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Making and breaking contacts: the cellular biology of cadherin regulation

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Cadherin-mediated cell–cell interactions are dynamic processes, and cadherin function is tightly regulated in response to cellular context and signaling. Ultimately, cadherin regulation is likely to reflect the interplay between a range of fundamental cellular processes, including surface organization of receptors, cytoskeletal organization and cell trafficking, that are coordinated by signaling events. In this review we focus on recent advances in understanding how interplay with membrane trafficking and other cell–cell junctions can control cadherin function. The endocytosis of cadherins, and their post-internalization fate, influences surface expression and metabolic stability of these adhesion receptors. Similarly, at the surface, components of tight junctions provide a mode of cross-talk that regulates assembly of adherens junctions.

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Current Opinion in Cell Biology 2007, **19**:508–514

This review comes from a themed issue on
Cell to cell contact and extracellular matrix
Edited by Lawrence Shapiro and Barry Honig

0955-0674/$ – see front matter
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DOI 10.1016/j.ceb.2007.09.008

Introduction: cadherin regulation and tissue organization

Cadherin adhesion molecules, notably those of the classical cadherin subfamily, crucially determine tissue organization both in health and disease. Cadherin function is dynamic and regulated by developmental and cellular signals. Moreover, cadherins exert their functional impact at several different biological levels, ranging from conferring resistance to detachment, controlling the morphogenesis of contacts as cells integrate into populations and influencing tissue patterning and cohesion. Thus, it is perhaps not surprising that many signaling pathways and cellular processes affect cadherin biology. In this review, we will focus on recent developments in two areas that emphasize how interconnected mechanisms influence cadherin function: membrane trafficking and junctional cross-talk.

Cadherin trafficking and the metabolic fate of cadherins

It has long been recognized that membrane trafficking must link the biosynthesis of cadherins with their eventual degradation in lysosomes. Like other integral membrane proteins, newly synthesized cadherins are packaged in the Golgi, transported to the cell surface and eventually internalized and degraded [1,2]. But in-between is a complex network of trafficking pathways that provide alternative routes for the endocytosis and subsequent fate of internalized cadherin (Figure 1).

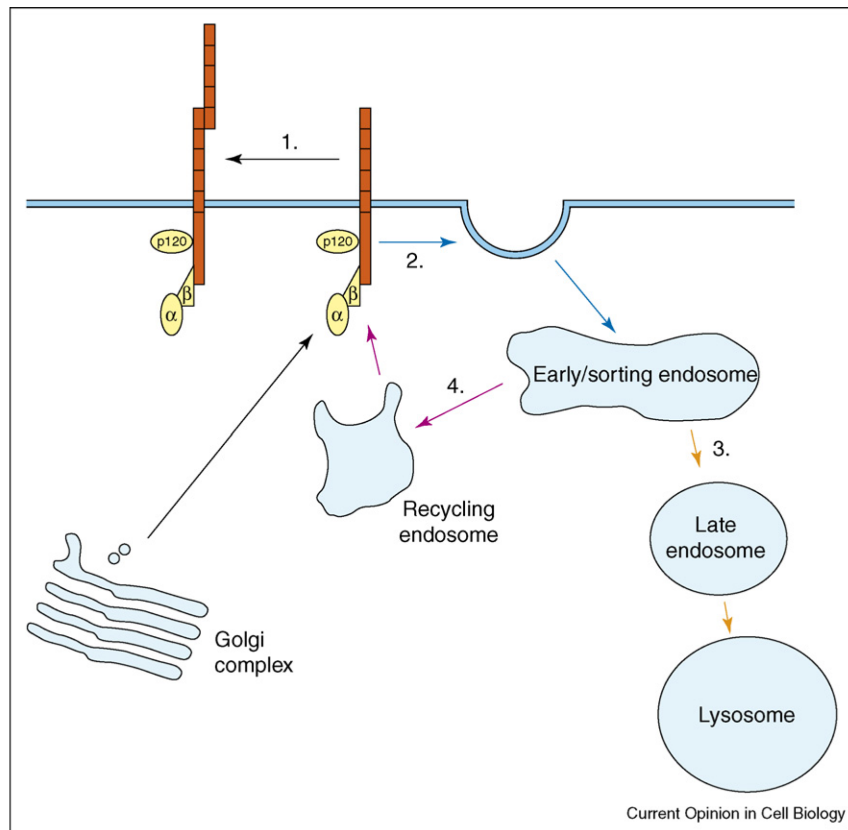
Regulating internalization: the yin and yang of adhesion and endocytosis

Constitutive endocytosis of a number of classical cadherins has been observed in cells that display apparently stable cell–cell contacts [3,4]. Basal levels of cadherin internalization would be expected to support their metabolic turnover and perhaps contribute to local remodeling of contacts. Endocytosis can also be substantially altered in response to external stimuli. For example, E-cadherin internalization is increased when subconfluent epithelial cultures are treated with EGF [5] while VEGF induces the internalization of VE-cadherin [6••]. Conversely, uptake of N-cadherin in cultured hippocampal neurons was decreased upon stimulation of NMDA receptors [7•]. This stabilized N-cadherin in synapses with the potential to affect synaptic plasticity. Thus, cadherin endocytosis is itself a dynamic process that can be physiologically, and perhaps pathologically, altered depending on cellular context.

Moreover, cadherin internalization may occur through different pathways. Constitutive endocytosis of E-cadherin and VE-cadherin appears to occur via a clathrin-mediated pathway [3,8•,9], but a number of clathrin-independent pathways for cadherin endocytosis have also been identified [5,10,11]. Notably, macropinocytosis is implicated in EGF-stimulated internalization of E-cadherin in subconfluent epithelial cells [5]. Different pathways may then dominate in different cellular contexts; it is further possible that cadherin endocytosis may be regulated by activating different uptake mechanisms. The extent to which this occurs remains to be defined. Even so, it is clear that cadherin internalization can be influenced by negative and positive mechanisms.

Perhaps the best characterized inhibitor of cadherin endocytosis is its associated protein, p120-ctn. One major mechanism for p120-ctn to support cadherin adhesion is by inhibiting clathrin-mediated endocytosis [8•,12],

Figure 1



The trafficking itineraries of classical cadherins. Newly synthesized cadherins are trafficked from the Golgi to the plasma membrane. Upon delivery to the surface, cadherins may engage in productive adhesive interactions **(1)** or be endocytosed **(2)**. Following internalization, cadherins are transported to early or sorting endosomes. Two alternative routes are available from this compartment: recycling back to the cell surface **(3)**, a path may commonly involve an intermediate step in the recycling endosomal compartment or transport through late endosomes for degradation in the lysosome **(4)**. Which of these alternatives is taken can influence surface expression and metabolic stability of the cadherin.

thereby stabilizing cadherins at the cell surface [13,14]. Additionally, Rac activity and actin cytoskeletal reorganization through the actin cross-linker, IQGAP, inhibited internalization in a cell-free model of E-cadherin endocytosis [9]. How these molecules block cadherin endocytosis, and whether they are mechanistically linked, remains unknown. One possibility is that dynamic actin reorganization is necessary to facilitate clathrin-mediated endocytosis [15], consistent with the observation that actin-disrupting agents promoted E-cadherin endocytosis [9]. Interestingly, p120-ctn can regulate Rac signaling with the potential to signal to the actin cytoskeleton [16].

Conversely, endocytosis is a complex, multi-step process that involves discrete events such as cargo selection, budding and fission of vesicles that are subject to tight regulation [17]. A key step is the recognition of target proteins by the endocytic machinery. The cytoplasmic tails of several classical cadherins bear a number of dileucine and tyrosine-based motifs that are potentially recognized by adaptor proteins of the clathrin-based endocytic apparatus [1,2]. Agents that inhibit cadherin endocytosis, such as p120-ctn [2,12], might then work by preventing these motifs being recognized and bound by the endocytic machinery. Alternatively, signals that can downregulate cadherin function might stimulate cadherin internalization by inducing pro-endocytic modifications to the cytoplasmic tail. A striking example occurs in endothelia, where VEGF activates a signaling cascade involving Rac and PAK that leads to the phosphorylation of a conserved serine motif in the cytoplasmic tail of VE-cadherin [6••]. This then recruits β-arrestin leading to the clathrin-dependent uptake of the cadherin. Monoubiquitylation can also promote endocytosis [18], and the E3 ligase, Hakai, binds E-cadherin in a tyrosine kinase-dependent fashion [19], providing an alternative mechanism to target cadherins for internalization. At this point it is noteworthy that Hakai did not bind N-cadherin; this emphasizes that while much work has been done with E-cadherin, the trafficking of other cadherins may have significant differences. Indeed, the serine residue necessary for β-arrestin-mediated internalization

of VE-cadherin is not found in either E-cadherin or N-cadherin [6••].

Net cadherin internalization is thus likely to reflect the balance between signals that promote endocytosis and those that inhibit it. This then raises the question whether there are dominant factors that control its internalization. Indeed, cadherin adhesion itself appears to be one such factor. This was first suggested by the observation that cadherin internalization increased when cell–cell contacts were broken by chelating extracellular calcium [3,20]. Similarly, EGF induced internalization of E-cadherin by macropinocytosis in subconfluent, but not confluent, cultures [5]. Consistent with this, Izumi *et al.* reported that it was the free cadherin, rather than the cadherins engaged in adhesion, that underwent endocytosis in cell-free assays. Moreover, homophilic ligation of cellular cadherins with recombinant ligands inhibited internalization in their experiments [9]. Together, these observations suggest that productive adhesive ligation of cadherins prevents their endocytosis, perhaps by activating Rac signaling and remodeling the actin cytoskeleton. Adhesion and endocytosis would then be closely opposed processes: Endocytosis would tend to decrease cadherin function by removing free cadherins from cell surfaces, making them unavailable to engage in adhesive interactions. Productive cadherin ligation, on the contrary, would stabilize the cadherin by preventing endocytosis, thereby prolonging its residence time on the cell surface.

Alternatively, Troyanovsky *et al.* recently proposed that endocytosis actively inhibits the cadherin adhesive machinery. The key observations were that manoeuvres, such as hypertonic stress, that inhibit cadherin internalization prevented the disassembly of cadherin *trans*-dimers that occurred upon chelation of extracellular calcium [21•]. Such *trans*-dimers are thought to reflect the adhesive interactions of cadherins on neighbouring cells, implying that endocytosis targets the adhesive machinery itself. This contrasts with the first model, where endocytosis would target the unliganded cadherin. It should be noted, however, that many non-specific manoeuvres that inhibit endocytosis can influence cadherin function by alternative mechanisms. For example, hypertonic stress induces tyrosine phosphorylation of cortactin [22], which contributes to cadherin junction integrity [23,24] (Helwani *et al.*, unpublished). These disparate models thus remain to be resolved and warrant further investigation. Nonetheless, they both emphasize the close functional and mechanistic relationship between cadherin adhesion and endocytosis.

Controlling the fate of cadherins after internalization

Once internalized, cadherins may either be recycled back to the cell surface or trafficked to late endosomes and lysosomes for degradation [1]. Traffic along these disparate pathways is also subject to cellular regulation [17]. Traffic to lysosomes entails passage through Rab5-enriched and Rab7-enriched compartments, and dominant-negative mutants of either of these GTPases block transport of E-cadherin to lysosomes [25•]. By contrast, membrane proteins can be recycled to the plasma membrane by two routes: directly from sorting endosomes shortly after internalization or after transport to the recycling endosome, a longer lived compartment that is characteristically enriched in the Rab11 GTPase [26,27•]. Rab11 and the exocyst complex appear to participate in a pathway for cadherin recycling. Disruption of the sec5 subunit of the exocyst caused Drosophila E-cadherin to accumulate in Rab11-enriched endosomes [28•], while Rab11 interacts with components of the exocyst providing a potential molecular basis for this functional relationship [28•,29•]. Thus, transport through recycling endosomes appears to constitute one, but not necessarily the sole, pathway for cadherin recycling. Subsequent transport to the cell surface is likely to involve microtubule-based transport [30] and phosphoinositide signaling [31]. Interestingly, in mammalian and Drosophila epithelial cells, the recycling pathway may be important for targeted delivery of E-cadherin to the cell surface. Expression of Rab11 mutants caused the apical mis-expression of E-cadherin in MDCK cells [27•] while disruption of the sec5 subunit of the exocyst in flies prevented transcytosis of DE-cadherin to adherens junctions in the dorsal thorax of the pupae [28•].

Faced with such disparate alternative pathways, what determines which route is taken after cadherins are internalized (Figure 1)? One possibility is that different uptake pathways lead to different fates. For example, uptake of EGF receptor by clathrin-mediated endocytosis may principally mediate recycling, while clathrin-independent uptake may serve as the major pathway for its degradation [32]. However, E-cadherin that was internalized by EGF-induced macropinocytosis appeared to be recycled to the cell surface [5]. Moreover, cadherins that are internalized by either clathrin-mediated or clathrin-independent entry mechanisms often appear in early endosomes that stain for EEA-1 [4,11]. This suggests that cadherins may enter a common compartment shortly after endocytosis, implying that the choice of fates is decided at, or after, exit from this early endosomal compartment.

Recent analysis of how Src signaling affects E-cadherin transport may be informative in analysing how such decisions may be made. Palacios *et al.* demonstrated that activation of signaling by a temperature-sensitive (ts) Src mutant downregulated E-cadherin expression in MDCK epithelial cells [25•]. This involved shuttling of internalized cadherin to lysosomes for degradation. Thus, one mechanism for mutant Src to inhibit E-cadherin would be to promote its traffic for degradation, presumably at the expense of recycling to the cell surface. Interestingly, ts-Src affected multiple steps along the pathway leading

to lysosomal degradation, including promoting GTP loading of Rab5 and Rab7 [25•]. As well, the authors reported that mutation of a predicted ubiquitylation site in the cadherin cytoplasmic domain prevented cadherin degradation without affecting its initial endocytosis. This suggests that for E-cadherin, like other membrane proteins [18], ubiquitylation may be an important signal for transport to lysosomes.

These observations thus carry two implications. Firstly, they suggest that there may be overarching cell signals that determine the post-endocytic fate of cadherins to define the choice between recycling and lysosomal shuttling. These will ultimately influence the metabolic half-life of the cadherin and its steady-state levels in the cell, independent of any changes in gene expression or protein synthesis. On the basis of the example of other membrane proteins, the decision to follow a path to lysosomal degradation may be key, as recycling is often a default pathway [17]. Secondly, such overarching signals may act by targeting multiple regulatory points found along the trafficking pathway of the cadherin, rather than necessarily at a single decision point, thereby biasing traffic along a pathway.

Taken together, cadherin trafficking – notably regulation of its post-endocytic fate – provides a very attractive mechanism to influence cadherin function. Indeed, it has been invoked in a number of morphogenetic processes where dynamic remodeling of cell–cell interactions is thought to happen [33•,34•]. Several limits to our current knowledge need to be noted, however. Firstly, it is difficult to specifically regulate cadherin trafficking. Our armamentarium to date has been limited to global manipulation of molecules such as Rab5 and sec5, whose activity is not specific for cadherin trafficking. Thus, the functional significance of altered cadherin traffic seen in these circumstances is formally correlative, rather than causal. Ongoing efforts to define the molecular mechanisms of cadherin traffic will be essential for progress in this area.

Secondly, we do not yet know how extensive is the contribution of regulated trafficking to dynamic control of cadherin function. Cadherin trafficking has the clear potential to affect both the surface expression and metabolic stability of cadherins. However, in vertebrate cells, cadherin adhesion can be significantly reduced without demonstrable changes in either the total or surface levels of cadherin, as occurs when either Myosin II or microtubule dynamics are acutely perturbed [35,36]. Changes in cadherin trafficking are unlikely to be responsible for the altered cadherin function seen under these circumstances. A key parameter may be the biological time scale. Functionally significant alterations in cadherin trafficking have been most commonly observed associated with changes in cell state (such as induction of epithelial-to-mesenchymal transformation by expression of mutant Src) or during morphogenesis in fast-developing organisms, such as Drosophila. It is possible that altered cadherin trafficking principally regulates cadherin function on relatively longer time scales, whereas rapid regulation occurs by other mechanisms, such as lateral clustering or cytoskeletal regulation.

Finally, although we have focused on the trafficking of the cadherin adhesion molecule itself, it is also quite plausible that membrane trafficking mediates the local delivery and turnover of other molecules necessary for cadherin biology. E-cadherin can bind elements of the exocyst complex [28•], providing the capacity for it to mark sites for vesicle docking and delivery [29•,37]. Exocytosis can deliver molecules such as Rho GTPases to the cell surface [32], with the potential to regulate cell signaling and cytoskeletal organization at cadherin adhesions. It is therefore possible that, rather than solely controlling cadherin transport and expression, membrane traffic could also affect cadherin function through the accessory proteins that functionally couple signaling and cytoskeletal activity to cadherin adhesion.

Tight junctions and cadherin regulation: cross-talk at the surface

Just as there is cross-talk between trafficking and cadherin function, similar functional interplay occurs between junctions at the cell surface. It has long been thought that cadherin function contributes to the assembly of other specialized cell–cell junctions, notably elements of the apical junctional complex in epithelia (tight junctions, gap junctions, desmosomes) [38]. But, as well, there is increasing evidence to suggest that tight junction (TJ) components influence cadherin-based adherens junctions (AJ).

This is exemplified by the ZO (Zonula occludens) family of MAGUK scaffolds, peripheral membrane proteins that were amongst the first components of the TJ to be identified [39]. That these are necessary for TJ assembly was recently established by Tsukita and co-workers who derived epithelial cells lacking all members of this family (ZO1-3) in which TJs are totally absent [40••]. Surprisingly, although cadherin contacts appeared intact in fully established monolayers of ZO-deficient cells [40••], cells also showed a defect in the efficiency with which they formed AJ [41•]. In a Ca^{2+} switch assay, these cells show a delay in the transition from spot-like AJs to more belt-like structures with associated linear actin cables. Initial accumulation of spots occurred with normal kinetics, along with the recruitment of afadin and Par-3/Par-6/Tiam-1, suggesting that the defects are specific to junctional maturation. That this requirement for ZO proteins may be specific to AJ-related functions was bolstered by structure–function studies, which suggested that a distinct region of ZO-1 is required for spot-to-belt matu-

ration as opposed to claudin polymerization [41•]. This suggested a role for ZO proteins in the dynamic process of AJ maturation.

A similar theme emerges from recent work on tracheal development in Drosophila, whose genome is predicted to contain one orthologue of ZO-1, *polychaetoid/pyd* [42]. Interestingly, Pyd seems to be important in determining the fate of tracheal cells that normally undergo branch fusion [43•], a role consistent with studies in vertebrates, where ZO-1 appears to sequester the proliferation-promoting transcription factor, ZONAB (reviewed in reference [44]). In addition, however, *pyd* mutants show defective rearrangement of tracheal cells [43•]. Dynamic analysis with a Pyd::GFP construct reveals that it localizes to AJs. Taken together, these data suggest that Pyd regulates remodeling of AJs during tracheal morphogenesis.

How, then, might TJ components, and perhaps TJs themselves, affect cadherin function and AJ assembly? Although it is still early, two possibilities already present themselves. Firstly, such cross-talk may represent interactions between cadherin complexes and TJ components in early cell–cell adhesions, before definitive junctions have formed. ZO-1 can bind α-catenin and has often been seen to co-localize with E-cadherin in early spot-like cell–cell contacts. Only later, as TJs form, does ZO-1 shift apically [45]. The ZO-1-binding region of α-catenin appears to be important for strong cadherin adhesion [46]. Moreover, ZO-1 can bind actin filaments [47] and could possibly serve as a cadherin-associated protein to link nascent adhesions to the actin cytoskeleton. Along similar lines, MAGI (*MAG*UK with *i*nverted domain structure)-1, is also found both in nascent adhesions and in TJs. Recently, Sakurai *et al.* [48•], reported that MAGI-1 accumulates at VE-cadherin contacts, coinciding with activation of the small GTPase Rap1, which can regulate cadherin adhesions [49]. Of note, MAGI-1 can bind the Rap1 activator, PDZ-GEF, and also associate with the actin-binding proteins, synaptopodin and α-actinin-4, providing other ways of linking to the cytoskeleton.

Alternatively, definitive TJ components may affect the biogenesis or maintenance of cadherin contacts themselves. Here it is noteworthy that the polarity determinants, *Drosophila* Stardust (Std) and its mammalian orthologue, PALS1/MPP5, are found in septate junctions and TJs, respectively [50]. These are best understood for their roles in determining apical polarity, but it has been known for many years that *Std* mutants also have defects in AJs [51,52]. Very recent work has uncovered a similar role for MPP5/PALS1 in zebrafish cardiac development [53]; loss of MPP5 function perturbs the cohesion of cardiac precursors, again suggesting that MPP5 somehow affects adhesion. How might a complex that does not physically colocalize with AJs in mature epithelia regulate AJ formation? Recent work from the Margolis laboratory using a strong PALS1 knockdown (KD) cell line suggests one clue [54•]. AJs, as well as TJs, are perturbed in PALS1(KD) cells and AJ components accumulate in cadherin-positive vesicles that appear to fail to undergo exocytic delivery to the surface. Moreover, exocyst components are also mislocalized in the KD cells. Thus, depletion of this TJ component appears to affect E-cadherin exocytosis. An interesting speculation is that MPP5/PALS1 spatially regulates the exocyst, perhaps forming with Stardust/Crumbs/PATJ part of a 'nascent polarizing' apparatus upstream of the exocyst that is required when cells first make cell–cell contacts. Dynamic analysis of the Crumbs complex vis-à-vis TJ and AJ components in nascent epithelia would help to resolve this question.

Conclusions

In summary, we sought in this review to highlight the ways in which cadherin function is determined by, and regulated through, the interplay between the adhesion receptor and the cellular systems of membrane traffic and tight junctions. Of course, these are not the only processes that influence cadherin biology. We have elided issues such as surface clustering of receptors, cytoskeletal activity and cell signaling in our discussion. Here, it may be useful to emphasize that cadherin function encompasses biophysical, cellular and tissue levels. Nor need these be commensurate; for example, inhibiting Arp2/3-dependent actin nucleation perturbs the morphogenesis of cadherin contacts without significantly affecting surface adhesion [55]. Thus, different cellular processes may regulate distinct functional parameters as cells use cadherins to interact with one another. Ultimately, a comprehensive understanding of cadherin regulation will need to encompass this diversity, both of effector mechanisms and biological outcomes.

Acknowledgements

We thank all the members of our laboratories for their support, advice and good cheer. We especially thank Tina Tuskey for helpful discussions regarding ZO proteins and Robert Parton and Jenny Stow for trying to teach us about membrane traffic. JH is supported by NIH grant GM58038; ASY and MSC are funded by the NHMRC (Australia).

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