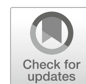

Structural-Mechanical and Biochemical Functions of Classical Cadherins at Cellular Junctions: A Review and Some Hypotheses

9

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Abstract

This article begins with a general review of cell adhesion molecules (CAMs) and narrows the focus down progressively to the cadherins (calcium binding-dependent CAMs), classifications of subfamilies of the cadherins, type I (E- and N-) cadherins, evolutionary relationships amongst cadherins, structural-mechanical and functional consequences of calcium binding to the cadherins, differential molecular interactions involving the extracellular (ecto) and intracellular (cytoplasmic) domains of the cadherins, multiple adherence-related homophilic and heterophilic interactions and associated functions of E- and N-cadherin in organismal development and disease and cadherin trafficking and membrane rafts. It ends by summarizing multiple perspectives and hypotheses concerning different aspects of cadherin structure, stability and function.

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Keywords

Cell adhesion · Epithelial cadherins · Neuronal cadherins · Cadherin interactions · Cadherin trafficking · Calcium binding · Cancer · Metastasis · Development

9.1 Cell Adhesion Molecules (CAMs)

A fundamental determinant of the formation, and maintenance, of diverse three-dimensional assemblies of cells within biological tissues or organs, is the mechanism by which any cell manages to adhere to its neighbouring cells. The surfaces of cells in multicellular organisms display a variety of cell adhesion molecules (CAMs) that work to keep the trillions of cells in the organism tied together into a composite and singular 'whole', mediating and regulating cell-cell interactions.

CAMs bind either to the molecular components of the extracellular matrix within which a cell happens to be embedded or to other CAMs displayed on the surfaces of neighbouring (or juxtaposed) cells. CAMs thus play very important roles in determining cell shape and integrity. Notably, CAMs also play very important roles in cell-cell and environment-cell signalling by linking up with proteins like the catenins, which are associated with the actin-based cellular cytoskeleton. Consequently, CAMs also play

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roles in cellular homeostasis and tissue morphogenesis.

CAMs can be broadly classified into (i) calcium binding-dependent and (ii) calcium binding-independent CAMs. Molecules such as the selectins or the cadherins are examples of calcium binding-dependent CAMs, whereas molecules such as the integrins or the immunoglobulins (including various members of the larger immunoglobulin superfamily) are examples of calcium binding-independent CAMs.

Amongst the calcium binding-independent CAMs, the integrins are CAMs that interact either with components of the extracellular matrix, such as fibronectin, collagen or fibrinogen, or with CAMs on the surfaces of other cells. The other calcium-independent CAM mentioned above, i.e. the immunoglobulin superfamily, is a group of cell surface glycoproteins consisting of immunoglobulin (Ig)-like structural domains consisting of 70–110 amino acids each. These Ig-like domains exhibit both heterophilic and homophilic interactions. To take some examples, ICAMs, or intercellular cell adhesion molecules, engage in heterophilic interactions and bind to other CAMs such as integrins, whereas N-CAMs, or neural cell adhesion molecules, engage in homophilic interactions and participate in cell-cell adhesions through interaction with other N-CAMs (Cruse et al. 2004).

Amongst the calcium binding-dependent CAMs, the selectins are a group of cell surface glycoproteins which bind to fucosylated carbohydrates. Different types of selectins turn out to be expressed on different types of cells, e.g. P-selectin is expressed on the surfaces of platelets and leukocytes; L-selectin on the surfaces of leukocytes, monocytes, neutrophils and eosinophils; and E-selectin on the surfaces of endothelial cells. The functions of the selectins vary considerably. For example, they range from leukocyte trafficking to signal transduction (Lodish et al. 2000).

Like the selectins, the molecules which are the focus of this review, i.e. the cadherins, constitute a distinct group of calcium binding-dependent CAMs. The cadherin superfamily is a multigene family of proteins with diverse structures and functions. Cadherins tend to localize at ‘adhesion junctions’ which are the dominant structural features visible at cell-cell adhesion interfaces.

9.2 The Cadherins: Calcium Binding-Dependent CAMs

The cadherins mediate cell adhesion in a calcium binding-dependent manner. All cadherins are multidomain proteins. The extracellular domains of the cadherins are a series of domain repeats, displaying considerable mutual sequence as well as structural similarities, suggestive of an origin based on gene duplication. Three calcium ions bind to each of the linker peptides separating any two extracellular cadherin domains. The length of each extracellular (or ecto) domain of a cadherin is about 110 amino acids. Every molecule of a cadherin is a membrane-displayed protein consisting of a cytoplasmic domain, a single-pass transmembrane region and multiple extracellular ‘repeat’ domains known as ‘ectodomains’. In mammalian cadherins, the number of the extracellular repeat domains available for adhesive interactions usually varies from 2 to 34 contiguous domains. The classical cadherins and protocadherins, however, have only about five to seven ectodomains (Suzuki and Hirano 2016). Each cadherin ectodomain has a fold similar to that of the Ig or Ig-like domains, i.e. the typical cadherin ectodomain contains seven β-strands arranged into two β-sheets that associate into a beta-sandwich fold, exactly as is seen in any Ig or Ig-like domain, however with distinct topological differences, i.e. with different contact schemes of the constituent β-strands from that seen in the Ig-like domains.

Unlike the ectodomains, the cytoplasmic domains of the cadherins have only a few conserved motifs, and these are seen amongst specific subfamilies of cadherins involved in binding or interaction(s) with cytoplasmic proteins. For example, β-catenin and catenin p120 (p120ctn) bind to two catenin-binding motifs in the cytoplasmic domains of the classical cadherins. Since these cytoplasmic domains are exceptionally diverse, they form the basis of the

categorization of the cadherin superfamily into various subfamilies (Suzuki and Hirano 2016).

9.3 Different Subfamilies of Cadherins

The cadherin superfamily is divided into four major subfamilies. These are (a) classical cadherins, (b) protocadherins, (c) desmosomal cadherins and (d) atypical cadherins (Priest et al. 2017). Below, we provide a brief description of each of these subfamilies, before we bring this review's focus primarily on to the classical cadherins.

(a) *Classical cadherins.* The classical cadherins constitute a major subgroup of the cadherin superfamily. In most higher organisms, the classical cadherins are characterized by the presence of a five domains-long extracellular region, a transmembrane region and a cytoplasmic region which consists of a folded domain that interacts directly with p120 and β-catenin and indirectly with α-catenin, to link up to actin filaments through protein-protein associations. The classical cadherins are further subclassified primarily into types, I, II, III and IV. Type I and type II classical cadherins are present only in vertebrates. They are classified on the basis of the tissue within which they were first identified. In vertebrates, there are 6 type I cadherins and 13 type II classical cadherins. Type III classical cadherins are found both in vertebrates and invertebrates, but not in mammals. Below, we provide a brief overview of these three types of classical cadherins. Type I classical cadherins consist of proteins like epithelial cadherin or E-cadherin which is known as CDH1 and neuronal (or neural) cadherin, known as N-cadherin, or CDH2. The type I classical cadherins display a conserved HAV tripeptide motif and a conserved tryptophan at the second position in the most distal domain, EC1, located farthest from the plasma membrane (Shapiro et al. 1995). The type I cadherins have five main members, which include CDH1 (E-cadherin, epithelial), CDH2 (N-cadherin, neuronal), CDH3 (P-cadherin, placental), CDH4 (R-cadherin, retinal) and CDH15 (M-cadherin, myotubule) (Suzuki and Hirano 2016). Type II classical cadherins consist of proteins like vascular endothelial (VE)-cadherin which is known as CDH5 and kidney (K)-cadherin which is known as CDH6 (Gumbiner 2005; Leckband and Prakasam 2006). Type II classical cadherins are characterized by the presence of two conserved tryptophan residues at the second and fourth positions (Trp2 and Trp4) in the most distal domain, EC1, but they lack the HAV tripeptide which is present in the type I cadherins (Shapiro and Weis 2009). There are currently 13 named type II cadherins, these being CDH5 (VE-cadherin, vascular endothelium), CDH6 (K-cadherin, foetal kidney), CDH7, CDH8, CDH9 (T1-cadherin, testis), CDH10 (T2-cadherin, testis), CDH11 (OB-cadherin, osteoblast), CDH12, CDH18, CDH19, CDH20, CDH22 and CDH24. The type III classical cadherins possess a variable number of ectodomain repeats (Oda et al. 2002; Tanabe et al. 2004). They also possess a conserved region called the primitive classical cadherin domain (PCCD) which lies between the cadherin repeats and the transmembrane helix. For the maturation of E-cadherin in *Drosophila*, proteolytic cleavage of PCCD is required (Oda and Tsukita 1999), indicating that type III and type I cadherins interact. Type IV cadherins have seven ectodomains.

(b) *Protocadherins.* More than 80 members of the cadherin superfamily together constitute another group of cadherins known as protocadherins. These are mainly expressed in the developing and mature vertebrate nervous system, although low levels of expression are also seen in the lungs and the kidney. The protocadherins possess six or

seven ectodomain repeats. These are highly conserved amongst the protocadherin subgroup but show low sequence homology with other members of the cadherin superfamily (Hulpiau and Van Roy 2009). As with other cadherins, in addition to ectodomains, the protocadherins have a single-pass transmembrane domain and a very distinct and specific cytoplasmic domain which, however, lacks motifs for catenin binding (Sano et al. 1993; Wu and Maniatis 1999; Nollet et al. 2000; Vanhalst et al. 2005). Based on their genomic organization, protocadherins are further classified into clustered and non-clustered protocadherins.

(c) *Desmosomal cadherins.* Like the classical cadherins, desmosomal cadherins also possess a highly conserved extracellular region consisting of five repeat domains (Boggon et al. 2002; Delva et al. 2009; Shapiro and Weis 2009). The cytoplasmic domain in these cadherins interacts with the β-catenin-related protein, armadillo, with plakoglobin and also with the plakophilins which are associated with the intermediate filaments (Hatzfeld 2007; Carnahan et al. 2010; Al-Amoudi et al. 2011). Desmosomal cadherins known as the desmogleins and desmocollins are highly expressed in epithelial tissues and cardiac muscle (Nollet et al. 2000; Green and Simpson 2007; Hulpiau and Van Roy 2009). Desmosomal cadherins exhibit both homophilic and heterophilic interactions (Green and Simpson 2007; Thomason et al. 2010). Desmosomal adhesion is crucial for the stability of adhesion junctions in epithelial cell sheets and in the regulation of epidermal differentiation (Garrod et al. 2002).

(d) *Atypical cadherins.* The main atypical cadherins are Dachsous (Ds), Fat, and Flamingo (Fmi). These are required for planar cell polarity signalling (Halbleib and Nelson 2006). Unlike classical and desmosomal cadherins, each of which have five extracellular or ecto (EC) domains, Ds and Fat are characterized by the presence of 27 and 34 extracellular repeat domains, respectively. The cytoplasmic domains of Ds and Fat show sequence homology with the catenin-binding motifs present in classical cadherins (Mahoney et al. 1991; Clark et al. 1995). In mammals, Fat1 interacts with Ena/VASP, a family of proteins involved in regulation of actin cytoskeleton assembly and dynamics (Moeller et al. 2004; Tanoue and Takeichi 2004). Fmi-1 is quite unique as it contains a seven-pass transmembrane region and nine extracellular domain repeats (Nakayama et al. 1998), causing it to be one of the most atypical cadherins yet known. Another much-discussed atypical cadherin is cadherin-23, or CDH23, which has 27 extracellular repeat domains and plays a role in hearing involving stereocilia (Siemens et al. 2004).

9.4 Evolutionary Relationships Amongst the Cadherins and Their Ectodomains

Several classifications have been made in respect of the cadherins. One of the earliest classifies them broadly into classical cadherins, protocadherins, desmosomal cadherins and cadherin-related genes or atypical cadherins (Suzuki 1996), even as summarized in the immediate previous section. In embryos undergoing development, the role of the classical cadherins is particularly important. Their evolutionary origins and connections with other cadherins have, therefore, been of some interest.

Invertebrate Cadherins DNA sequencing techniques and sequence comparisons have helped to trace the origins of the cadherins. In organisms like *Branchiostoma floridae* (lancelet), *Nematostella vectensis* (sea anemone) and *Trichoplax adhaerens* (primitive placozoan) which occupy key positions in studies of metazoan evolution, sequencing reveals the presence

of 30, 16 and 8 cadherin genes (or cadherin-like genes), respectively, in their genomes. The genome of the sea urchin, *Strongylocentrotus purpuratus*, contains 14 cadherin-like genes. The worm, *Caenorhabditis elegans*, has 12, and the fly, *Drosophila melanogaster*, has 17. In the closest known relative of the metazoans, *Monosiga brevicollis* (a unicellular non-metazoan choanoflagellate), 23 putative cadherin-like genes have been identified (Murray and Zaidel-Bar 2014). Members of cadherin families, lefftyrin, coherin and hedgling, were present in the last common ancestor of choanoflagellates and metazoans. Mainly present in choanoflagellates and sponges, these may have evolved by domain shuffling and lateral gene transfer. These genes are speculated to have adhesive functions in these organisms (Nichols et al. 2012). Cadherins containing extracellular domain repeats linked to Src homology 2 (SH2), Hedgehog N-terminal peptide (N-hh), immunoglobulin (Ig) and von Willebrand type A domains are seen in *M. Brevicollis* and *Amphimedon queenslandica*. Cadherins which are now classified as Fat cadherins are also observed in sponges and sea urchins. The conserved cadherin cytoplasmic domain containing β-catenin binding sites is also observed in *N. Vectensis* (Abedin and King 2008). The function of cadherins in these unicellular organisms is largely unknown, but they are found to be localized in the apical, collar and basal pole of these cells. They play a role in cell shape and polarity and facilitate intracellular processes by taking cues from extracellular environment.

Human Cadherins The human genome encodes 114 cadherins. Although many arise through alternative splicing of mRNA, the presence of such a sizeable repertoire of genes has caused them to be classified as 'cadherin main branch' and 'cadherin-related major branch'. The cadherin-related major branch mainly consists of protocadherins, whereas the cadherin main branch consists of classical (type I) cadherins and atypical (type II) cadherins. Evolution of such a large superfamily of proteins appears to have mainly resulted from whole-genome duplications, individual gene duplications and diversification of duplicated genes. Type I cadherins consist of CDH1/E-cadherin/epithelial cadherin, CDH2/N-cadherin/neuronal cadherins, CDH3/P-cadherin/placental cadherins, CDH4/R-cadherin/retinal cadherin and CDH15/M-cadherin/myotubule cadherins. Each of these consists of the same number (five) of extracellular domains and a highly conserved tryptophan at position 2 (used for adhesion), and cytoplasmic domains are used for association with other proteins of the armadillo family and β-catenin (Hulpiau and Van Roy 2010).

Origins of the Five Ectodomains Bioinformatics-based analyses of DNA and protein sequences from divergent organisms reveal that an ancestral five repeat cadherin gene arose before divergence into paralogs. Repeated duplication of the extracellular domains appears to have led to the formation of a classical cadherin prototype in which introns were inserted because the introns in all cadherin genes are present in exactly the same locations. After divergence of this basic linear gene structure of the cadherin gene into different organisms, mutations could have occurred at fixed rates. Of the five classical cadherins, N-cadherins show the least rate of change because of a selection pressure placed on it due to its presence in the nervous systems. The existence of duplicates of gene paralogs could generate a wider scope for intragenomic recombination. It could also lower selection pressure on copies due to greater redundancy. The somatic morphology of organisms changes dramatically in vertebrates. So, the E-cadherins are placed under much less selection pressure and appear to have evolved faster than N-cadherins (Gallin 1998).

The domains I and II (reckoning from the N-terminal) of E-cadherins only share 25% sequence identity with each other, whereas domains III, IV and V show no significant similarity in their sequences. Conservation is observed at different residue positions amongst

different domains. Position A5 is occupied by Glu in all sequences of domains I, II and III, while this position is shared by Gln and Asp in case of domains IV and V. A1 position in EC I of all cadherins is shared by hydrophobic residues, and EC II shares Gly and Ala residues. The position D1 is also conserved for hydrophobic residues for domain I and conserved for hydrophobic and aromatic position for domains II and IV. Domain V is the least conserved domain amongst classical cadherins. The gaps (deletions and insertions) in sequences of ectodomains are almost always found at the borders of strands or helices (Kister et al. 2001). Various studies have suggested that the five and seven extracellular domains of type I and type IV cadherins have independently evolved from a common ancestral cadherin that is represented by type III cadherins. EC1 of type I cadherin and the EC6 of type IV cadherin appear to have evolved from the same extracellular cadherin domain in the common precursor (Nishiguchi et al. 2016).

Relationships with Other Cadherins Phylogenetic studies reveal that the different cadherins, namely, N-, E-, R- and P-, group together more closely than do cadherins from a single species. This indicates that they are paralogs which originated before the divergence of mammals, birds and amphibians. Studies suggest that E- and P-cadherins belong to one paralog group, while N- and R-cadherins belong to another group. In the E-/P-cadherin group, domains I and II are most closely related and so are domains III and IV. In the N−/R-cadherin paralog group, domains I and V are most closely related and so are domains II and IV (Gallin 1998). A total of 72 amino acids at the C-terminal end of the cytoplasmic domains of E-, P- and N-cadherins are highly conserved (Niessen and Gumbiner 1998). Protein sequence similarity searches demonstrate sequence similarities of the outermost extracellular domain, EC1 of N-, R- and P-cadherins to the EC1 of E-cadherins, to be 77–78% (Nollet et al. 2000). In terms of adhesive properties, in simulation studies, R-cadherins appear to have greater homophilic as well as heterophilic binding affinities than E-cadherins (Vendome et al. 2014).

9.5 The Type I Classical Cadherins, E-Cadherin and N-Cadherin

The classical cadherins are a large family of cell surface glycoproteins essential for tissue morphogenesis and development (Takeichi 1995). In both vertebrates and invertebrates, they are characterized by the presence of extracellular (EC) domains, each consisting of 110 amino acid residues folded into a β-sandwich structural motif. The calcium binding regions are highly conserved amongst species (Nollet et al. 2000; Posy et al. 2008). The type I classical cadherins occur only in vertebrates. In this review, our focus is primarily on two type I classical cadherins: epithelial cadherin (E-cadherin) and neuronal or neural cadherin (N-cadherin).

Once an E-cadherin or N-cadherin molecule has begun to be synthesized and emerges from a ribosome, the signal peptide at its N-terminus facilitates the molecule's secretion into the lumen of the endoplasmic reticulum. This causes the five extracellular domains (EC1–EC5) of the cadherin to be serially and progressively transported across the membrane. Transport stops with the transmembrane (single-pass) region crossing the membrane and getting retained and not allowed to be secreted, causing the domain that follows it, i.e. the cytoplasmic domain, to fold on the cytoplasmic side of the membrane. The folded cytoplasmic domain then binds to catenins, at some point. The catenins are linked to the actin cytoskeleton. Prior to the completion of this entire assembly, the budding of the ER into vesicles takes place. These vesicles travel to the cell membrane and fuse with it, and this causes the cadherins to appear on the cell surface, where they can perform their adhesive function (s) involving the extracellular matrix or other cells, with their extracellular domains, or ectodomains, facing the outside of the cell. Prior to their appearance on the cell surface, the signal

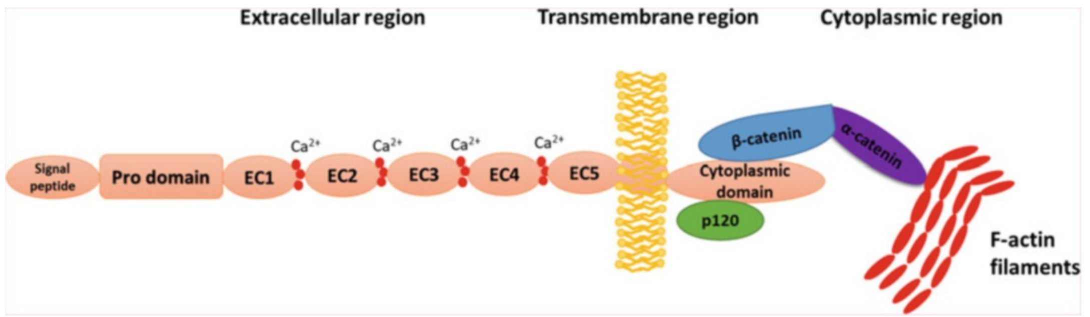

Fig. 9.1 Schematic diagram representing an E- or N-cadherin and its interactions with the cytoskeleton. The entire translated polypeptide is shown; however, the signal peptide and the pro-domain are removed through protease action, prior to adhesive function, and this removal presumably also precedes the interaction with actin for most molecules

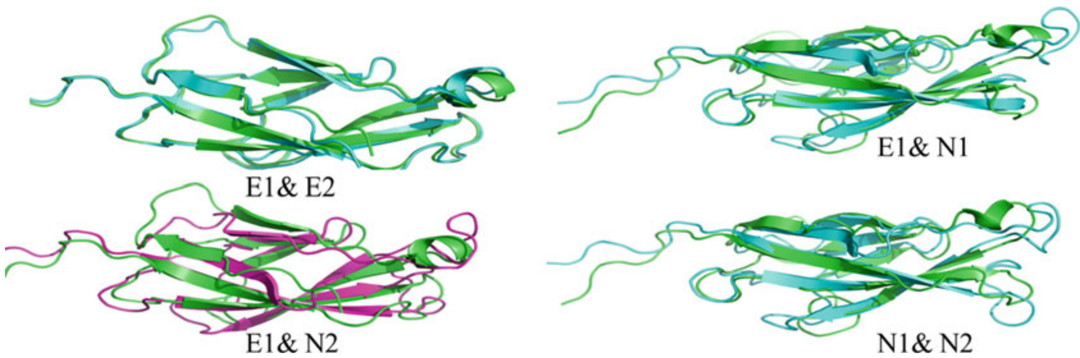

Fig. 9.2 Backbone ribbon diagram representations displaying the structural similarity and superimposability of various pairs of the first two ectodomains, EC1 and EC2, of E-cadherin (E1, E2) and N-cadherin (N1, N2). The figures were generated using the software PYMOL using PDB ID 3Q2V for mouse E-cadherin and PDB ID 3Q2W for mouse N-cadherin. Structure superimposition was also done using PYMOL

peptide and the pro-domain immediately following the signal peptide are required to be excised and removed at some stage, by proteases present in the lumen of the ER (as occurs with most secreted proteins).

Figure 9.1 shows a representation of a classical (E- or N-) cadherin molecule incorporated into the endomembrane system, prior to the proteolytic removal of the signal peptide and the pro-domain, i.e. as the entire translated polypeptide, with a putative interaction having already occurred with the catenins. This diagram must not be taken literally, in that it is rather unlikely, although not impossible, for cadherins to interact with the cytoskeleton prior to the removal of the N-terminal regions of the molecule. Following excision and removal of the signal peptide and the pro-domain and following transport to the cell surface, the outermost section of the cadherin which then comes into contact with similarly processed cadherins on the surfaces of juxtaposed cells is the domain known as EC1.

The E- and N-cadherin ectodomains show sequence homology as well as structural homology with each other (Patel et al. 2003; Chen et al. 2005; Posy et al. 2008). As shown below in Fig. 9.2, the structures of the EC1 and EC2 ectodomains of E- and N-cadherins are perfectly superimposable, both in cases in which these domains are drawn from the same cadherin and

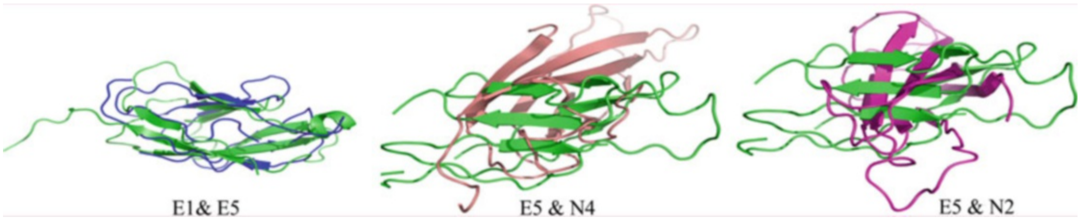

Fig. 9.3 Backbone ribbon diagram representations displaying the lack of structural similarity and superimposability for some of the ectodomains, e.g. EC1, EC2 and EC5, of E-cadherin (E1, E2, E5) or N-cadherin (N2, N4). The figures were generated using the software PYMOL using PDB ID 3Q2V for mouse E-cadherin and PDB ID 3Q2W for mouse N-cadherin. Structure superimposition was also done using PYMOL

also where they are drawn from different cadherins, for comparison.

Notably, however, this structural homology does not extend to all five ectodomains. The structures of EC3, EC4 and EC5 are quite unlike the structures of the EC1 and EC2 domains. As shown in Fig. 9.3, the structures of EC1 and EC5 of E-cadherin (E1, E5) are not as similar to each other as the structures of EC1 and EC2 of E-cadherin (E1, E2) shown superimposed in Fig. 9.2. Figure 9.3 also shows (i) how dissimilar the structures of the other cadherin domains (which are more membrane-proximal) tend to be, in respect of the E- and N-cadherins, as well as (b) how the membrane-distal and membrane-proximal domains tend to be dissimilar, although all of these ectodomains (or EC domains) consist of similar beta-sandwich folds.

9.6 Effects of Binding of Calcium to E- and N-Cadherins

Of all the CAMs, the cadherins, in particular, show a unique dependence on extracellular calcium for their activity, i.e. for the occurrence of homophilic interactions with other CAMs. There are three calcium-binding sites present in each inter-domain region, showing sequential binding to three calcium ions suggestive of a cooperative mechanism of calcium binding. The calcium ions are bound by the negatively charged, aspartate and glutamate side chains and by the backbone oxygen. The inter-domain regions possess three copies of the consensus sequence, DXNDNXP, acting as the master binding motifs for the three calcium ions. Within these, the calcium-binding sequences, PENE (residues 10–13), LDRE (residues 66–69) and DAD (residues 134–136), utilize their aspartate and glutamate residues to bind to calcium, and these sequences are conserved amongst all inter-domain regions in all classical cadherins, and not just the Type I classical cadherins. Figure 9.4 shows a representative inter-domain region and its calcium-binding sites.

The binding of calcium is reported to impart structure and stability to the ectodomains (Prasad and Pedigo 2005), in that the binding of three calcium ions to each inter-domain region causes ‘rigidification’ of that section of the cadherin and facilitates homophilic interactions by aligning the five EC domains into a rigid ‘rod-like’ arrangement. Monovalent cations, e.g. potassium and sodium, can also bind to the cadherins. However, such binding induces no conformational change (or rigidification) in the ectodomains or in their geometric dispositions towards each other. Electron microscopic studies have shown that treatment of full-length (EC1-EC5) epithelial cadherin [or fusion constructs comprising the first two domains (EC1 and EC2) of E-cadherin] with physiological concentrations of calcium induces changes in structure, causing the arrangement of the domains to become more rod-shaped. The absence of calcium, in contrast, makes the structure more compact and flexible, as well as more susceptible to proteolytic cleavage (Takeichi 1990). A higher vulnerability to proteases has also been observed where mutation(s) exist in

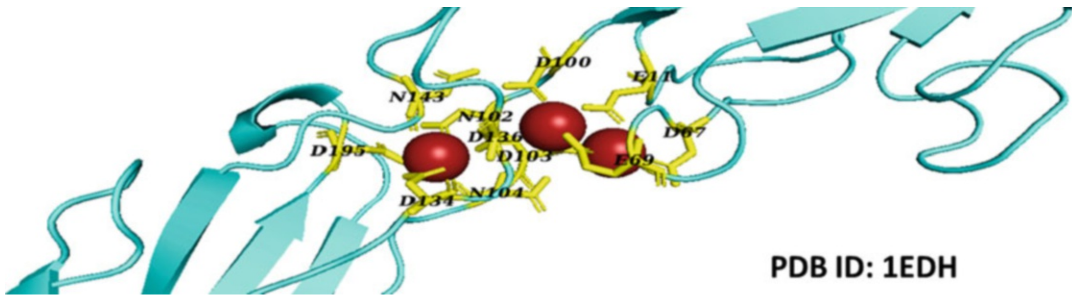

Fig. 9.4 Linker regions separating any two cadherin extracellular (EC) or ecto repeat domains are capable of binding to three calcium ions (shown in red, with residues involved in the binding shown in yellow). The detail shown is specific to the linker separating the EC1 and EC2 domains of E-cadherin). The figure was generated using the software PYMOL using PDB ID 1EDH

certain amino acid residues involved in calcium binding, such as Asp-134 (Ozawa et al. 1990a, b). Circular dichroism studies have shown that the ectodomains undergo a secondary structural alteration, from a randomly coiled conformation to a conformation with greater β-sheet content, upon calcium addition (Prasad and Pedigo 2005). X-ray crystallographic studies reveal that a twofold symmetric dimer of the N-terminal EC domains of E-cadherin forms in the calcium-bound state, but not in the absence of calcium (Nagar et al. 1996). Molecular dynamics simulations suggest that there is a significant change in the structural dynamics of an EC1-EC2 fusion construct when solvent-exposed calcium ions are removed from a calcium-bound state (Cailliez and Lavery 2005). Thus, various computational and biophysical studies have confirmed that the apo-cadherin (the unbound state) shows significant conformational flexibility and that calcium-bound cadherin ectodomains display significant rigidity of conformation and restriction of conformational flexibility involving inter-domain regions. In terms of function(s), the absence of calcium also appears to abolish the adhesive function of the cadherins and makes them more susceptible to proteases. The increased rigidity of ectodomains upon calcium addition also causes molecules to come into proximity and exhibit interactions with each other to form dimers, either between molecules on the same cell (*cis* dimers) or between molecules on the surfaces of cells which are juxtaposed to each other (*trans* dimers). It could be conceived that binding of calcium cooperatively makes the otherwise flexible (and flippy-floppy) string of extracellular domains into a rodlike unit behaving as a single entity and that the reduction in intra-chain motions and the stabilization of a single conformation facilitate weak intermolecular interactions which would otherwise have been disfavoured by the occurrence of strong chain motions.

The binding of calcium ions to the inter-domain regions occurs with affinities which are rather poor, in comparison with the affinities of many other molecular systems, ranging from micromolar to millimolar values of binding constants (Shapiro et al. 1995; Nagar et al. 1996; Tamura et al. 1998; Boggon et al. 2002; He et al. 2003; Prasad and Pedigo 2005; Abedin and King 2008; Harrison et al. 2010). Such a range of calcium-binding affinities suggests that the cadherins might exhibit a dynamic response to the changing calcium ion concentrations in the extracellular milieu. Such a dynamic response could potentially facilitate the transmission of information about the junctional calcium status into the interior of the cell via interactions of cadherin cytoplasmic domains with the catenins and the cytoskeletal network in the cytoplasm. The binding of calcium ions to the inter-domain regions in ectodomains not only facilitates the rigidification and dimerization of these domains but also appears to stabilize them conformationally. Numerous cellular and biophysical studies have been performed to elucidate the strength

as well as the stability of cadherin dimerization. For E-cadherins, thermal and chemical denaturation studies employing CD spectroscopy observed a shift in the structure-melting or unfolding temperature of the protein upon the addition of calcium (10 mM), from 40 °C (without calcium) to 65 °C (with calcium). Thermal studies involving differential scanning calorimetry are also in agreement with these spectroscopic experiments (Prasad and Pedigo 2005) suggestive of a role for calcium in stabilizing some aspect of cadherin conformation.

9.7 Cadherin-Cadherin Interactions in the *Cis* and *Trans* Modes Involving Ectodomains

The structures of the cadherin domains elucidated through X-ray crystallographic studies reveal that homophilic interactions between the cadherin ectodomains involve the N-terminal domain, EC1, i.e. the domain which is most distal to the plasma membrane (Shapiro et al. 1995). When cadherin ectodomains on the same cell's surface interact in a 'side-by-side' manner, they form *cis* dimers. When cadherin ectodomains present on juxtaposed or opposing cells interact, they form *trans* dimers. Currently, it appears that the binding interface is formed by only the EC1 domains of interacting partner cadherins, regardless of whether the interaction happens to be a *cis* or *trans* interaction. Various crystallographic studies suggest that *trans* dimers are formed in two conformations: strand-swapped dimers (S-dimers) and X-shaped dimers (X-dimers) (Shapiro et al. 1995; Boggon et al. 2002; Parisini et al. 2007; Ciatto et al. 2010; Harrison et al. 2010; Vendome et al. 2011). The crystal structures of N-cadherins show naturally formed strand-swap dimers, which are formed during crystallization (Shapiro et al. 1995).

S- and X-trans Dimers A *trans* interaction of the S-dimer variety occurs through the exchange or swapping of the β-strands located at the N-terminus of the EC1 (Harrison et al. 2010), between two interacting EC1 domains. Physically, the formation of such a strand-swapped dimer takes place by the insertion of a tryptophan residue present at the second position from the beta-strand of one partner into the hydrophobic pocket of its interacting partner, and vice versa. All type I classical cadherins have one conserved tryptophan, Trp2, at residue position 2 in the chain, and this tryptophan anchors the exchanged strands between the interacting EC1 domains, whereas for type II cadherins, both Trp2 and Trp4 participate in anchoring the exchanged strands. In addition, in both type I and type II cadherins, the remaining residues at the positively charged N-terminus also participate in the formation of various intermolecular salt bridges that further stabilize the strand-swapped dimers which are initially formed by the insertion of the tryptophan residue(s) into hydrophobic pocket (s) in the interacting partner domain. Notably, after the insertion of cadherins into membranes, the proteolytic cleavage of the signal peptide and 'pro-region' is absolutely essential for such salt bridge interactions to occur (Häussinger et al. 2004), suggesting that this removal could regulate the timing and context of the function of the cadherin and that cadherins remain in an essentially non-functional state until this removal takes place. Mutation of Trp2 in E-cadherin as well as N-cadherin prevents cell aggregation through cell-cell adhesion, suggesting that the strand-swapping mechanism is essential for cell-cell adhesion involving classical cadherins (Tamura et al. 1998). It has been demonstrated that the strand-swap dimerization is driven by the release of some physical strain present in the anchored N-terminal β-strands on cadherin monomers. The ease of the release of the strain thus appears to determine the binding affinity observed, where measurements have been made. Further, it has been observed that alteration in the length of the strand, or mutation of anchoring amino acid residues, can significantly change binding affinities. Various studies have also proposed that the binding specificity in type I cadherins is

regulated by individual binding affinities and that there is a conserved Pro5-Pro6 motif which prohibits non-specific high-affinity interactions (e.g. heterophilic interactions) by preventing the formation of hydrogen bonds between the opposing β-strands. It is reported that mutations in the Pro5-Pro6 motif (involving one or both prolines) can result in the formation of not just high-affinity homodimers but also various heterodimers that do not otherwise form (Vendome et al. 2011).

As opposed to S-dimers, the classical cadherin X-dimers are formed by surface interactions between two monomeric cadherins along the inter-domain region between the first two ectodomains EC1-EC2 in a trans fashion that resembles an 'X' shape (Harrison et al. 2010). The crystal structure of two engineered EC1-EC2 domains of human E-cadherin containing some additional N-terminal amino acid residues provides evidence of the formation of X-dimers through the obstruction of the strand-swapped mode of interaction (Nagar et al. 1996; Pertz et al. 1999). Similarly, various biophysical and structural studies have shown the presence of X-dimers in P-cadherins and nonclassical T-cadherin (Ciatto et al. 2010; Vendome et al. 2014). Nuclear magnetic resonance (NMR) studies along with some single-molecule fluorescence resonance energy transfer (FRET) experiments involving E-cadherins have confirmed that the mutation of Trp2 to alanine leads to the formation of X-dimers, through inhibition of the formation of strand-swapped dimers (Sivasankar et al. 2009; Li et al. 2013). Early studies proposed X-dimers to be a transient intermediate in the formation of S-dimers (Sivasankar et al. 2009), but recent biophysical studies suggest that the X-dimer and the S-dimer conformations constantly alternate during interactions of the ectodomains (Manibog et al. 2016), transforming from one into the other in a dynamic fashion. Nothing is yet known about how calcium affects this equilibrium, i.e. whether the concentration of calcium in the vicinity of the cadherin determines the frequency of the interconversion or the lifetime of either the X-dimers state or the S-dimer state.

Despite being involved in cell-cell adhesion, classical cadherins have remarkably high dissociation constants in solution. The dissociation constants (K_d) for the majority of the classical cadherins are in the micromolar range which indicates weak interactions. For instance, the K_d for the full-length ectodomains EC1-EC5 of C-cadherins interacting in homophilic fashion, determined by analytical ultracentrifugation, is 64 μM (Chappuis-Flament et al. 2001). Similarly, K_d values for the EC1-EC2 domain fusions of E-, N-, R-, C- and P-cadherins, obtained from such experiments, are only about 97 μM, 26 μM, 14 μM, 127 μM and 31 μM, respectively (Vendome et al. 2014), i.e. they are all in the micromolar range. In E-cadherins, the mutations that exclude possibilities of strand-swapping show an exceptionally larger K_d value (around 916 μM) suggesting that the monomers forming X-dimers have indeed very weak affinity for each other and that they are less favoured than S-dimers (Harrison et al. 2010).

Through thermodynamic and simulation studies, binding free energies for S-dimers and X-dimers have been calculated. These studies revealed that the S-dimers are more stable and have larger interacting surfaces than X-dimers. It has been proposed that the staggered interface in X-dimers could just be a crystal contact rather than an actual interacting (functional) surface. Since cadherin-cadherin interactions are quite weak (Koch et al. 1997) and are characterized by a small but concentrated interface, it would not be prudent to neglect the evidence of a staggered interface obtained from the crystal structure studies. Through analysis of dimeric interfaces, it has been stated that the specificity of interaction in the S-dimers must depend on subtle thermodynamic or kinetic factors. The strand-swapped interface is identical for E- and C-cadherins but varies a little from that in the N-cadherins (Cailliez and Lavery 2006).

Cis Interactions In addition to the strong *trans* interactions, weak *cis* (lateral) interactions occurring in the other regions of EC1 and some regions of the EC2 domain are also reported. It is not yet

known whether the remaining domains, i.e. EC3, EC4 and EC5, play a role in *cis* interactions. The *cis* dimeric interface, proposed from the crystal structure of C-cadherin, is conserved in terms of residue content and geometry in E-cadherin as well as in N-cadherin (Boggon, Murray et al. 2002). The *cis* dimer interface possesses an asymmetric junction between the EC1 ectodomain of one cadherin monomer and the EC2 ectodomain of its interacting partner; mutation of the interfacial residues involved abolishes the *cis* dimer assembly. Various biophysical techniques have suggested that *cis* dimerization interactions are either weak or transient (Häussinger et al. 2004; Harrison et al. 2011).

Monte Carlo simulations suggest that *trans* dimerization could be a prerequisite for *cis* dimerization because *trans* interactions lower the entropic penalty associated with *cis* dimer formation by reducing the conformational flexibility of the interacting cadherin domains (Wu et al. 2010, 2011). Recent studies have shown that both cells expressing E-cadherins, as well as liposomes coated with E-cadherins, tend to form ordered arrangements of 2D cadherin lattices at intercellular or inter-liposome junctions; however, mutants which abolish *cis* interactions fail to form such arrangements. This suggests that *cis* interactions must not only occur to facilitate the formation of ordered cadherin lattices but also that they could play a crucial role in forming cadherin clusters on membranes which stabilize cell-cell adhesions (Harrison et al. 2011).

It is believed that slow mechanisms such as exocytosis and endocytosis, as well as rapid mechanisms such as the lateral diffusion of cadherins to form cadherin clusters, could facilitate increased avidity of interactions between cellular surfaces which could potentially compensate for the poor affinity of cadherin-cadherin interactions and create the required total strength of collective cadherin-cadherin interactions to facilitate substantive cell-cell interactions (Iino et al. 2001; de Beco et al. 2009; Zhang et al. 2009). If this is the case, *cis* interactions must also be of consequence.

However, there are some conflicting studies which challenge the role of *cis* dimers in the formation of cadherin clusters. A recent study of E-cadherins embedded in liposomal bilayers suggests that cadherin clusters could be stabilized by intracellular linkage to the F-actin cytoskeleton, and not really by the formation of *cis* dimers. Further, there is no consensus yet about whether prior *trans* dimerization is required to facilitate *cis* interactions or whether the opposite is true, i.e. whether prior *cis* dimerization is required to occur to facilitate *trans* dimerization. Indeed, the two could be interdependent and operate through cooperative feedback mechanisms. Various computational and super-resolution microscopic studies have shown that glycosylation of ectodomains could also facilitate *cis* dimerization, indicating that the importance of *trans* dimerization prior to the formation of *cis* dimers might apply mainly to cadherins produced without any glycosylation (e.g. in bacterial cells for in vitro studies), whereas glycosylation could facilitate *cis* interactions in vivo. Towards the end of this review, a summary figure regarding possible *cis* and *trans* modes of interactions of cadherin ectodomains is included; this figure incorporates both currently known and hypothesized modes of interactions and also certain new hypotheses regarding such interactions.

9.8 Cadherin-Cytoskeleton Interactions Involving the Cytoplasmic Domain

The classical cadherins possess a cytoplasmic domain which is involved in direct binding to p120-catenin and β-catenin and indirect binding to α-catenin, a member of the vinculin superfamily (Shapiro and Weis 2009). The p120 catenin binds with the juxtamembrane domain (JMD) of the cadherin. It imparts stability to this domain by inhibiting internalization and degradation of the domain (Davis et al. 2003; Xiao et al. 2003). Cadherins are linked with the actin cytoskeleton via salt bridge-based interactions of α-catenin with cadherin-bound β-catenin, on the one side,

and F-actin on the other (Gates and Peifer 2005; Kwiatkowski et al. 2010). This suggests that α-catenin could bridge cadherin-bound β-catenin to actin. However, there is no substantial evidence to back up this possibility, and there have been challenges of the mechanism suggesting simultaneous binding of α-catenin to β-catenin and F-actin (Drees et al. 2005; Yamada et al. 2005). Nonetheless, various studies corroborate the proposition that α-catenin linkage is essential for adhesion and junction assembly, without specific reference to any mechanistic details regarding this contention (Pokutta and Weis 2007; Hartsock and Nelson 2008; Kwiatkowski et al. 2010; Yonemura et al. 2010; Taguchi et al. 2011; Yonemura 2011).

The central region of the β-catenin, containing armadillo repeats (each repeat consisting of 40 amino acid residues), binds to the cytoplasmic domain of the cadherin (Hülsken et al. 1994; Funayama et al. 1995). β-catenin just acts as a linker, or intermediary, between α-catenin and the cytoplasmic domain of the E- or N-cadherin. According to a study involving an engineered cadherin construct in which the cytoplasmic domain of the cadherin is replaced by α-catenin, there is an alteration in cell adhesion (compared to the cell adhesion mediated by the whole protein complex) even in the absence of β-catenin (Nagafuchi et al. 1994). It is noteworthy that cadherin expression levels regulate catenin expression post-translationally. Surprisingly, when c-DNAs encoding E-, N-, or P-cadherins were transfected to L-cells, catenin expression levels increased significantly, without affecting the mRNA content (Nagafuchi et al. 1991).

A molecule known as γ-catenin, or plakoglobin, is associated with the desmosomal cadherins (Korman et al. 1989; Witcher et al. 1996). This shares significant structural and functional similarity with β-catenin and armadillo and can, therefore, replace β-catenin in the cadherin-catenin complex (Hülsken et al. 1994); however, γ-catenin associates to form weak complexes that dissociate more readily than β-catenin-cadherin complexes (Haegel et al. 1995). The deletion of the γ-catenin gene is lethal to heart structure formation and results in early death of the embryos, ostensibly due to the disruption of the strong association of γ-catenin with desmosomal cadherins (Bierkamp et al. 1996). The cytoplasmic domain of the classical cadherins is also known to interact with other proteins like tyrosine phosphatases (Brady-Kalnay et al. 1995; Kypta et al. 1996).

Numerous experiments have elucidated the functional significance of the cytoplasmic domain. It has been reported that the deletion or overexpression of the catenin-binding site or the complete cytoplasmic domain abolishes cell-cell adhesion mediated by the cadherins (Ozawa et al. 1990a, b; Nagafuchi et al. 1994). On the other hand, some mutation-based studies have also demonstrated that the presence of the cytoplasmic domain is not essential for cadherin-mediated cell-cell adhesion. In these studies, the cytoplasmic domain is substituted for desmoglein-3, one of the desmosomal cadherins that cannot bind to the catenins. The mutations did not affect the cadherin-mediated cell adhesion in cultured cells indicating that catenin association is not the sole mechanism that regulates cell adhesion (Roh and Stanley 1995). In principle, this assertion is not unreasonable. Since many in vitro experiments demonstrate that the EC1 domains of the cadherins are fully capable of engaging in *cis* and *trans* interactions by themselves, without the presence of the remaining EC domains and even without the presence of the membrane and cytoplasmic domains, it is conceivable that cadherins lacking the cytoplasmic domain can mediate cell-cell adhesion; of course, signalling into the cell would be expected to be significantly affected.

9.9 Adherence-Related Functions of E- and N-Cadherins in Organismal Development and Disease

Cell Sorting and Segregation It is natural to assume that differences in cadherin-cadherin binding affinities and specificities must be somehow used by organisms to facilitate the sorting and segregation of cells expressing these

cadherins differentially, into different tissues. Given their extreme structural and conformational similarities, it would be interesting to understand the differences between them which regulate the highly specific homophilic pairing, heterophilic pairing and cell patterning behaviour seen in organisms. We know that cells expressing different cadherins can either be sorted homotypically (forming separate aggregates based on the type of cadherin expressed on these cells) or heterotypically (forming intermixed aggregates of different types of cadherin-expressing cells). However, due to our poor understanding of the relationship between cellular binding specificity and free energies of intermolecular binding for different types of cadherins and cadherin pairs, good models (including molecular models) are not yet available for cadherin-mediated cellular patterning, despite our knowledge of the structures of the domains of some of these cadherins and some structural details of their interactions.

Even so, some recent studies have shown that E- and N-cadherin bearing cells first form distinct homotypic cell aggregates and then interact heterotypically through the interfaces between the two distinct cell aggregates. Studies using analytical ultracentrifugation and surface plasmon resonance have determined the homophilic and heterophilic affinities, respectively, for both full-length E-cadherins and N-cadherins (Katsamba et al. 2009). The free energies for N- and E-cadherins turn out to be comparable, at 6.5 kcal/mol for N-cadherin and 5.3 kcal/mol for E-cadherin. The K_d values from ultracentrifugation studies determined for N- and E-cadherins were 22.6 μM and 160 μM, respectively, i.e. the homo-dimerization affinity for N-cadherins was found to be approximately tenfold greater than that for E-cadherins. Similarly, surface plasmon resonance experiments have provided the K_d for the heterophilic affinity between E- and N-cadherins, and this has been found to be intermediate to that applying to the two homophilic dimerization affinities. Many reports are suggestive of significant heterophilic interactions between E- and N-cadherins (Volk et al. 1987; Niessen and Gumbiner 2002). It may be argued that the small differences in affinity of homophilic and heterophilic interactions can be compensated for (or competed out) with high avidities and that this can play a significant role in determining cell adhesion behaviour and apparent specificity of interaction. Within a population of cells, subpopulations required to engage in differential interactions with other cells can express different numbers of different cadherins, on different facets of the cell's surface, and this detail can get smeared out in studies assuming that all cells of a particular type express the same numbers of the same types of cadherins. Thus, we may believe that based on the dimerization affinities and availabilities of E- and N-cadherins in requisite numbers, the equilibrium between homotypic and heterotypic interactions would be established according to the requirements of cells (and their genetic programming) and would not only facilitate cell sorting, segregation and formation of separate tissue layers but also allow the adhesion of these layers onto one another.

The cadherin superfamily plays a fundamental role in tissue morphogenesis, development and homeostasis, simply because cadherins are crucial for maintaining cellular contacts that can either directly participate in signalling or facilitate signalling involving other molecular interactions which are facilitated by cell-cell contacts. For example, reduced expression levels of epithelial cadherin (E-cadherin) are associated with enhanced invasion and metastasis in many tumours. Through their stable cell-cell adhesive interactions, cadherins mediate 'contact expansion' by reducing the 'interfacial tension' at the junctional interfaces of cells. Another way in which cadherins help to release interfacial tension or cortex tension is via the signal transmission to the actomyosin cytoskeleton through interactions with catenins. Cadherins also resist mechanical forces that attempt to disrupt cell-cell contacts. Also, cadherins are reported to be involved in the signal transduction processes that regulate cell fate (Stephenson et al. 2010; Lorthongpanich et al. 2012; Sarpal et al. 2012), cell polarity

(Wang et al. 2010; Bosveld et al. 2012) and cell proliferation (Nelson and Chen 2003; Kim et al. 2011; Schlegelmilch et al. 2011). One of the intriguing adhesive variants of the E-cadherin, known as uvomorulin, has the ability to adhere cells to each other during early stages of embryogenesis involving the morula. Since the loss in expression levels and functions of E-cadherins is correlated with tumour metastasis and their re-expression is associated with the decrease in proliferative and invasive capacity, they are proposed to function as tumour suppressors and metastasis suppressors.

Interactions with Growth Factors The N-cadherins have been reported to exhibit heterophilic interactions with a wide variety of proteins, including some growth factor receptors and some cell matrix proteins. One such protein is the fibroblast growth factor receptor (FGFR). The regulatory mechanism for the interactions between FGFR and N-cadherin is not known. However, these interactions can potentiate signalling in cancer cells either by the aid of endogenous FGFs, stabilizing the cell surface receptors, or by forming a higher-order complex which does not require FGF for downstream signalling (Nakamura et al. 2001; Nourse et al. 2007). Recent studies have also proposed a direct crosstalk between E-cadherin and EGF receptors (EGFR). EGFR is known to disrupt cell adhesions by destabilizing cadherin-catenin interactions, downregulating cadherin expression and exocytosis. The engagement of E-cadherin in newly formed cell-cell contacts appears to stimulate the rapid activation of EGFR in an EGF-independent manner. Notably, EGFR-initiated signalling pathways enhance cell proliferation and cell survival through MAP kinase, PI3-kinase and Rho GTPases. Future studies on the understanding of the molecular mechanisms underlying the interactions between EGFR and E-cadherin in normal and tumour epithelial cells can provide new insights into the development of suitable therapeutics for cancer treatment (Gavard and Gutkind 2008).

Interactions with Matrix Proteins It has been reported that dimeric E-cadherin interacts in a heterophilic manner with the cell matrix protein, integrin αEβ7, in a calcium-dependent manner (Corps et al. 2001). Since integrin αEβ7 has only one ligand, i.e. E-cadherin, high specificity of binding is ensured. This restricts autoreactive mucosal (cytotoxic) T cells in their specific locations. Both the integrin subunits, αE and β7, participate in the interaction with E-cadherins. In addition, the integrin α2β1 also shows interactions with both E- and N-cadherins but in somewhat different modes of interactions. In adhesion networks involving N-cadherins, the integrin α2β1 interacts with type I collagen and is involved in melanoma cell invasion and metastasis. Independently, E-cadherin/α2β1 integrin adhesion networks are also thought to regulate cell-cell adhesion in a type I collagen-independent manner (Siret et al. 2015).

Interactions with Other Cadherins and Receptors Various in vitro studies and site-directed mutagenesis-based studies have thrown up evidence of heterotypic interactions between N-cadherin and R (retinal)-cadherin. These studies support the S-dimer model for *cis*-heterodimerization of N- and R-cadherin molecules on the surfaces of the same cells. In vitro studies have demonstrated that the two cadherins are co-expressed in neurons and show co-localization at certain neural synapses, implying significance in cell adhesion in the neural retina (Shan et al. 2000).

Some biochemical studies have shown that N-cadherin interacts with the N-terminal domain of the glutamate receptor, GluR2, in both *cis* and *trans* fashions. In hippocampal neurons, N-cadherin and GluR2 form a synaptic complex that stimulates presynaptic development and function and also promotes dendritic spine formation (Saglietti et al. 2007). N-cadherin is also known to show robust interactions with another cadherin known as protocadherin-19 (Pcdh19). Bead aggregation studies examining the interactions of beads bearing proteins have

revealed that the heterophilic Pcdh19–N-cadherin complex forms along with homophilic complexes, suggesting the usefulness of this interaction as a switch, converting between distinct binding specificities (Emond et al. 2011).

An atypical E3 ubiquitin ligase, Fbxo45, which facilitates ubiquitin-mediated degradation of proteins, has also been identified as an interacting partner for N-cadherin. The binding interface for this interaction overlaps with the calcium-binding motifs as well as with the dimerization interface, such that these interactions are disrupted by the addition of calcium, implying an 'either-or' mechanism of switching of interactions. N-cadherin proteolysis is also substantially enhanced by RNAi-mediated Fbxo45 gene silencing, leading to the impairment of neuronal differentiation and reduced expression of N-cadherins. Surprisingly, Fbxo45 prevents the calcium depletion-induced proteolysis of N-cadherin and R-cadherin and promotes neuronal differentiation by directly interacting with either N- or R-cadherins (Chung et al. 2014). The wide gamut of interactions of N-cadherin with different proteins suggests that it plays important regulatory and switch-like roles, sensing different situations and responding like a node in a protein interaction network.

Cadherin Gene Organization and Regulation of Expression During Development There are around 114 different types of cadherins encoded by the human genome. Not all of this diversity arises from the presence of a comparable number of genes; rather the diversity owes significantly to alternative RNA splicing mechanisms responsible for producing multiple splice variants from a smaller group of genes. The encoded classical cadherins include E-cadherins, N-cadherins, P-cadherins and cadherin 12 (Type2 cadherin). Several genes encoding classical cadherins have been identified and sequenced.

The gene *cdh1* encodes E-cadherin (CDH1). It is localized in the 16q22.1 region of chromosome 16. This gene has 14 splice variants. One of these encodes a preproprotein which is proteolytically processed to yield the mature glycoprotein. The transcription of the CDH1 gene is directly regulated by methylation of CpG islands present within the gene's promoter. Methylation of these CpG sites by DNA methyltransferase enzymes leads to the downregulation of the CDH1 encoding gene. Also several transcription factors such as Snail, Slug, E12/E47, ZEB-1 and SIP-1 are known to regulate E-cadherin expression through interactions with the enhancer boxes present upstream of the gene (Bolós et al. 2003). A substantial part of the *cdh1* gene consists of introns, the largest amongst which is the second intron. This aberrantly large intron is known to contain *cis* regulatory elements that regulate E-cadherin expression during development (Stemmler et al. 2005). The expression of E-cadherins begins very early during embryonic development, probably at the 1-cell stage, i.e. the zygote (Ogou et al. 1982). The differentiation and polarization of epithelia occur early, during the morula stage, in ontogenic terms, when there is a compaction of the embryo and each cell undergoes polarization along its apicobasal axis, generating an 'epithelial-like' phenotype. E-cadherins are thought to assume a critical part in the compaction of the morula, since functionally interfering antibodies against the E-cadherins have been shown to decompact the morula (Riethmacher et al. 1995). However, the source of E-cadherins at this stage is very likely to be maternally encoded (i.e. using proteins encoded from maternal mRNA), as embryos that are homozygous for an E-cadherin mutation develop normally up to the morula stage and have been shown to compact properly despite the mutations being present in the germline. In any case, most zygotic genes do not express this early during development, and cellular characteristics are essentially maternally encoded. The mutant morula cells become initially polarized, based on their maternally inherited cadherins, but very soon the embryo appears to become severely distorted, owing ostensibly to the mutation in the zygotically encoded version of the E-cadherin protein once this begins to be produced. This establishes that E-cadherin expression plays a

very important role in maintaining cell polarity during the early stages of development.

At the stage of implantation, all embryonic cells express E-cadherin. The molecule, however, disappears from some cell layers as the differentiation of cell occurs, into various cell types. The best known example is the epithelial to mesenchymal transition (or EMT) which occurs during the formation of the mesoderm. The cells of the mesoderm migrate into the space between the ectoderm and the endoderm and lose E-cadherin during migration. During invagination of the neural plate, this region also loses E-cadherin. Other regions of the ectoderm continue to express E-cadherin, as do all endodermal cells. In older embryos, all ectoderm-derived cells express E-cadherin, barring certain terminally differentiated, non-proliferating epithelial cells such as lens fibre cells and keratinized epidermal cells, which lose E-cadherin from the membrane. Mutations in the *cdh1* gene leads to various diseases in humans which include cancers of the breast, colo-rectum, ovaries and many more due to increased proliferation, or occurrence of metastasis.

The N-cadherins are encoded by the *cdh2* gene in humans which maps to the 18q21.1 region of the chromosome 18. N-cadherins are known for their role in neural and mesodermal development. N-cadherins first appear in some ectodermal cells at the time of gastrulation. These are the most abundant type of cadherins in the neural tube at the early stages of development; however, during the process of differentiation of the central nervous system, they become regionally localized followed by the complete loss of N-cadherins from the neural retina and some layers of the cerebellum. Also the early neural tube expresses N-cadherin, in the dorsal-most region where neural crest cells are generated. However, the expression of this cadherin is downregulated in neural crest cells migrating from the neural tube; they instead begin to express cadherin-7 (Nakagawa and Takeichi 1998). When dorsoventral migration is concluded, N-cadherin tends to be upregulated in neural crest cells just before their their differentiation into the dorsal root ganglia (DRG) and sympathetic ganglia. After dorsolateral migration, dermal melanocytes express N-cadherin, facilitating contacts with fibroblasts in the skin dermis. Following the formation of the ganglion, N-cadherin appears at various places: on the apical surface of the neural tube, at the basolateral surface of the floorplate, upon the neuronal cells which are localized ventrally or laterally within the neural tube, upon fibrous axonal processes and in the ventral root and sympathetic ganglia.

When organisms turn into adults, N-cadherin is observed in neural tissue, endothelial cells, fibroblasts, the retina, as well as myocytes and osteoblasts (Taneyhill 2008). N-cadherin is also asymmetrically expressed in the chicken embryo; its activity is required during gastrulation to establish the left-right axis. Blocking the function of N-cadherin alters the expression of transcription factors, Snail and Pitx2, both downstream components of the cascade of factors regulating establishment of left-right asymmetry (García-Castro et al. 2000). N-cadherin mutations in the heart lead to the disassembly of the intercalated disc structure in the adult myocardium (Kostetskii et al. 2005). Mutations in the *cdh2* gene are also associated with the arrhythmogenic right ventricular cardiomyopathy (ARVC) which has been identified as the cause for sudden cardiac arrest in young people (Mayosi et al. 2017).

Cadherins and Their Connection to the Epithelial to Mesenchymal Transition (EMT) EMT is a core process in embryonic development, cancer progression and fibrosis (Kalluri and Weinberg 2009). By definition, EMT is the process of conversion of the epithelial cell type into the mesenchymal type under the influence of epigenetic mechanisms. The plasticity of epithelial cells entering the transition is highlighted by loss of ZO-1 and E-cadherin expression and upregulation of the proteins, fibroblast-specific protein 1 (FSP-1) and vimentin (Zeisberg and Kalluri 2004; Liu 2010). Such 'transitional' cells interact less with the extracellular matrix at its basal surface and gain the ability to invade it. Through this plasticity-marking trait, highly proliferative epithelial cells tend to develop the mesodermal tissue by undergoing the transition

(Tsai and Yang 2013). EMT thus appears to be an energy-conserving and resource-scavenging mechanism designed to quickly deliver myofibroblast- or fibroblast-type cells urgently required by an organism during a response to an injury and in organ development. However, this fluidity of state is also associated with pathophysiological evils like fibrosis and tumour formation, and cells undergoing EMT can enter into these states instead of undergoing transdifferentiation (Kalluri 2009).

The transdifferentiation process in EMT starts with the cell-cell contacts at tight, adherence and gap junction followed by a loss of cell polarity. E-cadherin along with other signatures of epithelial phenotype disappears from the cell surface during this transition through repression of expression of E-cadherin at the transcriptional, translational and post-translational levels. A variety of injury-causing factors and growth factors, including transforming growth factor-β, basic fibroblast growth factor (BFGF) and hepatocyte growth factor, are known to induce this process (Chen et al. 2005; Farrell et al. 2014). N-cadherin is produced in some carcinomas that have lost or downregulated E-cadherin. Unlike E-cadherin, N-cadherin exhibits weaker adhesive interactions. Thus there is a correlation between the switch from E- to N-cadherin and invasive cellular behaviour. The switch from E- to N-cadherin and the associated higher degree of invasiveness are linked to higher tumorigenicity of E-cadherin-negative cell lines and also to the poorer prognosis of E-cadherin-lacking tumours. Similarly, reversal of EMT by constitutive E-cadherin expression is shown to inhibit neoplasticity and invasiveness in tumours. Usually, differentiated tumours show E-cadherin expression, and levels of expression are inversely related to the grade of cancer in solid-organ malignancy (Lombaerts et al. 2006; Wheelock et al. 2008). Many signalling pathways converge to cause EMT via inhibition of E-cadherin expression, and these include Wnt, TGF-β and Notch/delta signalling (Son and Moon 2010; Tsai and Yang 2013). In the mammary gland's epithelial cells, integrin-linked kinase activity stimulates Wnt signalling through the Snail and Slug transcription factors, repressing E-cadherin and further enhancing tumorigenicity (Tsai and Yang 2013). Furthermore, hypoxia-inducing factor also directs the expression of Snail and Slug, and TGF-β (which is responsible for extracellular matrix remodelling) induces Snail leading to repression and loss of E-cadherin (Wang et al. 2013). Collectively, all inducers of EMT converge to cause multiple and pleiotropic effects, and the loss of E-cadherin is the most common effect. Below, we focus some more on cadherins and neoplasia/cancers (Thiery et al. 2009).

Cadherins and Their Connection to Cancers Dysfunction of classical cadherins has been suggested to be a hallmark in the origin and progression of neoplastic diseases (Kawanishi et al. 1995). E-cadherin, being a prime marker (and potentially also a prime maintainer) of the epithelial phenotype, has received significant attention in cancer literature (Thiery 2002). There are clinical correlations between aberrant E-cadherin expression and tumour prognosis. There are also observations of E-cadherin dysfunction in tumour progression in in vitro and in vivo models. These combinedly suggest an important role for E-cadherin (Vleminckx et al. 1991; Kowalski et al. 2003). Overall, dysfunction or reduced E-cadherin function has been reported in carcinoma of the breast, nasopharyngeal cavity, pancreas, lung, stomach, GI cavity, kidney and prostate. Considering the source or origin, mechanisms of E-cadherin dysfunction or dysregulation can be genetic, epigenetic, transcriptional and translational or post-translational, i.e. mechanisms of E-cadherin's loss of function can include proteolytic cleavage of ectodomains, proteasomal degradation of E-cadherin upon endocytosis, miRNA-induced downregulation, transcriptional repression involving gene hypermethylation or expression of repressors through signalling, germline and somatic mutations, aberrant TGF-signalling and/or loss of heterozygosity. These mechanisms were observed and reported in a variety of cancers,

suggestive of a multilevel association of loss of E-cadherin function with the origin and propagation of neoplasms (Berx and van Roy 2009). Cadherin switching is another recently discussed mechanism associated with the E-cadherin null phenotype of most cancers (Wheelock et al. 2008). Below, we focus on genetic and epigenetic associations, transcription factor involvement and other processes.

Genetic Modifications Loss of heterozygosity (LOH) at the chromosomal level and loss of function (LOF) through mutations at the protein level are two ways in which the expression of E-cadherin is downregulated genetically. LOH is a common genetic aberration found in cancers in which the functional tumour suppressor gene is absent due to cross chromosomal events. The loss of heterozygosity of chromosome 16q21-22, where the E-cadherin gene is located, has been demonstrated in a variety of tumours including carcinoma of the breast, gastric, prostate and oesophageal cancer (Wijnhoven et al. 1999; Cleton-Jansen 2002; Corso et al. 2013). Similarly, progressive accumulation of somatic mutations in E-cadherin can lead to the process of carcinogenesis. Loss-of-function mutations in E-cadherin genes are known in diffusive gastric cancer, but these are rare events; these include in-frame deletion, truncation and splice-site-type mutations (Carneiro et al. 2008). Although rare in nature, E-cadherin mutations are known to cause familial aggregation of the diffusive form of gastric cancer. Most of these cause the occurrence of a stop codon resulting in premature cessation of expression. Polypeptide truncating germline mutations are also known to occur along the entire length of E-cadherin and can be associated with lobular breast carcinoma (Masciari et al. 2007). Whether the loss of E-cadherin is merely an effect is a moot point, since clearly the loss of the protein increases invasiveness, indicating that it can be a cause. There might also be other cause-effect relationships. It would be interesting to see whether the loss of E-cadherin can be a sole triggering event of a causal nature, in cancer. Of course, in cells that have lost E-cadherin, there are so many other changes, including EMT-type changes that it is difficult to establish which constitute ‘cause’ and which constitute ‘effect’.

Epigenetic Modifications Many types of epigenetic modifications regulate gene expression, including methylation, acetylation, sumoylation, phosphorylation and ubiquitylation. Of course, methylation is the easiest to study with the existing technology and, therefore, the best known and most explored (Weinhold 2006). Hypermethylation of the E-cadherin 5′ proximal promoter has been found to result in reduced E-cadherin expression. Methylation-induced downregulation of E-cadherin is seen in many types of carcinoma, with a clear negative correlation between the levels of methylation and E-cadherin expression (Yoshiura et al. 1995). Hypermethylation leads to methyl-CpG binding proteins, MeCP2, and MBP2, interacting with the E-cadherin promoter, resulting in histone deacetylase (HDAC) recruitment, leading to the compaction of chromatin and suppression of transcription of the E-cadherin gene (Bhatt et al. 2013).

Other than hypermethylation, expression of the E-cadherin gene is regulated by Snail, Slug (Snail2), Twist, ZEB1 and ZEB2 which repress E-cadherin expression. Snail binds to E-box elements and recruits HDACs, triggering the cascade leading to chromatin compaction. Elevated Snail expression is common in invasive ductal carcinoma of the breast, and its higher expression is correlated with high-grade and lymph node-metastasized mammary tumours (Blanco et al. 2002). Similarly, Twist (a member of the basic helix-loop-helix family) recruits histone-lysine N-methyltransferase to the E-cadherin promoter to repress E-cadherin expression while inducing N-cadherin expression, giving rise to poor prognosis in cancer (Lamouille et al. 2014).

Other Mechanisms of Cadherin Downregulation Endocytosis-based uptake, shedding of ectodomains through extracellular cleavage of E-cadherin and intracellular cleavage of the linker

region between the transmembrane section and the cytoplasmic domain, all singly or in combination, correlate with malignancy. Physiological recycling of E-cadherin involves endocytic pathways mediated by clathrin, caveolae and micropinocytosis, through which E-cadherin is recycled to new sites in cell-cell junctions. Endocytosis and recycling of E-cadherin are significantly increased in cells devoid of stable cell-cell contacts due to either low confluency or depletion of extracellular Ca^{2+} by a chelating agent (Le et al. 1999). Additionally, abnormal phosphorylation of a tyrosine residue in the cytoplasmic domain, induced by over-activation of proto-oncogenes like EGFR, Met and Src, results in internalization and ubiquitin-mediated proteasomal degradation of E-cadherin. Additionally, in the cytoplasmic domain of E-cadherin, where β-catenin binds, there are a number of serine and threonine residues which become putative sites for phosphorylation by a diversity of kinases. Phosphorylation of these sites in E-cadherin may alter its binding to β-catenin. On the other hand, phosphorylation of β-catenin by Src kinase results in disassembly of the cadherin-catenin complex, leading to loss of cell-cell adhesion and migration of β-catenin into the nucleus. Similarly, growth factors like epidermal growth factor and scatter factor can also lead to similar effects (Roura et al. 1999; McEwen et al. 2014). Another catenin, known as P120 catenin, is also known to regulate the expression of E-cadherin, presumably through feedback and sensing mechanisms, by ensuring the stability of the protein. Loss of P120 catenin is seen in a variety of cancers.

The regulation of what happens to the ectodomains also decides the fate of adhesion junctions between cells in cancers (Strumane et al. 2006). The proteolytic degradation of E-cadherin by zinc-dependent matrix metalloproteinases is known. Increased expression of these proteases correlates with the progression of cancer and inflammatory diseases. The ectodomain of E-cadherin near the plasma membrane is cleaved by these metalloproteinases, causing free E-cadherin fragments to be found circulating in the serum of patients with neoplastic diseases. Fascinatingly, soluble fragments of E-cadherin are also found to stimulate the migration of cells grown in collagen matrix under in vitro conditions (Nawrocki-Raby et al. 2003). Serine proteases like kallikrein 6 and 7 have also been found to be overexpressed in pancreatic and squamous cell carcinoma. Kallikrein 6 is known to modulate the protease activity of other proteinases like disintegrin which leads to the shedding of the extracellular domain of E-cadherin, resulting in metastasis of tumour cells (Johnson et al. 2007; Klucky et al. 2007).

9.10 Cadherin Trafficking, Association with Membrane Rafts and Non-association with Organelles

Trafficking of Cadherins Changes in cellular morphology and interactions and rearrangements accompanying physiological changes require changes in cadherin composition and constitution (Kowalczyk and Nanes 2012). Endocytosis, degradation and recycling of cadherins do occur, with proteins constantly being removed from the plasma membrane through endocytosis and recycled back into the membrane through exocytosis. E-cadherin is known to be internalized through clathrin-mediated endocytosis (Le et al. 1999), as well as through growth factor-induced pathways utilizing non-clathrin-mediated endocytosis like Rac1-dependent micropinocytosis (Watanabe et al. 2009). Once E-cadherin is internalized, it enters a Rab5 + ve compartment meant for sorting transmembrane proteins (Zerial and McBride 2001). Vesicles bud off from these compartments, mediated by the GTPase dynamin (Doherty and McMahon 2009). These can either be recycled back to the plasma membrane or marked for lysosomal degradation in a polarized manner (Woichansky et al. 2016). The amount and location of cadherin already present on the cell surface appear to determine whether a cadherin gets degraded after endocytosis or recycled back to the cell surface. The molecular mechanisms governing the recycling of cadherins

have not been fully explored, and whatever is known is largely about E-cadherin, with virtually nothing being known about N-cadherin recycling. Several studies suggest that p120 catenin inhibits endocytosis. Several amino acid motifs have been identified as being responsible for cadherin internalization. In certain cases, adaptor proteins mediating endocytosis have been identified (Cadwell et al. 2016). Processes requiring cell migration are dominated by endocytic trafficking, and so trafficking affects healing of wounds after injury, tumour metastasis and angiogenesis, as already mentioned in the section dealing with neoplasia/cancer. This is supported by the finding that endothelial cell migration is inhibited by mutations in DEE endocytic motifs in the molecule, VE-cadherin. Thus identification of all such motifs would be of use in revealing how endocytic signals contribute to adhesion, migration and cell patterning in tissues.

Association of Cadherins with Rafts Lipid rafts are an integral part of the plasma membrane which exist as liquid-ordered regions. Rafts are small in size and are abundant in cholesterol and glycosphingolipids. Despite having somewhat distinct protein and lipid compositions, rafts are not identical in terms of composition of their constituents in all cells and all situations. Various proteins, especially those involved in cell signalling, have been shown to be present in rafts (Pike 2003). N-cadherin present at cell junctions is colocalized with lipid rafts, and disruption of lipid rafts results in the inhibition of cell-cell adhesion, without any modification of the interaction of N-cadherin and catenins to its plasma membrane. This suggests that lipid rafts might be an important site for the dynamic assembly of classical cadherins like N-cadherin at cell junctions; in fact, lipid rafts appear to stabilize cadherin-dependent adhesion complexes (Causeret et al. 2005). The presence of E-cadherin in lipid rafts has been shown to be necessary for the initial interaction of *Listeria monocytogenes* with cells, in order for it to gain entry into host cells (Seveau et al. 2004). Proteins in lipid rafts are sometimes interaction sites for the entry of pathogens, and it is interesting that E-cadherin associated with rafts plays a role in listeria infection.

Non-association of Cadherins with Exosomes and Organelles Hardly anything is known about whether cadherins are present on exosomes, or about whether they affect the fusion of the exosome with other cells, or play any role in cell-cell fusion which is either stimulatory or inhibitory. Similarly, almost nothing is yet known about whether cadherins are present on the membranes covering intracellular organelles such as the nucleus, or lysosomes, or whether they play any causative roles in endocytosis or exocytosis, or phagocytosis, rather than just being the subjects of these processes in terms of their trafficking. Given that the entire membranous pool of the cell is commonly described as the 'endomembrane system', with evidence of the rapid exchange of lipids between the plasma membrane at the cell surface and the membranes covering all intracellular organelles, it is interesting that there is no evidence of the association of any cadherin, including any of the classical cadherins, with the entire endomembrane system, with organelles or with exosomes. Of course, the lack of evidence in this regard cannot be assumed to be the evidence of lack and it might be a good idea to examine whether indeed organelle membranes lack cadherins and other CAMs and also how these are restricted and removed. It may be said, in jest, that the endoplasmic reticulum is associated with cadherins; only that they face the lumen of the ER and not the cytoplasm. What about the other organelle membranes? Can the presence or location of cadherins in these tell us something about the nature of their equilibrium with the plasma membrane?

9.11 Hypotheses, Perspectives and Questions

Do Cadherin Domains Act Like Extendable 'Unfoldable-Refoldable' Springs? Anyone familiar with the studies of the mechanical unfolding of the repeat domains of the muscle protein 'Titin' performed over a decade ago

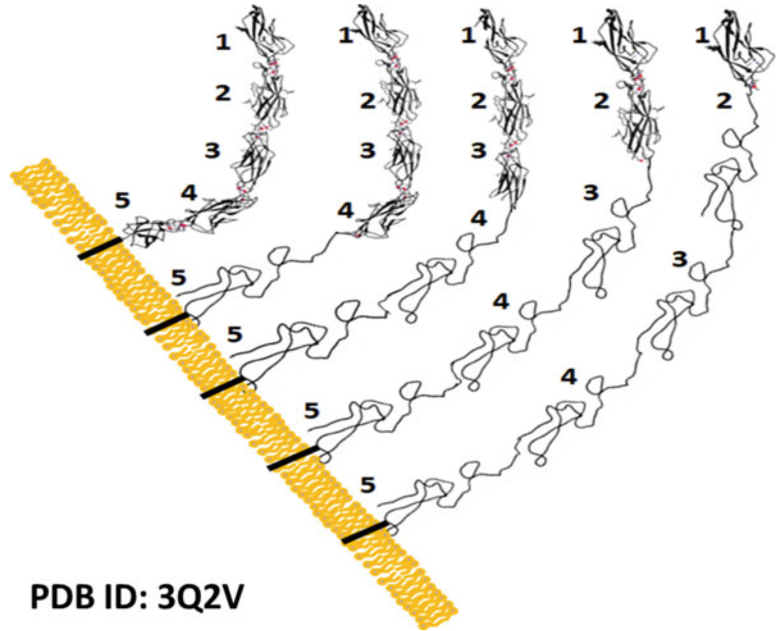

Fig. 9.5 As long as EC1 remains folded and capable of engaging in intercellular contacts, other domains could undergo unfolding in order to undergo lengthening and allow cells to move away and towards each other while remaining in contact through the unfolding and refolding of domains. The structures of the folded domains in this schematic figure were generated using the software UCSF Chimera and the PDB ID 3Q2V for mouse full-length E-cadherin

(in order to examine how many piconewtons of force are required to unfold individual domains, as well as whether domains refold when allowed to do so) would be likely to wonder whether the repeat domains of cadherins undergo mechanical force-induced unfolding as cells move away from each other. It is conceivable that the mechanical forces exerted upon the cadherins by the movements of cells could indeed result in the unfolding of the ectodomains. In particular, with cadherins which have many more than five repeat domains, e.g. cadherin-23, it is even conceivable that cells displaying such cadherins on their surface, which appear not to be in contact, happen to actually still remain in contact (despite having moved away from each other) or be already in contact (long before they have physically touched each other). Their still-folded (and still interacting) outermost EC1 and EC2 domains could very well have 'snaked' away from their surfaces, due to the existence of other unfolded repeat domains in the polypeptide chain, right up to the domains that lie next to the membranes. It is even conceivable that where there are enough of such partially unfolded cadherin polypeptides on a cell's surface, these could trap cells at large distances prior to the actual physical contact and then 'reel in' such cells into coming into close contact through the progressive calcium binding-aided folding of unfolded domains. This concept is schematically illustrated in Fig. 9.5, for a classical cadherin containing five ectodomains, e.g. E-cadherin or N-cadherin.

Are Longer Cadherins Used for Long-Distance Cell Contacts and Looser Adherence Junctions in Cancers? As a corollary to the above, in certain situations, e.g. in cancers in which cadherin expression profiles are altered and cadherins with larger numbers of repeat domains, e.g. cadherin-23, tend to be overexpressed, could the membranes of juxtaposed cells appear to be farther away from each other than in the case of adhesions based on classical cadherins? What implications might such a situation have for the cells in question? Would the adherence junctions be much more accommodating, in terms of allowing greater contact with the extracellular fluids, nutrients, cytokines and other factors?

Are N-cadherin's Outermost Ectodomains Likely to Be More Refoldable than Those of E-cadherin? Although it might seem like a specious argument at first sight, it could be argued that different kinds of cadherins have evolved for the mutual attachments of neuronal and epithelial cells because neurons are required to very quickly 'make or break' synaptic connections with other neurons, whereas contacts between epithelial cells tend to be somewhat more long-lived (and in certain instances, e.g. in tissues, lifelong contacts). The question is whether this is likely to have a biophysical correlate, in terms of the unfolding and refolding characteristics of the EC1 and EC2 domains of the E- and N-cadherins. If one were to make a prediction, one could argue that since neurons often need to rapidly separate away from other neurons in order to join yet other neurons, the mechanical forces involved in such rapid separation could result in the unfolding of the ectodomains of N-cadherin. If such domains were completely incapable of undergoing rapid refolding and no time were available to replenish the relevant regions of the neuron's surface with freshly synthesized and folded cadherins, neurons could become incapable of breaking contacts and making new contacts. In contrast, in epithelial cells, there would presumably be time available to replenish E-cadherins destroyed through the unfolding of ectodomains. Thus, the prediction would be that N-cadherin's ectodomains, especially domains EC1 and EC2, must be far more amenable to undergoing unfolding and refolding than those of E-cadherin. It would be interesting to verify whether this is indeed the case.

Are Homophilic Contacts Between N-cadherins Less Strong than Those Between E-cadherins? For reasons entirely similar to those laid out in the perspective mentioned immediately above, it could be argued that, on balance, contacts between N-cadherins would be weaker, in terms of dissociation constants, than contacts between E-cadherins, because neurons have a greater need, and tendency, to dissociate. On the other hand, it could also be argued that the areas of contacts between neurons tend to be smaller than those between epithelial cells and that, therefore, fewer cadherin molecules could be involved in building cell-cell contacts, with these contacts being much stronger than those between epithelial cells. It would be interesting to more fully examine which of these scenarios are true, given that there is already some evidence reviewed in this article to suggest that the former scenario is true.

Does Avidity Compensate for Differences in Affinity? In general, it might be argued that differences in the affinities of homophilic contacts between cadherins might not be extremely relevant, because it is conceivable that cells overexpress a cadherin that employs weaker homophilic contacts, if necessary, to ensure that there are a much larger number of molecules involved in making contacts, with greater avidity compensating for lower affinity. Of course, there is only a finite amount of area available on the cell surface, and cadherins and other CAMs have to share it with a multitude of other cell surface proteins and receptors. It would be interesting to understand how cells manage these issues of avidity versus affinity, based on their shapes and surface areas.

Do Calcium Channels and Transporters Regulate Cadherin Function and Cell Separation in Development and Cancer? Calcium channels and transporters on the cell membrane can be thought to underlie a region of the cell's surface which is engaged in cadherin-based cell-cell adhesive interactions, much like the underbrush on a forest's floor underlies the tall trees of a forest. Since cadherins have relatively weak (micromolar to millimolar) affinities for calcium, it is likely that there occurs a significant dissociation and reassociation of calcium, allowing cadherins to remain associated for long durations only when the equilibrium concentrations of calcium are sufficiently high to overcome the poor affinity of cadherins for calcium. Under such circumstances, if the replenishment of calcium from the serum were to be restricted, such that a requisitely high, equilibrium concentration of

calcium was slow to be re-established, ions transported away from the vicinity of cadherins by channels or transporters could facilitate rapid dissociation of cells in a region of the surface through rapid calcium depletion-aided loss of cadherin-cadherin *trans* contacts. Such a mechanism would be particularly effective if there were insufficient scope for the rapid replenishment of calcium owing to the restriction of ion movement by molecular crowding, calcium trapping or continued calcium removal by channel/transporter action. From a design and engineering viewpoint, one very efficient way to cause rapid cell-cell separation would be to upregulate the presence or activity of calcium channels and transporters, in a 'hit-and-run' mode, rapidly sucking up the available calcium and transporting it into the cell until cadherin-cadherin separation is achieved. Separation would, of course, immediately allow replenishment of calcium from the serum and extracellular fluids, but presumably by this time cells would have already separated. Intriguingly, there is evidence that verapamil which blocks calcium transport also does reduce cellular metastasis, which requires cell-cell separation (Tsuruo et al. 1985). There is also considerable evidence available now to suggest that metastasising cells do have upregulated levels of at least three different calcium channels (Mo and Yang 2018), suggesting that the above scenario is likely to be true. The concept is explained in Fig. 9.6 below.

Do Endogenous Proteases Function in Cell Separation? Cell-cell separations in cell culture experiments are achieved by adding trypsin and EDTA. Presumably, the EDTA chelates calcium away from the cadherins, making them more susceptible to proteolysis by trypsin in a non-calcium-bound state, and this allows trypsin to then selectively act on the cadherins to rapidly cut them away from each other and break up residual cadherin-cadherin contacts. If, indeed, this is how cell-cell separations are achieved in cell culture, it is conceivable that the same could also apply to cells in vivo, i.e. cells could produce and secrete trypsin whenever necessary, in the vicinity of the regions of a cell's surface attempting to dissociate from surrounding cells, to facilitate the dissociation process along with the mechanism outlined above about upregulation of calcium channels and transporters. It must be mentioned here that originally, it was assumed that trypsin is only made by acinar cells in the pancreas; however, for over two decades now, it is known from in situ hybridization, immunohistochemistry and reverse-transcription PCR that trypsin is expressed widely in epithelial cells in the oesophagus, stomach, skin, lung, small intestine, liver, kidney and extrahepatic bile duct and also in neuronal and splenic cells, as well as in the brain. Also, many cell types have receptors for trypsin on their surfaces. So, basically, the hypothesis is that a cell wishing to exit from a tissue, or alter its contacts, could simply produce and secrete trypsin to act in autocrine fashion.

Is There a Division of Labour Amongst Cadherin Domains for Cis (EC3, EC4 and EC5) and Trans (EC1 and EC2) Interactions? While there is now much evidence that EC1 and EC2 engage in adhesive interactions, not much is known about the role of the remaining ectodomains, i.e. EC3, EC4 and EC5, besides some information which is available about interactions of such domains with certain growth factors and some receptors. Our proposal is that EC3, EC4 and EC5 are domains that engage in *cis* interactions amongst cadherins on the same surface, with EC1 and EC2 are engaged primarily in *trans* interactions between cells. We further propose that *cis* and *trans* interactions cooperate and are interdependent. It is already known that EC1 and EC2 form monomers and dimers and nothing larger, either individually or in fusion constructs. We propose that EC3, EC4 and EC5 will turn out to form large multimolecular complexes consisting of folded polypeptide chains, individually and in fusion constructs, suggesting that they have a natural tendency to associate and cluster together and that they could bring cadherins together through *cis* interactions.

Do Cis Cadherin Interactions Contribute to Cell Surface Flatness? Calcium binding straightens up the entire set of five ectodomains

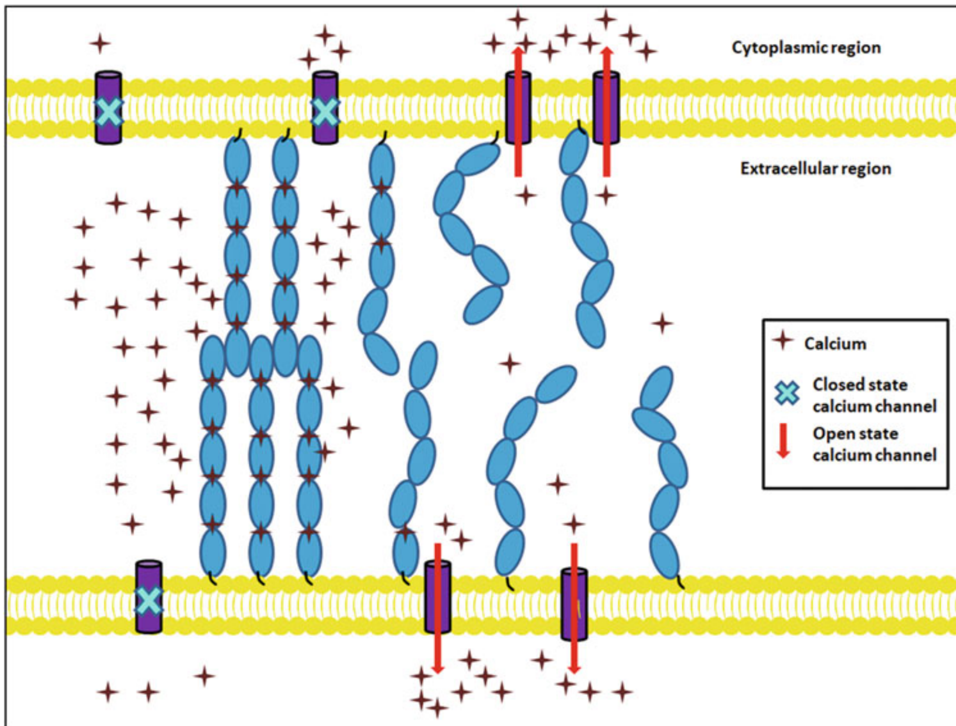

Fig. 9.6 Two adjacent regions of an adherence junction are shown. The one on the left is shown to have fewer calcium channels/transporters. These are also shown as being inactive and/or fewer in number. This allows calcium association-dissociation from the cadherins in the region to occur in such a manner that a high equilibrium concentration of calcium can be maintained in the region, with available calcium ions remaining titrated within the region by binding dissociation with cadherin. As a consequence, cell-cell contacts also remain stable. In contrast, in the region on the right, there are larger numbers of calcium channels transporting calcium into cells and/or higher numbers of functionally active channels/transporters. This results in the available calcium being depleted through transport into the cell and cellular organellar stores. This is proposed to result in a poorer (suboptimal) equilibrium calcium concentration in the region, owing to the slowness of replenishment of calcium from the serum. Consequently, the loss of the requisite calcium concentration in the region leads to the loss of cadherin-cadherin associations and separation of cells. The above is proposed as a mechanism for cell-cell separations during metastasis

into a rodlike rigid shape. Above, we have hypothesized that this can stimulate cis interactions between cadherins displayed on the same cell's surface, involving interactions of the EC3-EC3, EC4-EC4 and EC5-EC5 varieties, especially where a high concentration of calcium-bound cadherins pre-exists on the cell's surface allowing molecules to collide and associate. Here we propose that this is the primary mechanism for causing a cell's surface to be flat, i.e. by causing the formation of two-dimensional lattices of cadherins that then hold the plasma membrane in a flat shape. Of course, this could then be further supported by actin cytoskeletal dynamics involving the catenins. This concept is shown in Fig. 9.7, which shows how a combination of *cis* and *trans* interactions between E-cadherins can lead to the formation of a rigid adherens junction that ensures the flatness of the cell's surface. Of course, such a flat interface region between cells would need to be supported adequately by the formation of intracellular contacts of the cadherins with suitably disposed catenins and the actin cytoskeletal network.

Summary of Concepts Figure 9.7a also shows schematically some differences between E- and N-cadherin-based contacts involving cuboidal

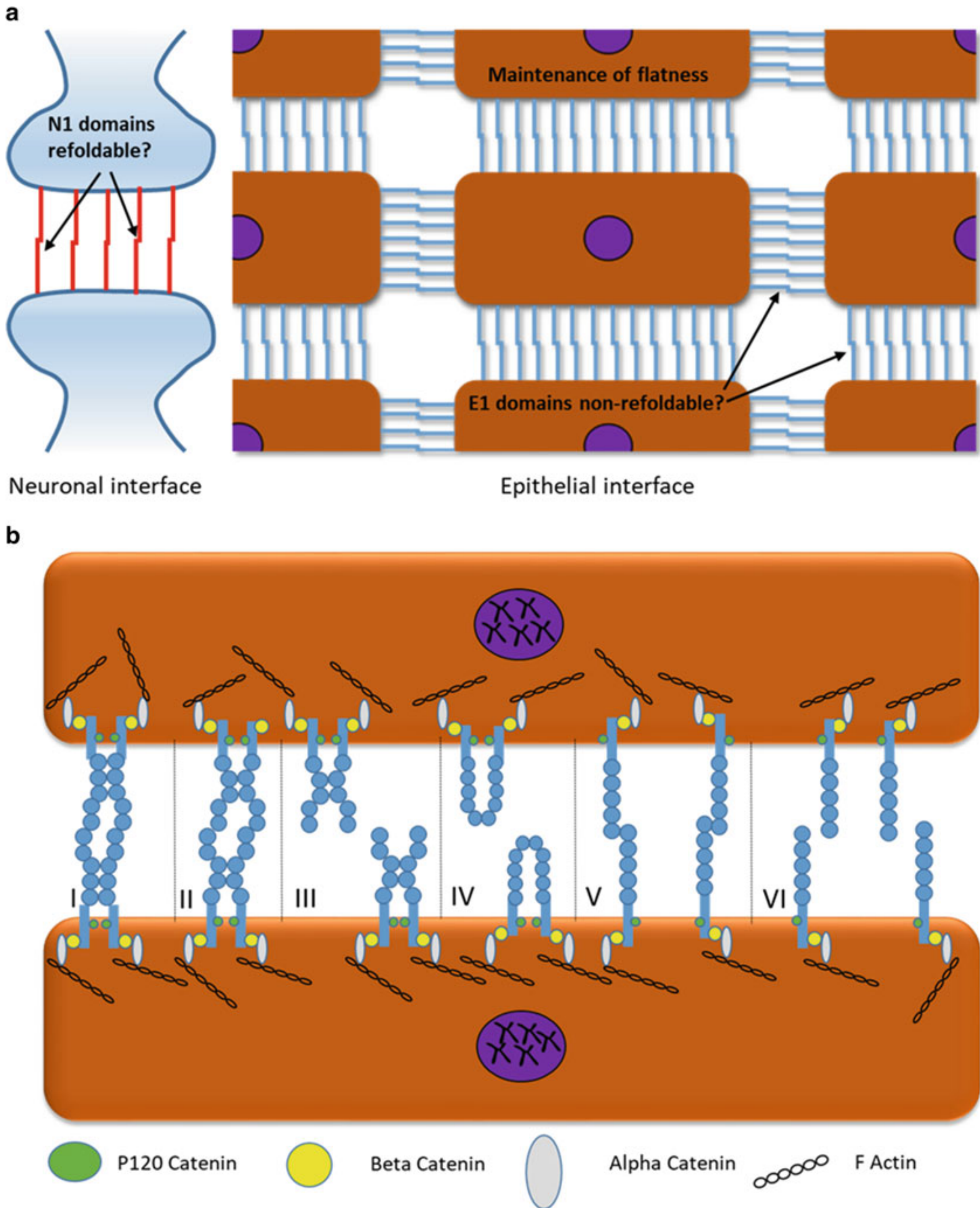

Fig. 9.7 *Panel* (**a**) Likely differential behaviour of N- and E-cadherin at neuronal cell-cell interfaces and epithelial cell-cell interfaces, respectively. It is proposed that the outermost ectodomains of N-cadherin must interact with lower affinity, there must be fewer N-cadherins present, and also the outermost ectodomains of N-cadherin must unfold and refold in a facile manner, to facilitate the rapid breaking and remaking of connections between neurons. On the other hand, it is proposed that the outermost ectodomains of E-cadherin must interact with higher affinity, there must be more E-cadherin molecules present and the outermost ectodomains of E-cadherin may not be amenable to unfolding and refolding (but require replenishment after breaking of contacts), to facilitate long-lived contacts. The figure also emphasizes that high-density E-cadherin contacts could facilitate maintenance of cell surface flatness. *Panel* (**b**) Six likely different modes of existence of the ectodomains of cadherins at cell-cell

epithelial cells and neuronal synapses, respectively. Our hypothesis is that the latter type of contacts involve EC1 and EC2 domains which are more easy to unfold and refold (refer to the concept outlined in Fig. 9.5) because neurons are required to frequently dissociate and reassociate. Thus, the EC1 and EC2 domains of N-cadherin are likely to be amenable to multiple rounds of unfolding and refolding. In contrast, epithelial cells engage in contacts that are far more long-lived, on the average. Therefore, their EC1 and EC2 domains could have evolved in a manner that would not allow them to undergo multiple rounds of unfolding and refolding. Also, these differential needs of neuronal and epithelial cells could be serviced by a lower density of cadherins at the cell-cell interface, as well as a lower strength of the EC1-EC1 trans interaction in the case of neurons. All these possibilities are summarized in Fig. 9.7a, which would be fascinating to explore experimentally. Figure 9.7b serves the purpose of summarizing all the different modes of cadherin-cadherin interactions that have been discussed in this review.

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Fig. 9.7 (continued) interfaces. Mode I shows *cis* interactions involving EC3, EC4 and EC5 and *trans* interactions involving primarily EC1, supported by EC2. Mode II shows an interaction similar to mode I, except that *cis* interactions involve only EC3 and EC4. Mode III shows only *cis* interactions involving EC3 and EC4 with no *trans* interactions. Mode IV also shows only *cis* interactions; however, these involve only EC1 with no *trans* interactions. Mode V shows *trans* interactions involving EC1 with no *cis* interactions. Mode VI shows cadherins with neither *cis* nor *trans* interactions

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