction of these core components results in an increase of E-cadherin at the plasma membrane. Dynamin is a large, multidomain GTPase that assembles into helical structures along invaginating membranes and drives the scission of endocytic vesicles through a cycle of oligomerization and GTP hydrolysis (Fig. 2) (Schmid and Frolov 2011; Kirchhausen et al. 2014). Dynamin-mediated endocytosis is required for E-cadherin redistribution at mature AJs of MDCK and MCF7 cells, but also plays an important role in AJ turnover during *Drosophila* epithelial morphogenesis (Classen et al. 2005; Georgiou et al. 2008; Leibfried et al. 2008; de Beco et al. 2009; Levayer et al. 2011). A key observation of the *Drosophila* in vivo studies is that E-cadherin endocytosis is locally enhanced along the planar axis or along the apico-basal axis of epithelial cells and that this local E-cadherin turnover has an instructive role in tissue morphogenesis. For example, polarized endocytosis of E-cadherin is crucial for cell intercalations in the elongating *Drosophila* embryo whereby epithelial cells change neighbors through the shrinkage of planar polarized junctions along the dorsoventral axis. Blocking of clathrin-mediated endocytosis causes the loss of E-cadherin planar polarization and a block of cell intercalations (Levayer et al. 2011). Similar observations were made in *Drosophila* pupal epithelia (Classen et al. 2005; Georgiou et al. 2008; Leibfried et al. 2008; de Beco et al. 2009). Wing epithelial cells become hexagonally packed through the shrinkage of individual AJ by polarized E-cadherin turnover (Classen et al. 2005; Warrington et al. 2013). Consistently, loss of E-cadherin or dynamin function disrupts the planar polarized organization of the wing epithelium (Classen et al. 2005; Fricke et al. 2009). Polarized endocytosis is also necessary for local E-cadherin removal during embryonic wound repair (Hunter et al. 2015). Blocking of endocytosis on wounding disrupts not only AJ remodeling but also prevents the assembly of contractile actomyosin cables at the wound

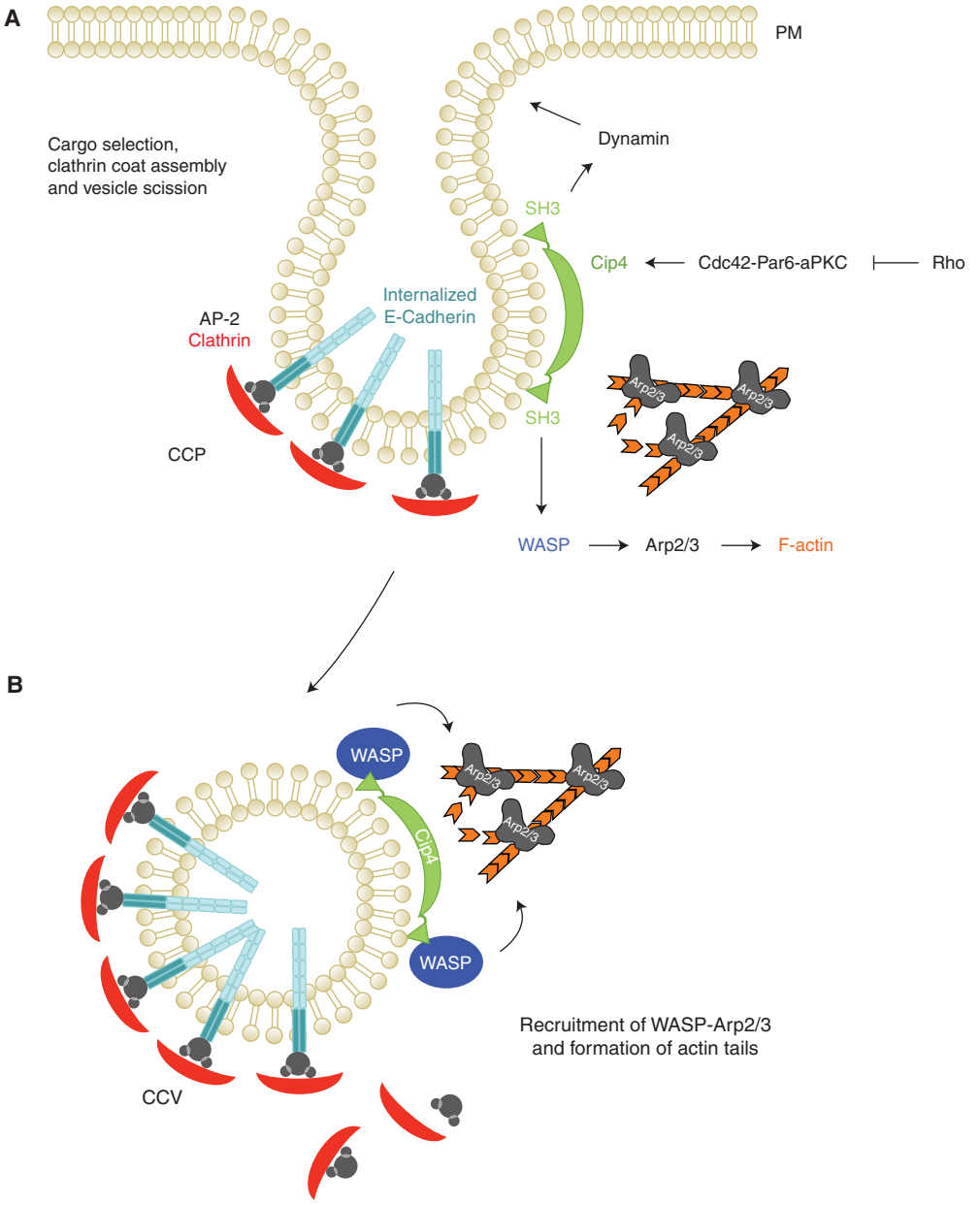

Figure 2. The Cdc42-Par6-aPKC polarity complex promotes E-cadherin endocytosis by recruiting the Cip4-WASP-Arp2/3 actin machinery. (*A*) E-cadherin is actively internalized by clathrin-mediated endocytosis. Vertebrate E-cadherin contains an AP-2 binding motif. Once E-cadherin is selected and bound by AP-2 or other cargo-adapter proteins (e.g., Numb, see main text), the clathrin coat is assembled. Cdc42–Par6–aPKC recruits Cip4 to the site of E-cadherin endocytosis. F-BAR proteins such as Cip4 are thought to facilitate the scission by recruiting Dynamin to the neck of a nascent vesicle. This recruitment requires the SH3 domain that binds to Proline-rich motifs of Dynamin but also the Arp2/3 activator Wiskott–Aldrich syndrome protein (WASP). (*B*) WASP-Arp2/3-mediated actin polymerization has a supportive function in vesicle budding and promotes actin-comet-tail-based movement of newly formed clathrin-coated vesicles. The GTPase Rho seems to suppress cadherin endocytosis by antagonizing Cdc42–Par6–aPKC functions (Warner and Longmore 2009).

margin that dramatically slows down wound closure (Hunter et al. 2015). Thus, E-cadherin endocytosis appears to be a necessary step for the cytoskeletal rearrangements driving wound repair. An important requirement in coupling endocytosis-driven junctional remodeling and actomyosin contractility has also been observed in *Drosophila* dorsal closure and in zebrafish epiboly cell movements (Mateus et al. 2011; Song et al. 2013).

p120 CATENIN, AN INHIBITOR OF VERTEBRATE E-CADHERIN ENDOCYTOSIS

Interestingly, the acidic dileucine motif in the juxtamembrane domain of vertebrate E-cadherin overlaps with the binding site for p120 catenin, a well-known inhibitor of cadherin endocytosis (Davis et al. 2003). In the absence of p120 catenin, cadherins are rapidly internalized and degraded in the lysosome (Miyashita and Ozawa 2007b; Xiao et al. 2007). Recent data strongly suggests that p120 catenin binding masks the endocytic signal and may thereby compete for AP-2 binding to cadherins in mammals (Nanes et al. 2012; Perez-Moreno and Fuchs 2012). Supporting this notion, the binding affinities of p120 catenin and AP-2 for the acidic dileucine motif are similar (Fig. 1) (Nanes et al. 2012). However, a physiological role of AP-2 function in the initiation of clathrin-mediated endocytosis of E-cadherin has so far only been shown in *Drosophila* germband extension, a major morphogenetic movement during fly gastrulation (Levayer et al. 2011). Remarkably, *Drosophila* E-cadherin lacks the endocytic motif and p120 catenin function is not required for internalization of E-cadherin for degradation (Myster et al. 2003; Bulgakova and Brown 2016). Different from mice, worms and flies lacking p120 catenin are viable and fertile and display no striking defects in the structure or function of adherens junctions (Myster et al. 2003; Pettitt et al. 2003; Davis and Reynolds 2006; Perez-Moreno et al. 2006). However, loss of *Drosophila* p120 catenin function strongly enhances phenotypic traits in E-cadherin and β-catenin mutants, suggesting an important supportive role of p120 catenin in junctional cell adhesion (Myster et al. 2003). Surprisingly, Bulgakova and Brown (2016) recently found that *Drosophila* p120 catenin rather promotes clathrin-mediated endocytosis of E-cadherin and propose that the inhibitory function of p120 catenin has been newly acquired in vertebrates during evolution. Thus, the key role of p120 catenin as a master regulator of cadherin stability may represent a vertebrate-specific adaptation in response to an increased complexity in tissue morphogenesis and architecture during evolution (Carnahan et al. 2010). It also implies the existence of additional ancient key principles in regulating the stability of AJs present in invertebrate and vertebrates.

POSTTRANSLATIONAL MODIFICATIONS REGULATING E-CADHERIN TURNOVER

Phosphorylation might represent such an ancient regulatory mechanism controlling cadherin internalization. Early cell culture studies have already revealed that cell–cell junctions are prominent targets of tyrosine phosphorylation (Maher et al. 1985). Stimulation of epithelial cultures by growth factors (e.g., epithelial growth factor [EGF] or human growth factor [HGF]) promotes tyrosine phosphorylation of several junctional proteins followed by a rapid internalization of junctional complexes. Within the past decades, numerous studies in cultured cells have unraveled a series of tyrosine, but also serine phosphorylation events and identified diverse kinases and phosphatases acting on E-cadherin/catenin complex integrity (reviewed in Daniel and Reynolds 1997; Roura et al. 1999; Lilien and Balsamo 2005; Bertocchi et al. 2012; Coopman and Djiane 2016). The picture emerging from these studies of how AJ functions and phosphorylation interplay is still very incomplete, especially because many of these phosphorylation events strongly depend on the cell type and the stimulus such as growth factor treatment (reviewed in Bertocchi et al. 2012; Coopman and Djiane 2016).

Members of the Src protein family have emerged as critical cytoplasmic tyrosine kinases that affect E-cadherin dynamics and E-cad-

herin/catenin complex integrity in vertebrates and invertebrates. The activation of Src kinases and subsequent phosphorylation of AJ components in cultured epithelial cells generally results in a disruption of cell–cell adhesions and increased invasiveness (Behrens et al. 1993; Boyer et al. 1997; Calautti et al. 1998; Owens et al. 2000). However, depending on the signal strength, Src can also act positively on E-cadherin-mediated cell adhesion (McLachlan et al. 2007). On Src activation E-cadherin is phosphorylated at two specific tyrosine residues in the juxtamembrane domain, thereby creating a binding surface for the c-Cbl-like ubiquitin ligase Hakai (Fig. 1) (Fujita et al. 2002). Since the binding sites for Hakai and p120 catenin are closely apposed, it has been suggested that p120 catenin binding might also compete with Src or Hakai and hence might inhibit ubiquitination and degradation. Consistently, ubiquitination of the juxtamembrane domain prevents p120 catenin binding to E-cadherin and results in proteasomal degradation of E-cadherin (Hartsock and Nelson 2012). However, tyrosine phosphorylation-defective mutants of E-cadherin can still be internalized and Hakai-mediated ubiquitination might rather serve as a sorting signal for its trafficking to lysosomes (Palacios et al. 2005). Thus, ubiquitination does not seem to directly regulate the internalization of E-cadherin. Hakai is highly conserved in metazoans, and flies lacking Hakai function also show severe defects in epithelial integrity, as well as defects in cell specification and cell migration (Kaido et al. 2009). A functional interaction between Hakai and Src kinases has never been observed in *Drosophila*. Src42A, one of the two Src kinases in *Drosophila*, forms a ternary complex with E-cadherin and β-catenin (Armadillo [Takahashi et al. 2005]). Src42A activation has a dual effect on E-cadherin-based AJs. On the one hand Src42A increases E-cadherin turnover by phosphorylation of β-catenin and thereby, destabilizing AJ complex integrity during tracheal epithelial morphogenesis in *Drosophila* (Shindo et al. 2008). On the other hand it also results in a transcriptional activation of E-cadherin by released β-catenin that acts as a well-known transactivator of the Tcf/Lef family of transcription factors (Shindo et al. 2008; Langton et al. 2009). Recent FRAP experiments of green fluorescent protein (GFP)-marked E-cadherin at *Drosophila* AJs further revealed that Src42A promotes the recycling of E-cadherin and is required for polarized cell-shape changes during epithelial tube elongation (Forster and Luschnig 2012). Thus, Src-mediated tyrosine phosphorylation has an important conserved role in E-cadherin turnover. However, the functional consequences and the underlying regulatory mechanism of tyrosine phosphorylation seem to differ remarkably between different cell types and species.

The posttranslational addition of a small ubiquitin-related modifier (SUMO) protein to E-cadherin seems to be essential for E-cadherin recruitment to AJs and for the maintenance of its interaction with the actin cytoskeleton in *Caenorhabditis elegans* (Fig. 1) (Tsur et al. 2015). SUMO modification is a reversible process analogous to ubiquitylation: Sumoylation is mediated by the concerted actions of E1, E2, and E3 enzymes, whereas desumoylation is promoted by SUMO specific proteases (Flotho and Melchior 2013). A key finding is that sumoylation on a conserved lysine residue of the E-cadherin cytoplasmic tail reduces its interaction with β-catenin (Tsur et al. 2015). Both the loss and overexpression of SUMO proteases resulted in similar defects in AJ assembly, suggesting that balanced sumoylation-desumoylation events are important to sustain AJ plasticity. Sumoylation is essential for most organisms and mammals express three SUMO precursor proteins, whereas *C. elegans*, *Drosophila,* and yeast only express a single SUMO protein (Flotho and Melchior 2013). Thus, it will be important to determine whether transient sumoylation acts as a conserved molecular switch of AJ dynamics in other species.

LOCAL E-CADHERIN ENDOCYTOSIS DEPENDS ON THE Cdc42-Par6-aPKC POLARITY COMPLEX

The spatially controlled endocytosis of E-cadherin depends on evolutionarily conserved polarity proteins including the PDZ domain

protein Par6, the atypical protein kinase C (aPKC) and the Rho-GTPase Cdc42, which is also a well-known regulator of the actin cytoskeleton. Par6 and aPKC form, together with the scaffold protein Par3 (Bazooka), the Par complex, a universal module defining apico-basal cell polarity in metazoans (Tepass 2012). Cells of the pupal thorax epithelium of *Drosophila* lacking either the Par6-aPKC or Cdc42 function show a defective internalization of E-cadherin characterized by malformed endocytic membrane tubules, reminiscent of vesicle scission defects in *dynamin* (shibire) mutant cells (Georgiou et al. 2008; Leibfried et al. 2008). The Par complex seems to act as an effector of Cdc42 in controlling endocytosis of E-cadherin, because constitutively active aPKC can partially suppress the AJ disruption of *cdc42* loss-of-function (Fig. 2). An important requirement of Cdc42 and the Par6-aPKC module in the endocytic turnover of E-cadherin has also been found in mammalian cell culture and in *C. elegans* (Balklava et al. 2007). Recent studies in mammals as well as in flies further suggest that other polarity proteins, including Lethal giant larvae (LgL), Scribble (Scrib), and Crumbs, may also play an important role in the maintenance of AJs (Goldstein and Macara 2007; Nance and Zallen 2011). However, these effects could also be indirect because these proteins are also crucial for the maintenance of apico-basal polarity and therefore tissue integrity (Tepass and Knust 1990, 1993).

How is the Cdc42-Par6-aPKC polarity complex linked to the endocytic machinery promoting E-cadherin internalization? Cdc42 is also a well-known activator of the Wiskott-Aldrich syndrome protein family (WASP), which, in turn, promotes actin nucleation through the Arp2/3 complex, a major actin nucleator in eukaryotic cells (Fig. 2) (Symons et al. 1996; Rohatgi et al. 2000; Pollard and Beltzner 2002). Cdc42 directly binds WASP and relieves an autoinhibitory contact between the GTPase-binding domain and the carboxy-terminal Arp2/3-activating VCA module (Kim et al. 2000). On activation of WASP by Cdc42 branched actin filaments are formed at the rim of endocytic pits that are thought to facilitate the invagination and scission of the underlying membrane (Fig. 2) (Merrifield and Kaksonen 2014; Kaksonen et al. 2006). WASP-Cdc42-induced actin polymerization at endocytic sites further depends on the membrane-curvature promoted by F-BAR proteins such as Cip4 (Cdc42-interacting protein 4) (Ho et al. 2004; Tsujita et al. 2006; Takano et al. 2008). The F-BAR domain dimer of Cip4 forms a crescent-shaped surface that binds and deforms the membrane, whereas the carboxy-terminal Src homology 3 (SH3) domain binds Dynamin and WASP (Fig. 2) (Heath and Insall 2008; Aspenstrom 2009; Chen et al. 2013). Thus, F-BAR proteins such as Cip4 are prime candidates to couple WASP-Cdc42-mediated actin polymerization to Dynamin-mediated vesicle scission in endocytosis and vesicle movement (Fig. 2) (Fricke et al. 2010). In *Drosophila*, thorax epithelia lacking either WASP or Cip4 function display AJ breaks and the formation of long tubular endocytic structures that are very reminiscent of *dynamin* mutant cells in which vesicle scission is blocked (Georgiou et al. 2008; Leibfried et al. 2008). A dynamin-related phenotype has been observed on overexpression in the fly wing epithelium of a dominant-negative Cip4 protein lacking the Dynamin-interacting SH3 domain (Fricke et al. 2009). Here, Dynamin function is required for junctional remodeling during epithelial repacking (Classen et al. 2005). Thus, a model has been proposed in which activated Cdc42 acts through the Par complex and recruits Cip4-WASP to promote both Dynamin-mediated vesicle scission and branched actin nucleation at endocytic vesicles (Leibfried et al. 2008; Fricke et al. 2009). Despite the conserved function of Cip4 in regulating early steps in E-cadherin endocytosis, flies, worms, and mice lacking Cip4 function are viable and develop largely normally, suggesting additional compensatory or redundant functions that are likely fulfilled by other members of the F-BAR protein family (Fricke et al. 2009; Giuliani et al. 2009; Feng et al. 2010; Rolland et al. 2014). Supporting this notion, double mutants lacking Cip4 and the Cip4-like protein Nostrin show reduced viability and fertility (Zobel et al. 2015). Double mutant flies show

defects in wing polarization defects and egg chamber encapsulation caused by an impaired turnover of E-cadherin (Zobel et al. 2015).

ENDOSOMAL SORTING OF E-CADHERIN

Once internalized by clathrin-dependent or -independent endocytosis, E-cadherin enters a Rab5 positive compartment, which is central for sorting of most cell surface transmembrane proteins (Fig. 3) (Pfeffer 2013; Wandinger-Ness and Zerial 2014). Within this early-endosomal (EE) compartment E-cadherin is either sorted back to the plasma membrane through recycling endosomes (RE) or it is sequestered into intraluminal vesicles of multivesicular endosomes (MVE) that finally fuse with lysosomes for degradation. How cadherins are selected from sorting endosomes for trafficking to recycling endosomes is not yet understood. Endosomal sorting of E-cadherin may involve the segregation of tubular endosomal subdomains

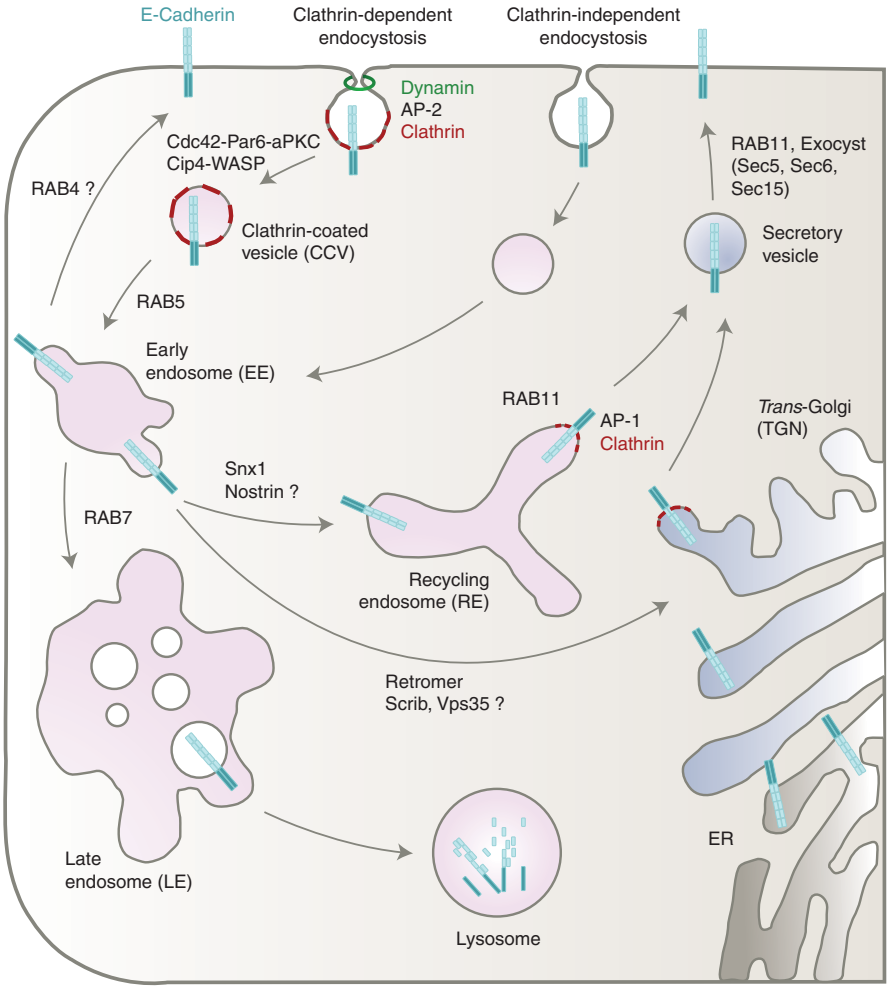

Figure 3. Trafficking pathways of E-cadherin. E-cadherin can undergo either clathrin-dependent or independent endocytosis. Internalized E-cadherin traffic through different endosomal compartments. Numerous molecules and endocytic machineries determine the fate of E-cadherin either to endosomal recycling back to the plasma membrane or to lysosomal degradation. Polarized sorting in both the biosynthetic pathway from the *trans*-Golgi network and recycling endosomes requires vesicle carriers such as AP-1 as discussed in the main text.

that recruit diverse microtubule motors driving endosomal membrane scission and trafficking (Hunt et al. 2013; van Weering and Cullen 2014). This function has been proposed for Nostrin (Zobel et al. 2015). Nostrin localizes at distinct subdomains of Rab5/Rab11 endosomal intermediates. A cooperative recruitment model has been proposed, in which Cip4 first promotes membrane invagination and early actin-based endosomal motility, whereas Nostrin is linked to microtubules through the minus end-directed kinesin motor Khc-73 for trafficking of recycling endosomes (Zobel et al. 2015). An important role of the mammalian orthologs Kif13A and KifC3 either in the formation of recycling endosomal tubules along microtubules or in the microtubule-dependent transport to AJs has recently been identified (Meng et al. 2008; Delevoye et al. 2014).

Known integral components of the tubule-based endosomal sorting mechanism are sorting nexins (Snx). Previous work has shown that the two Snx-Bar proteins Snx4 and Snx1 are involved in E-cadherin sorting in epithelial cell culture (Bryant et al. 2007; Solis et al. 2013). Depletion of Snx1 resulted in increased intracellular accumulation and turnover of E-cadherin internalized from the cell surface of MCF-7 cells (Bryant et al. 2007). Snx1 is a conserved component of the retromer complex, an endosomal protein sorting machinery regulating the recycling of endocytosed proteins from endosomes to the *trans*-Golgi network (TGN) or to the plasma membrane (Fig. 3) (Cullen 2008; Wang and Bellen 2015). Whether endocytosed E-cadherin can be recycled back to the TGN via the retromer is not known. Interestingly, *Drosophila* Vps35 (CG5625), which is part of the cargo-selective trimer of the retromer, was recently found in a genome-wide RNAi screen for genes required for E-cadherin-dependent cell–cell adhesion (Toret et al. 2014).

E-CADHERIN IS MAINLY RECYCLED THROUGH Rab11-POSITIVE ENDOSOMES

Recycling of internalized membrane receptors can either be facilitated by a rapid Rab4-mediated route or transit through a slow Rab11-positive recycling pathway back to the plasma membrane (Fig. 3) (Stenmark 2009; Scott et al. 2014). Several studies in epithelial cell culture and in diverse *Drosophila* epithelial tissues showed that E-cadherin is principally transported to a Rab11-positive recycling endosomal compartment (Fig. 3) (Classen et al. 2005; Lock and Stow 2005; Bogard et al. 2007; Desclozeaux et al. 2008; Roeth et al. 2009; Pirraglia et al. 2010; Hunter et al. 2015; Le Droguen et al. 2015; Loyer et al. 2015), whereas there is only little evidence showing that E-cadherin can also pass a Rab4-dependent rapid recycling route as recently found during *Drosophila* leg development (de Madrid et al. 2015). Consistently, disruption of Rab11-mediated recycling results in an abnormal intracellular accumulation of E-cadherin and junctional integrity is severely impaired.

POLARIZED EXOCYTOSIS OF E-CADHERIN BY A Rab11-EXOCYST COMPARTMENT

A striking intracellular accumulation of E-cadherin in enlarged Rab11-positive vesicles was also observed in mutant cells lacking single components of the exocyst complex such as Sec5, Sec6, and Sec15, indicating a defect in the targeted delivery of E-cadherin from the basolateral domain to the apicobasal AJs (Langevin et al. 2005). Further evidence suggest that the exocyst can regulate E-cadherin recycling by acting as a direct molecular link between the Rab11-recycling endosomes and the E-cadherin/catenin complex in tissue remodeling and collective cell migration (Fig. 3) (Classen et al. 2005; Langevin et al. 2005; Blankenship et al. 2007; Wan et al. 2013).

The exocyst is an evolutionarily conserved multi-subunit protein complex that is crucial to tether secretory vesicles derived from the *trans*-Golgi network (TGN) or from recycling endosomes to the plasma membrane for exocytosis (Wu and Guo 2015). In yeast, the exocyst is localized to defined regions of the plasma membrane where it mediates the polarized delivery of proteins and lipids required for polarized mem-

brane growth. This model has been adapted to epithelial cells, where targeted recycling of internalized junctional proteins to the apical membrane provides a mechanism controlling AJ remodeling during cell polarization (Grindstaff et al. 1998; Mostov et al. 2003). Consistently, Rab11 and β-catenin interact with the exocyst components Sec15, Sec5, and Sec10, respectively, and disruption of these interactions results in an intracellular accumulation of E-cadherin in recycling endosomes (Fig. 3) (Beronja et al. 2005; Langevin et al. 2005). Recycling endosomes can also function as an intermediate compartment for newly synthesized E-cadherin that is not directly transported from the TGN to the plasma membrane by the exocyst in cultured epithelial cells (Yeaman et al. 2004; Lock and Stow 2005). Remarkably, in polarized MDCK cells newly synthesized E-cadherin is transported as a complex with β-catenin and the formation of the E-cadherin/β-catenin complex is already important for efficient exit from the endoplasmic reticulum (Chen et al. 1999) Uncoupling the binding of β-catenin from E-cadherin by introducing corresponding mutations in the E-cadherin cytoplasmic tail results in an accumulation of the proteins in intracellular compartments and subsequent degradation in lysosomes (Miyashita and Ozawa 2007a). A recent in vivo study in the *Drosophila* follicular epithelium further supports a model in which the Rab11-exocyst interaction regulates targeting of vesicles with endocytosed as well as newly synthesized E-cadherin (Woichansky et al. 2016). The investigators further propose the existence of two exocytosis pathways for de novo synthesized E-cadherin, first a Rab11-independent pathway for exocytosis to the basal-lateral region, and second a Rab11-dependent pathway that targets exocytosis to apico-lateral AJs (Woichansky et al. 2016). In the same study a so far uncharacterized GTPase, RabX1, has been identified as a new critical component regulating E-cadherin recycling. In *rabX1* mutant epithelia endocytosed E-cadherin is not properly recycled, but rather accumulates together with Rab5 and Rab11 in a large compartment (Woichansky et al. 2016).

SORTING OF NEWLY SYNTHESIZED E-CADHERIN FROM THE *TRANS*-GOLGI NETWORK AND Rab11 RECYCLING ENDOSOMES

In a number of ways sorting at the TGN resembles the initial steps in endocytosis. Post-Golgi vesicles are coated by clathrin and polarized sorting requires clathrin adaptors of the family of heterotetrameric AP complexes such as AP-1 (Fig. 3) (Bonifacino 2014). Different from endocytic AP-2, AP-1 complexes localize at the TGN and REs and control either the biosynthetic sorting at the TGN (AP-1A) or the RE sorting (AP-1B) to the basolateral surface (Folsch et al. 2003; Gravotta et al. 2012; Folsch 2015). In MDCK cells, double knockdown of AP-1A and AP-1B results in missorting of many basolateral proteins including E-cadherin, and causes a dramatic loss of cell polarity (Gravotta et al. 2012). Interestingly, similar to AP-2, AP-1 complexes recognize as a basolateral sorting signal the same dileucine-based motif in the cytoplasmic tail of E-cadherin (Lock and Stow 2005; Ling et al. 2007; Mattera et al. 2011). Thus, AP-1 and AP-2 recognize almost identical sets of dileucine motif-containing membrane cargo proteins, but function at different intracellular sites (Fig. 3). Knockout studies in mammals, zebrafish, *Drosophila*, and *C. elegans* further confirmed an important conserved role of AP-1 in E-cadherin trafficking and loss of AP-1 function results in strong reduction of E-cadherin-mediated cell adhesion and epithelial disorganization (Shim et al. 2000; Zizioli et al. 2010; Burgess et al. 2011; Takahashi et al. 2011; Shafaq-Zadah et al. 2012; Zhang et al. 2012; Hase et al. 2013; Gariano et al. 2014; Gillard et al. 2015; Loyer et al. 2015).

Unlike vertebrates, invertebrates only contain a single AP-1 complex and *Drosophila* and *C. elegans* E-cadherin lack the dileucine-based AP-1-sorting signal in their cytoplasmic tails (Boehm and Bonifacino 2001). Thus, in invertebrates, interactions between E-cadherin and AP-1 complexes might not be direct, but may instead be mediated by adaptors such as phosphatidylinositol phosphate 4 kinases (PI4K) (Ling et al. 2007). Mammalian PIPKIγ directly

binds both E-cadherin and the μ subunit of AP-1, thus it might act as a signaling scaffold that links AP-1 complexes to E-cadherin. Depletion of PIPKIγ or disruption of PIPKIγ binding to either E-cadherin or AP complexes results in defective E-cadherin transport and blocks AJ assembly (Ling et al. 2007). Phosphatidylinositol-4-phosphate (PI4P) is a critical lipid involved in the progressive assembly of AP-1-specific clathrin coats at the TGN (Wang et al. 2003; Santiago-Tirado and Bretscher 2011).

In the *Drosophila* germline, loss of AP-1, Rab11, PI4KIIα, or of exocyst components disrupts nurse cell plasma membrane integrity and causes a striking multinucleation phenotype (Fig. 4) (Murthy and Schwarz 2004; Murthy et al. 2005; Bogard et al. 2007; Tan et al. 2014; Loyer et al. 2015). It has been suggested that this common phenotype is caused by a defective intracellular trafficking of membrane components, but the exact underlying cellular defect is not known thus far. A detailed phenotypic analysis of AP-1 mutant germline, however, recently revealed that AP-1 regulates the trafficking of E-cadherin to the ring canals (Fig. 4). These stable intercellular bridges between nurse cells and the oocyte are composed of noncontractile actin bundles, which are anchored to the plasma membrane by adhesive E-cadherin clusters or semicircular adherens junctions (Fig. 4) (Loyer et al. 2015). Thus, the loss of nurse cell membranes in all these mutants is likely caused by the detachment of the plasma membrane from ring canals followed by fragmentation of the membrane (Fig. 4) (Loyer et al. 2015).

Ring canals are also formed at E-cadherin-positive boundaries in mammalian germ cell cysts, but their functions are not well understood (Pepling et al. 1999; Mork et al. 2012). Unlike in flies, where these intercellular bridges allow the transport of nutrients to the oocyte, in mammals the ring canals are thought to play a role in the synchronization of mitotic divisions and the entry into meiosis (Haglund et al. 2011).

A number of additional players have also been identified that control E-cadherin trafficking and cell adhesion, such as Scribble (Scrib), a conserved polarity protein and tumor suppressor that defines the basolateral domain in epithelial cells (Navarro et al. 2005; Qin et al. 2005; Dow et al. 2007). A more recent study suggests that Scrib may stabilize E-cadherin/p120 catenin binding and blocks retrieval of E-cadherin to the Golgi (Lohia et al. 2012). An additional function of Scrib in blocking retromer-mediated diversion of E-cadherin to the Golgi has been discussed (Lohia et al. 2012). A substantial body of evidence also indicates an important regulatory role of glycosyltransferases involved in the remodeling of N-glycans on E-cadherin, a process that dramatically affects E-cadherin stability and localization (Liwosz et al. 2006; Zhao et al. 2008; Zhou et al. 2008; Pinho et al. 2011). N-glycosylation of E-cadherin has also been shown to be an essential posttranslational modification in the lateral epidermis during *Drosophila* germband extension (Zhang et al. 2014). Mutations in the *xiantuan* gene (*xit*) encoding a conserved ER glycosyl-transferase affect glycosylation and the intracellular distribution of E-cadherin, but not the total amount of E-cadherin protein. The phenotypic analysis of *xit* mutants further suggests that N-glycosylation is important for the distribution and clustering of E-cadherin within the plasma membrane. A similar role for O-mannosylation in E-cadherin distribution was recently described in mouse embryos (Lommel et al. 2013; Vester-Christensen et al. 2013).

CONCLUSIONS AND PERSPECTIVES

Cell biological approaches in cultured epithelial cells together with in vivo studies in genetically tractable model organisms such as *Drosophila* and *C. elegans* have greatly advanced our understanding of the molecular regulation of AJ dynamics and homeostasis. Here, we highlighted conserved players and mechanisms that regulate E-cadherin endocytosis, sorting, exocytosis, and recycling. Recent quantitative proteomic approaches using the proximity biotinylation technique further suggest a remarkable molecular complexity and identified additional conserved regulatory hubs controlling E-cadherin-mediated cell adhesion (Guo et al. 2014; Van Itallie et al. 2014a,b). These approaches expand

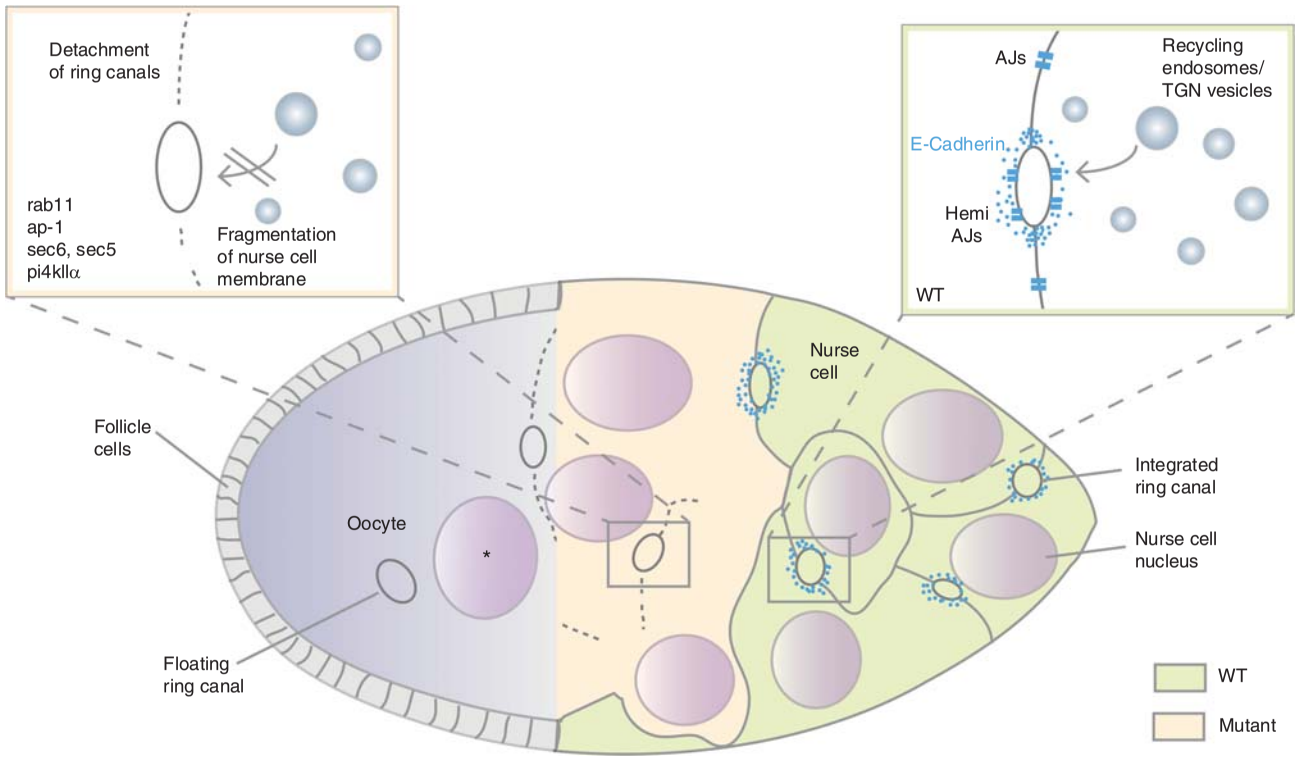

Figure 4. Polarized trafficking of *Drosophila* E-cadherin is required for the maintenance of ring canal anchoring to plasma membrane. *Drosophila* eggs arise from individual units called egg chambers consisting of two cell types, the germline cyst and the surrounding somatic monolayered follicle epithelium. The 16-cell germline cyst consists of one oocyte (blue) and 15 nurse cells (green and orange) that provide nutrients for oocyte maturation. Cytoplasmic bridges between nurse cells and the oocyte support the growth of the oocyte. These bridges are formed by F-actin-rich ring canals that allow the directed microtubule-mediated transport of all macromolecules and organelles from the nurse cells into the oocyte. E-cadherin clusters form circular "hemi-adherens junctions" around the ring canals mediating the anchoring of ring canals into the nurse cell membrane. During oocyte maturation, the volume of the oocyte increases by four orders of magnitude resulting in a dramatic increase of plasma membrane tension. A continuous secretory transport of E-cadherin is required to maintain ring canal anchoring to the plasma membrane in wild type (WT). In nurse cells mutant for Rab11, AP-1, Sec5, Sec6 or PI4KIIα (orange) the polarized trafficking of E-cadherin to ring canals is disrupted. Due to the increasing membrane tension ring canals detach from the membrane resulting in an immediate fragmentation of the membrane. As a consequence, floating ring canals and nurse cell nuclei in the oocyte (*) can be frequently observed in mutant egg chambers defective for E-cadherin trafficking.

the inventory of the E-cadherin interaction network of hundreds of uncharacterized proteins at the AJs. Numerous cytoskeletal proteins were found, in addition to many trafficking, signaling proteins, metabolic enzymes, and transcription factors. The candidates identified are unlikely to be unique to AJs and future studies need to address their functional and physiological relevance in E-cadherin-mediated cell adhesion. Subcellular localization data might be the first step to exclude potential false-positives of these initial screens. However, many of the most abundant candidates in the E-cadherin interactome, such as Filamin-A, Scrib, Discs large (Dlg), and Annexins, are multifunctional and/or are active in multiple subcellular compartments. A recent genome-wide RNAi screen for genes required for calcium- and cadherin-dependent cell–cell adhesion is a new step forward to identify conserved protein hubs that functionally complement the proximity biotinylation approaches mentioned above (Toret et al. 2014). In this study, the Vale and Nelson groups identified about 400 proteins that regulate the core E-cadherin/catenin adhesion complex using nonmotile and non-extracelluar matrix (ECM)-adherent *Drosophila* S2 cells in which all components required for integrin dependent-cell adhesion, cell spreading, and cell migration were eliminated. Selected candidates were further validated for defects in cell–cell adhesion in mammalian MDCK epithelial cells and in *Drosophila* for defects in E-cadherin-dependent oocyte positioning on germline-targeted RNAi. As might be expected, the largest group of proteins found in the E-cadherin interactome as well as in the RNAi screen are actin regulators including subunits of the WAVE regulatory complex (WRC), a major activator of the Arp2/3 complex that drives cell movements in most eukaryotes (Pollitt and Insall 2009). AJs are known to be tightly coupled to the actin cytoskeleton and this functional interplay is crucial for the junctional integrity, but also provides the mechanical forces coordinating individual cell-shape changes during tissue rearrangements in morphogenesis. However, the exact function of the Arp2/3 complex that is recruited to E-cadherin junctional complexes (Kovacs et al. 2002), in controlling E-cadherin dynamics in morphogenetic movements and epithelial remodeling, is still not well understood. Addressing how actin polymerization exactly acts on AJ integrity, internalization or intracellular trafficking of E-cadherin will be important for understanding tissue morphogenesis. Combining cell culture approaches with in vivo animal models has been applied very successfully. Advances in high-resolution light microscopy and in three-dimensional (3D) electron tomography will help us to better analyze the functional consequences of E-cadherin trafficking within a cell and within an animal. This will be not only important for a better understanding of E-cadherin biology, but also tissue remodeling in development and disease.

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