Proteolytic cleavage of cadherins: Functional role of the cleaved extracellular and cytoplasmic domains

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Abstract

Dynamic regulation of cadherin mediated cell-cell adhesion is crucial for morphogenesis and tissue homeostasis. Cadherin adhesive function can be regulated by distinct proteolytic cleavage events, resulting in release of either the ectodomain or cytoplasmic domain. However, it is unclear if the released fragments have biological activity by themselves. This thesis analyses the functional significance of the generated cadherin fragments.

Using *Xenopus laevis* development as model system, it was shown in this thesis that the C-cadherin ectodomain has the capacity to interfere with morphogenetic movements *in vivo*, since overexpression of this fragment in early *Xenopus* embryos resulted in severe gastrulation defects. The observed defect was due to inhibition of convergent extension movements, a process that requires downregulation of cadherin adhesiveness. However, ectoderm integrity was not affected by expression of the cadherin ectodomain, demonstrating that this fragment specifically interferes with processes that require a tight regulation of cadherin function. The EC1 repeat of the cadherin extracellular domain contained all the necessary information to interrupt gastrulation movements. This activity was not dependent on the amino acid tryptophan at position 2, which is crucial for an adhesive functional molecule. Surprisingly, overexpressing the ectodomain of other classical cadherins caused similar defects, indicating the capacity for heterophilic interactions.

The cadherin ectodomain may not only have a function during development, but could directly contribute to cancer progression. Indeed, a direct correlation was found between increased soluble E-cadherin levels in sera of melanoma patients and tumor mass.

The cadherin cytoplasmic domain was shown to serve as a substrate for the PS1/γ-secretase complex, raising the possibility that the released domain may translocate to the nucleus to regulate gene expression, in analogy to the Notch signalling pathway. Using full length cadherins fused to either Gal4 or Gal4VP16 transcription factors in combination with Gal4-dependent reporter assays, it was shown that cadherins translocate to the nucleus, but are unable to function as transcriptional activators. Interestingly, nuclear translocation appeared to be independent of PS1 and 2.

Together, the presented results suggest that proteolytic cleavage not only alters cadherin adhesive activity, but may also serve as a mechanism to signal changes in adhesiveness to the nucleus.

Zusammenfassung

Die dynamische Regulierung der Cadherin-vermittelten Zell-Zelladhäsion ist entscheidend für Morphogenese und Homöostase von Geweben. Ein Mechanismus zur Regulierung von Cadherinen ist die proteolytische Freisetzung der extrazellulären und cytoplasmatischen Domäne. Bislang ist jedoch unklar, ob die Spaltprodukte selbst über biologische Aktivität verfügen. Ziel dieser Arbeit ist, die funktionale Bedeutung der freigesetzten Cadherin-Domänen zu untersuchen.

Mit Hilfe des *Xenopus laevis* Modellsystems konnte in dieser Arbeit gezeigt werden, dass die extrazelluläre Domäne des C-Cadherins morphogenetische Bewegungen beeinträchtigen kann, da eine Überexpression dieser Domäne zu einer Störung der Gastrulation führte. Dieser Effekt beruht auf einer Inhibierung der konvergenten Extension, einem Prozess, der verminderte Cadherin-Aktivität erfordert. Die Integrität des Ektoderms blieb jedoch von einer solchen Überexpression unbeeinträchtigt. Daraus wird ersichtlich, dass die extrazelluläre Domäne spezifisch solche Prozesse beeinflusst, die eine strenge Regulierung der Cadherin-vermittelten Zelladhäsion benötigen. Es konnte gezeigt werden, dass die EC1 Domäne des C-Cadherins alle notwendigen Informationen zur Störung der Gastrulation enthält. Diese Aktivität ist unabhängig von der Aminosäure Tryptophan an Position 2, welche für die adhäsive Funktion von Cadherinen essentiell ist.

Die Expression von extrazellulären Domänen anderer klassischer Cadherine führte zu ähnlichen Defekten, was auf heterophile Cadherin-Interaktionen schließen lässt.

Um den Einfluß der extrazellulären Domäne von Cadherinen auf Tumorprogression zu untersuchen, wurde der Gehalt an löslichem E-Cadherin in Seren von Melanompatienten analysiert. In dieser Arbeit konnte eine direkte Korrelation der Mengen an E-Cadherin und S100, einem Marker für die Malignität von Melanomen, nachgewiesen werden.

In früheren Studien wurde gezeigt, dass die cytoplasmatische Domäne von Cadherinen als Substrat für den PS1/γ-Sekretase-Komplex dienen kann. Dies wirft die Frage auf, ob die freigesetzte cytoplasmatische Domäne, ähnlich dem Notch Signaltransduktionsweg, in den Zellkern gelangen und Transkription aktivieren kann. Um die Rolle des freigesetzten Fragmentes zu untersuchen, wurden Cadherine mit den Transkriptionsfaktoren Gal4 oder Gal4VP16 fusioniert. Transfektionsstudien mit einem Gal4-abhängigen Reporter zeigten, dass Cadherine in der Tat kerngängig sind, aber keine Aktivierungsfunktion besitzen.

Zusammenfassend weisen die in dieser Arbeit dargestellten Ergebnisse darauf hin, dass die proteolytische Spaltung von Cadherinen nicht nur der Regulierung von Adhäsion dient, sondern auch einen möglichen Mechanismus darstellt, Veränderungen in der Adhäsion zum Zellkern zu vermitteln.

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1. Introduction

The dynamic regulation of adhesive contacts between cells is crucial for both embryonic development and adult life. The cadherin family of adhesion molecules plays an important role in the establishment, maintenance, and regulated turnover of cell-cell contacts. As such, cadherins are involved in many cellular processes, including the sorting of cells into separate layers (Takeichi, 1995), cellular rearrangements and thus changes in the shape of tissues (Keller, 2002), establishment of epithelial sheets by formation of junctional complexes (Perez-Moreno et al., 2003), synapse formation (Uchida et al., 1996), epithelial to mesenchymal transition (Hay and Zuk, 1995), and cell migration (Godt and Tepass, 1998).

1.1 The cadherin superfamily

The first cadherins were identified for their ability to mediate Ca^{2+}-dependent adhesion in cultured cells and for their role in the development of early mouse embryos (Kemler et al., 1977; Takeichi, 1977). Cadherins are Ca^{2+}-dependent, transmembrane cell-cell adhesion molecules. They constitute a large protein family sharing the presence of extracellular cadherin repeats (EC domains) in their extracellular region as a common feature. This domain is approximately 110 amino acids long and contains several highly conserved sequences. The tandemly repeated EC domains are connected by Ca^{2+}-binding motifs. Upon Ca^{2+} binding they produce a rigidified, rod-like ectodomain projecting from the cell that interacts with a cadherin on an opposing cell (Gooding et al., 2004).

The number of cadherin repeats varies from five to more than 30 among the different cadherin family members. Based on structural differences in the EC domain and cytoplasmic domain, the cadherin superfamily can be further divided into different subgroups: type I (classical) and type II cadherins, desmosomal cadherins, protocadherins, atypical cadherins, and cadherin-related proteins (Patel et al., 2003)(Fig. 1.1).

Classical cadherins are the best characterized subgroup, which are often associated with various forms of adhesive junctions. Initially, these proteins were named after the tissue in which they were first identified, for example E (epithelial) -cadherin or N (neural) -cadherin, but it has become evident that the expression pattern of the different members is often more complex. Their extracellular domain consists of five EC domains with highly conserved motifs including a tryptophan at position 2 (W2) of the EC1 domain and an HAV sequence located in EC1 as well. They are part of a multiprotein complex, in which their cytoplasmic domain interacts with ß-catenin and is linked to the actin cytoskeleton

via α-catenin. The structure of the type I, classical cadherin complex will be discussed in detail below.

Type II cadherins are structurally very similar to type I cadherins, but differ in their extracellular domain in that they have two conserved tryptophan residues (W2 and W4) in the EC1 domain and lack the HAV motif. Type II cadherins are often upregulated in migrating cells, for example *Xenopus* cadherin-11 is highly expressed in migrating neural crest cells (Borchers et al., 2001).

Desmosomal cadherins (desmocollins and desmogleins) are adhesion molecules of desmosomal junctions, which interact with plakoglobin (γ-catenin) and desmoplakin, thus providing a linkage to intermediate filaments of the cytoskeleton (Garrod et al., 2002).

T (truncated) -cadherin belongs to a small group of atypical cadherins, which lacks the cytoplasmic domain as well as a part of the transmembrane domain and is linked to the membrane by a glycosyl-phosphatidylinositol anchor (Vestal and Ranscht, 1992). T-cadherin mediates Ca^{2+}-dependent cell adhesion by an as yet unidentified mechanism.

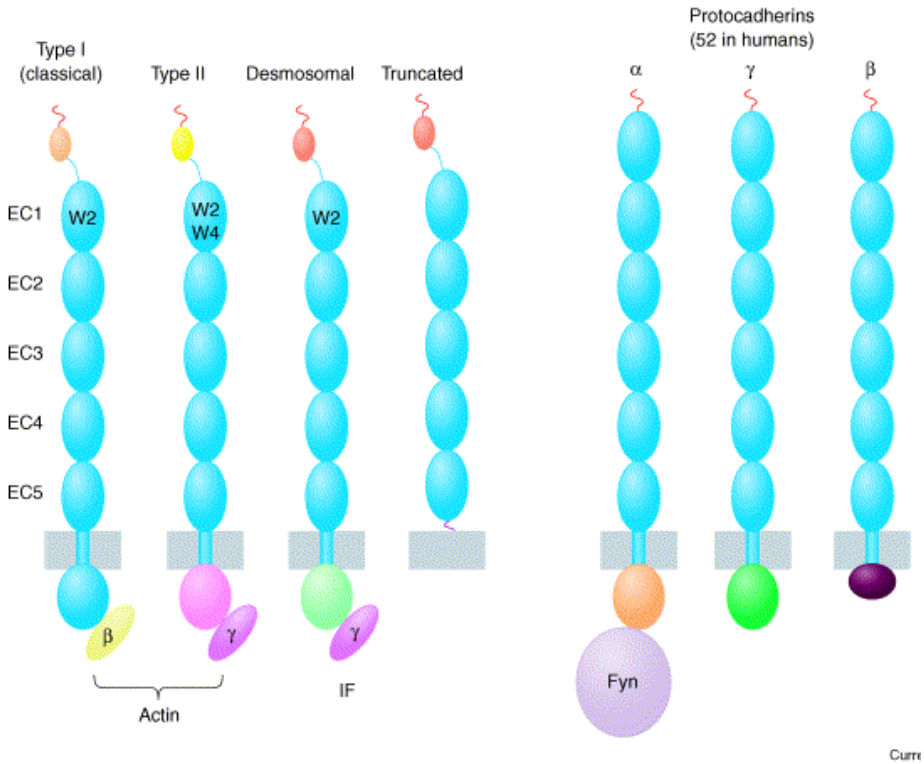


Figure 1.1: Schematic representation of the cadherin superfamily: Type I and II cadherins are linked to the actin cytoskeleton via catenins, desmosomal cadherins to intermediate filaments (IF). These subgroups contain five extracellular domain repeats (EC1-5) and share conserved tryptophan residues (W2; W4) in the EC1 domain, which are crucial for their adhesive activity. Truncated cadherin is a member of atypical cadherins due to the lack of a cytoplasmic and transmembrane domain. Protocadherins (α, β and γ subfamilies) each contain six EC domains and have diverse cytosolic binding partners (taken from Patel et al., 2003).

Protocadherins are only found in vertebrates and comprise a very large and divergent cadherin-related subgroup. In contrast to classical cadherins, these proteins contain up to seven extracellular domain repeats and have divergent intracellular domains that do not interact with β-catenin but instead with other proteins such as Fyn-kinase (Frank and Kemler, 2002). In the human and mouse genome protocadherin genes are arranged in three clusters (α, β and γ) that together encode more than 50 different protocadherin proteins (Wu and Maniatis, 2000; Wu et al., 2001), which are mostly expressed at synaptic junctions of the nervous system (Kohmura et al., 1998). Recent studies have given insight into the functional role of protocadherins in morphogenetic processes. The *Xenopus* paraxial protocadherin PAPC is expressed in the paraxial mesoderm of the gastrulating *Xenopus* embryo and promotes selective adhesion and convergent extension movements of the shaping embryo (Kim et al., 1998).
Many cadherin-related proteins have been identified that contain cadherin EC domains, but do not belong to a distinct subgroup, for example *Drosophila* Dachsous, Fat and Flamingo, which are involved in establishing planar cell polarity (Saburi and McNeill, 2005).

1.2 Classical cadherins and their association with catenins

Since this thesis mainly deals with classical cadherins, a more detailed description of this subfamily is given. Classical cadherins are single pass transmembrane proteins that consist of an extracellular domain with five EC repeats and a highly conserved cytoplasmic domain with approximately 150 amino acids. They are produced as immature proteins with a pro-domain at the N-terminus of EC1. The pro-domain is removed by furin family proteases, and this cleavage is essential for activation of the cadherin adhesive function (Haussinger et al., 2004; Ozawa and Kemler, 1990). The extracellular domain of classical cadherins forms a lateral, cis dimer that is required for homophilic binding and cell adhesion (Brieher et al., 1996).
The cadherin cytoplasmic domain has been shown to interact with a group of proteins termed catenins (Aberle et al., 1996): $p120^{ctn}$ binds the juxtamembrane region, whereas β-catenin interacts with the membrane distal region of the cadherin cytoplasmic domain. α-catenin associates with β-catenin and actin via two distinct domains and is therefore thought to provide a stable connection between the cadherin complex and the actin cytoskeleton (Fig. 1.2).

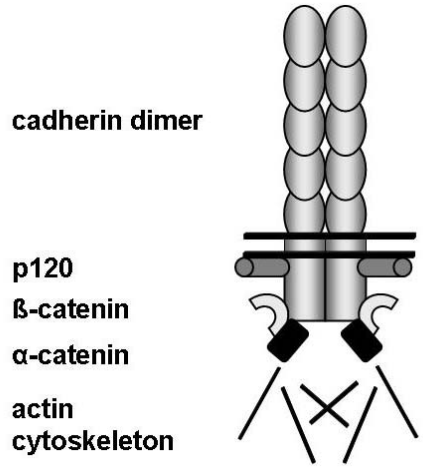


Figure 1.2: Schematic representation of the cadherin-catenin complex. The cadherin extracellular domain forms a lateral (cis) dimer required for trans binding and cell adhesion. The cytoplasmic domain interacts with catenins: $p120^{ctn}$ binds the juxtamembrane region, whereas β-catenin interacts with the membrane distal region of the cytoplasmic domain. α-catenin associates with β-catenin and actin via two distinct domains.

β-catenin is the mammalian homologue of the *Drosophila* segment polarity protein Armadillo (Riggleman et al., 1989). In addition to its function in cadherin-based cell adhesion, β-catenin also plays a key role in the Wnt signalling pathway (Hecht and Kemler, 2000): In the absence of Wnt signalling, levels of cytosolic β-catenin are kept low by phosphorylation of its N-terminus by a protein complex containing among others the glycogen synthase-3 β kinase (GSK-3 β). Subsequent ubiquitination results in targeted degradation of β-catenin by the proteasome. Binding of Wnt to its receptor frizzled leads to inhibition of the GSK-3 β-complex and thus accumulation of cytosolic β-catenin, which translocates to the nucleus and binds to TCF/LEF transcription factors to activate expression of target genes. Upregulation of β-catenin signalling has been implicated in the formation of various cancer types. Whether the roles of β-catenin in signalling and adhesion are independent of each other or whether these processes compete for a common pool of β-catenin is not fully understood yet (Gottardi and Gumbiner, 2004; Nelson and Nusse, 2004).

α-catenin is structurally unrelated to β-catenin. It shares homology with the actin associated protein vinculin, containing three different vinculin homology regions termed VH1 to VH3. Biochemical data showed that α-catenin can bind to β-catenin via VH1 and to actin via VH3 (Rimm et al., 1995). From these experiments it was concluded that α-catenin directly connects adherens junctions and the cytoskeleton by simultaneously binding to β-catenin and actin. However, recent studies provide evidence that α-catenin does not simultaneously associate to adherens junctions and actin, but rather is involved

in regulating actin dynamics, perhaps at sites of adherens junctions (Drees et al., 2005; Yamada et al., 2005).

$p120^{ctn}$ is another member of the armadillo repeat protein family. It was originally described as a substrate of p60v-Src protein kinase and is now known to exert diverse functions that will be discussed in more detail below.

In addition, many more proteins are associated with the cadherin complex, including receptor tyrosine kinases and phosphatases, the ubiquitin-ligase Hakai, and the immunoglobulin-like cell-cell adhesion molecule Nectin.

1.3 Cadherin binding and specificity

Tissue separation during embryonic development is mediated by cells sorting out from each other. One mechanism that is thought to drive cell sorting is the differential expression of cadherins, such as the switch from E- to N-cadherin expression in neural tube formation (Hatta and Takeichi, 1986), or differential cadherin expression in the brain that defines tissue borders. Indeed, improper expression of cadherins in neural crest cells or in motor neurons did alter proper cell sorting and targeting (Nakagawa and Takeichi, 1998; Price et al., 2002).

Initially, cadherin homophilic binding properties, which means that one type of cadherin expressed on a cell preferentially binds to an identical molecule on another cell, were thought to be the underlying mechanism for selective cell recognition and thus cell sorting. This conclusion came from *in vitro* experiments in which cells expressing different types of classical cadherins were used in aggregation assays and sorted out from each other to form separate cell aggregates (Nose et al., 1988).

However, there are further mechanisms contributing to sorting. Cell sorting can be mediated by different levels in the expression of a single cadherin (Friedlander et al., 1989; Steinberg and Takeichi, 1994). An *in vivo* example is the correct localization of the Drosophila oocyte, which is controlled by differential amounts of *Drosophila* E-cadherin expressed in germline cells and in associated follicle cells (Godt and Tepass, 1998), and which plays a crucial role in formation of the anterior-posterior body axis (Gonzalez-Reyes and St Johnston, 1998).

In addition, it has become evident that cadherins display a much wider array of binding specificities than was previously assumed and that the binding specificity does not necessarily determine cell sorting. This was shown using laminar flow assays with purified cadherin proteins and cell aggregation assays with cells expressing different classical cadherins. For example, cells expressing human N-cadherin or human E-cadherin bound equally well to a recombinant human E-cadherin substrate, but the two cell types did sort out from each other (Niessen and Gumbiner, 2002).

The molecular nature of the cadherin adhesive bond is still controversial. Structural studies of cadherin extracellular domains by crystallography provide evidence that interaction takes place between partner EC1 domains (Boggon et al., 2002; Shapiro et al., 1995). In fact, a crucial structural feature for the adhesive activity of cadherins is the highly conserved tryptophan at position 2 (W2) of the EC1 domain of all type I, II, and desmosomal cadherins. The W2 sidechain of one molecule is inserted into a hydrophobic pocket of another molecule and vice versa, thereby forming an adhesive interface called ´strand dimer` (Patel et al., 2003). The critical role for W2 in adhesion was highlighted by experiments using cadherin mutants in which the W2 was exchanged by alanine (W2A): cadherin-mediated aggregation of cells expressing this mutant was completely abolished (Shan et al., 2000; Tamura et al., 1998). In contrast, the involvement of the conserved HAV sequence of classical cadherins, which was initially implicated in mediating the adhesive interface, could not be confirmed by mutational analysis (Renaud-Young and Gallin, 2002).

Cadherins are able to form trans (adhesive) dimers between molecules from opposing cells or cis (lateral) dimers between molecules presented from the same cell, and the formation of lateral dimers has been shown to be required for initiation of adhesive strength (Brieher et al., 1996). Precipitation studies of epitope-tagged cadherins from transfected cells indicated that both cis and trans dimers can be mediated by the strand dimer interface (Klingelhofer et al., 2002).

The EC1 domain has also been implicated in conferring sorting specificity: P-and E-cadherin expressing transfectants sorted out into separate aggregates, but exchanging the EC1 domain of the two molecules also altered specificity of cell aggregation (Nose et al., 1990).

Some reports provide evidence that other domains besides EC1 are involved in adhesive binding. Direct measurement of intermolecular forces between cadherin molecules revealed several distinct force maxima, which could be explained by a complete overlap of cadherin ectodomains during homophilic binding (Sivasankar et al., 1999). Indeed, the study of Chappuis-Flament et al. (2001) demonstrated that multiple cadherin extracellular repeats are involved in homophilic binding and adhesion: Using different C-cadherin deletion constructs in bead aggregation assays and laminar flow adhesion assays, the authors showed that a minimum of three of the EC domains were required for effective binding and adhesion.

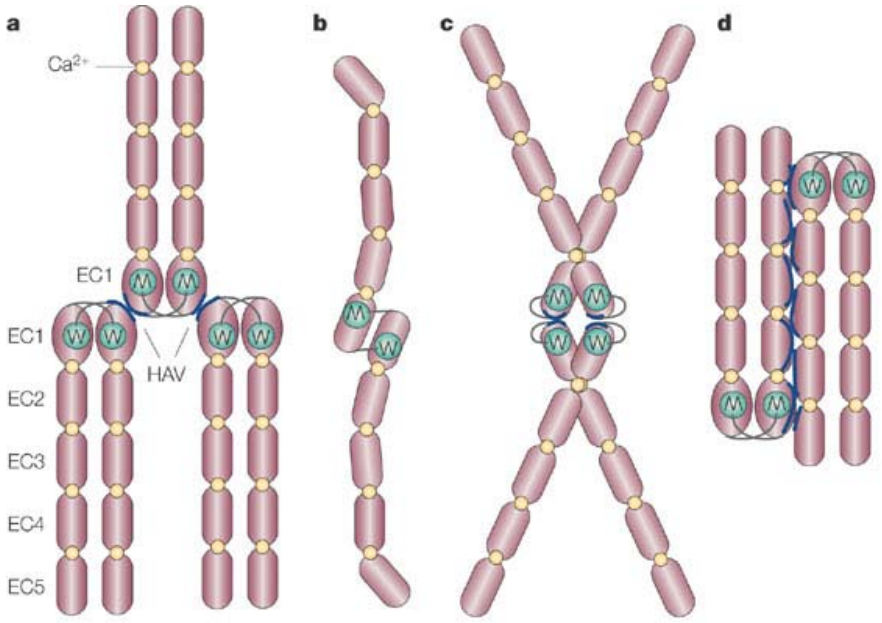


Figure 1.3: Several alternative models exist for the formation of the cadherin lateral and adhesive bond. (a) Lateral dimers are mediated by the trp 2 (W) strand dimer interface of EC1, adhesive binding is mediated via a different site, possibly the HAV motif. **(b)** Trp 2 mediates formation of adhesive, trans bonds. **(c)** Lateral dimers are formed via the Ca^{2+} -binding sites between EC1 and EC2, and trp 2 inserts into the hydrophobic pocket of the same molecule leading to a conformational change that activates trans dimerization. **(d)** Adhesive binding involves overlap of several EC domains (taken from Gumbiner et al., 2005).

Based on these findings several alternative models for the cadherin lateral and adhesive bond have been proposed (Gumbiner, 2005)(Fig. 1.3). In the ´linear zipper` model, which is based on the crystal structure of the N-cadherin EC1 domain, the strand dimer interface induces formation of lateral dimers that in turn engage in homophilic bonds via a different binding site (Shapiro et al., 1995). Analysis of the crystal structure of the complete C-cadherin extracellular domain indicated that the W2 strand dimer interface is responsible for formation of homophilic trans bonds (Boggon et al., 2002). In a revised model the flexible nature of the cadherin extracellular domain may allow the existence of cadherin cis dimers in equilibrium with trans dimers, consistent with the data that both are mediated by the W2 interface. In a third model, cis dimerization occurs at the Ca^{2+}-binding site between EC1 and EC2, and W2 insertion into the hydrophobic pocket of the same molecule leads to a conformational change that activates trans dimerization (Pertz et al., 1999). Another model implicates further EC domains in the formation of the homophilic bond upon lateral dimerization via the W2 interface (Chappuis-Flament et al., 2001; Sivasankar et al., 1999).

1.4 Regulation of cadherin adhesive activity

Various mechanisms have been shown to underlie the regulation of cadherin adhesive activity. A major form of regulation takes place at the level of cadherin gene expression. However, post-transcriptional regulatory mechanisms are necessary to ensure a rapid and dynamic change in the adhesive state of cadherins at the cell surface in response to various internal or external signals. Some main regulatory mechanism of cadherin activity will be discussed below.

1.4.1 Cadherin gene expression

Repression of E-cadherin has been extensively studied and the crucial role of repressor proteins such as snail and slug in epithelial to mesenchymal transition (EMT) and E-cadherin silencing during embryogenesis is now well established (Nieto, 2002). A yeast one hybrid screen using an E-cadherin promoter element as bait identified snail, slug and E12/E47 as factors directly binding to the E-cadherin promoter (Cano et al., 2000), and functional analysis of these factors revealed that they act as strong repressors of the mouse and human E-cadherin promoters. Indeed, knock-out mice for the different repressors of E-cadherin indicated that snail is required for induction of EMT during mouse embryogenesis, whereas E12/E47 and slug may be essential for maintenance of mesodermal tissue (Nieto, 2002).

1.4.2 Modulating the phosphorylation status of cadherins and catenins

Cadherins and signalling receptors are able to affect each others` function in a bidirectional crosstalk: On the one hand the function of the cadherin complex is regulated by modulation of its phosphorylation status. Cadherins as well as β-catenin and $p120^{ctn}$ can serve as substrates for many different receptor tyrosine kinases (such as EGFR, HGFR, VEGFR and FGFR) and non-receptor tyrosine kinases (such as c-Src). In general, phosphorylation results in disassembly of the cytoplasmic adhesive complex and disruption of cadherin mediated cell adhesion, whereas tyrosine phosphatases have been described to increase adhesion (Christofori, 2003). One mechanism by which E-cadherin activity can be downregulated in response to tyrosine kinase activation has been described by Fujita et al. (2002): in a yeast two-hybrid screen the authors identified a novel E3 ubiquitin-ligase (termed Hakai) that interacts with E-cadherin in a tyrosine phosphorylation dependent manner. Hakai mediates ubiquitination of tyrosine phosphorylated E-cadherin, leading to endocytosis of the E-cadherin complex and thus increased cell motility.

On the other hand cadherins can trigger activation of cadherin-associated growth factor receptors. For example, formation of cell contacts results in ligand induced EGFR

signalling upon association with E-cadherin, which in turn activates the MAPK pathway (Pece and Gutkind, 2000). N-cadherin has been shown to associate with different members of the FGFR family. Data suggests that N-cadherin may mediate binding of FGF to the corresponding receptor, but prevent subsequent internalization of the receptor and that would result in prolonged MAPK signalling and increased cell motility (Suyama et al., 2002). The vascular endothelial-specific VE-cadherin can associate with VEGFR-2 and downregulate VEGF-induced proliferative signalling: upon VE-cadherin expression and clustering at intercellular junctions, VE-cadherin interacts with VEGFR-2 and reduces VEGFR-2 phosphorylation, presumably by bringing VEGFR-2 in contact with phosphatases localized at the junctions (Lampugnani et al., 2002).

1.4.3 The role of small GTPases in cell adhesion

Recent findings have established an important role for small GTPases, especially the Rho subfamily including Rac, Rho, and Cdc42, in cadherin mediated adhesion and assembly of junctional complexes.

Braga et al. could show that Rho and Rac are necessary for the formation of cadherin-dependent cell contacts in keratinocytes (Braga et al., 1997), and that Rac functions in recruiting actin to sites of cadherin complexes. Rac was found to be involved in assembly of adhesive contacts in a reciprocal signalling mechanism: When *Xenopus* C- or human E-cadherin expressing cells were allowed to bind to the corresponding cadherin substrate, a rapid stimulation of GTP-Rac was observed (Kovacs et al., 2002; Noren et al., 2001). Activated Rac, in turn, resulted in accumulation of E-cadherin, β-catenin, and actin at sites of cell-cell contact and promoted assembly of adhesive complexes. The underlying mechanism is not fully analysed yet. However, the current model suggests that cadherin ligation at forming cell contacts activates Rac signalling, which leads to stimulation of actin assembly by the Arp2/3 actin nucleator complex that is critical for the extension of broader contact zones (Yap and Kovacs, 2003).

Several guanine nucleotide exchange factors (GEF) have been described to be associated with the cadherin complex, which might link cadherin ligation and Rac activation. Tiam-1 is an activator of Rac and was shown to inhibit HGF-induced scattering of MDCK cells by increasing E-cadherin mediated adhesion (Hordijk et al., 1997).

In some settings, Rho and Cdc42 were found to function in a similar way as Rac, but these data are less consistent (Gumbiner, 2005). Recently, another member of small GTPases, Rap1, was reported to regulate the formation of E-cadherin based cell-cell contacts (Hogan et al., 2004).

1.4.4 Cadherin turnover and trafficking – the role of p120^{ctn}

Recent studies have revealed the importance of intracellular trafficking as a means of regulating cadherin function. After synthesis cadherins are transported from the Golgi apparatus to the plasma membrane via sorting signals present in the cadherin cytoplasmic domain (Miranda et al., 2001). At the cell surface, cadherins do not exist in adhesive complexes in a static manner, but are constantly internalized into intracellular vesicles and subsequently recycled back to the cell surface via sorting endosomes or delivered to lysosomes for degradation (Le et al., 1999).

The exact mechanism of cadherin internalization is not well understood, but accumulating evidence suggests that p120^{ctn} serves as a key regulator in this process. Indirect analysis of p120^{ctn} function in adhesion by deleting the cadherin juxtamembrane region or mutating the p120^{ctn} binding site indicated that p120^{ctn} could have both negative and positive regulatory effects (Ozawa and Kemler, 1998; Thoreson et al., 2000; Yap et al., 1998). Using the p120^{ctn} deficient colon cancer cell line SW480, Ireton et al. demonstrated that restoring normal levels of p120^{ctn} in these cells resulted in increased E-cadherin levels and a concomitant rescue of epithelial morphology (Ireton et al., 2002). The underlying mechanism appeared to be post-translational since mRNA levels were unaffected and involved direct binding of p120^{ctn} to E-cadherin, resulting in an increase in E-cadherin half life. This indicated that p120^{ctn} binding promotes cadherin stability at the membrane. Further studies could then show that the stabilizing effect of p120^{ctn} was due to its ability to regulate cadherin turnover at the cell surface (Davis et al., 2003; Xiao et al., 2003). Reduction of p120^{ctn} by siRNA expression in human and mouse cell lines strongly reduced cadherin levels, but did not affect trafficking or processing of newly synthesized cadherins. Instead, cadherins were rapidly degraded at the cell surface in the absence of p120^{ctn}. This stabilizing effect of p120^{ctn} is common to all cadherins tested thus far (E-, VE-, N- and P-cadherin). Treatment of cells with inhibitors of lysosomes as well as proteasomes partly blocked degradation, implicating both pathways in this process. How exactly p120^{ctn} exerts its function is not known, but may involve proteins that compete with p120^{ctn} for cadherin binding, such as Hakai or presenilin 1 (PS1) (Fujita et al., 2002).

Besides stabilization of cadherins p120^{ctn} does have additional functions. After dissociation of the cadherin complex, p120^{ctn} accumulates in the cytoplasm, where it promotes cell migration by activation of Rac and inhibition of Rho (Anastasiadis and Reynolds, 2001). Furthermore, p120^{ctn} plays a crucial role in gene expression as well, by translocation to the nucleus and direct interaction with the transcriptional repressor Kaiso (Daniel and Reynolds, 1999). This interaction modulates both canonical and non-canonical Wnt signalling (Kim et al., 2004; Park et al., 2005).

1.5 Proteolytic cleavage of cadherins

In 1987, Wheelock et al. purified an 80 kD E-cadherin soluble fragment from serum free medium conditioned by MCF-7 cells. They further reported that this soluble fragment caused scattering of epithelial cells in culture, suggesting that it was able to interfere with cell adhesion *in vitro*. This was the first of a series of studies establishing the concept that distinct proteolytic cleavage of cadherins serves as a mechanism to rapidly regulate cadherin adhesive function at the cell surface, by releasing both the extracellular and cytoplasmic cadherin domain as soluble fragments and thus disrupting the cadherin complex. However, whether the released soluble fragments have biological functions of their own is still unclear.

1.5.1 Cadherin ectodomain shedding

Releasing large extracellular portions of substrate molecules by endoproteolytic activities, mainly mediated by metalloproteases, is a process called ectodomain shedding. Modulation of surface molecules via ectodomain shedding might inactivate the substrate protein by reducing protein concentrations at the cell surface, or activate it by generating a biologically active, soluble form of the substrate that acts in a paracrine way on neighbouring cells. Ectodomain shedding has been described for a wide range of proteins, including cytokines, growth factors and their receptors, and adhesion molecules (Dello Sbarba and Rovida, 2002).

First evidence that cadherin ectodomain shedding might play a role in development was published by Roark et al., showing that N-cadherin expression was specifically downregulated during chick retinal development, and that downregulation of the full length N-cadherin molecule as well as the concomitant accumulation of a soluble 90 kD fragment was inhibited using metalloprotease inhibitors (Roark et al., 1992). Purification and characterization of the soluble 90 kD fragment revealed that it retained biological function by promoting cell adhesion and neurite outgrowth (Paradies and Grunwald, 1993).

The biological function of the released cadherin ectodomain was addressed by Noe et al. (2000). E-cadherin was immunoprecipitated from MCF-7 and MDCK cells and incubated with the matrix metalloproteases (MMP) matrilysin and stromelysin-1, showing that these proteases directly cleave E-cadherin. To test whether the released extracellular fragment retained functional activity, *in vitro* studies were performed using conditioned medium of MDCK cells that had been preincubated with recombinant matrilysin or stromelysin-1, thus inducing the cleavage and release of E-cadherin ectodomain. The presence of conditioned medium containing increased E-cadherin ectodomain caused induction of MDCK cell invasion into collagen type I and inhibition of MDCK cell aggregation. These results led to the suggestion that the cadherin extracellular domain may retain biological

function after release from the cell surface to modulate cadherin activity in a paracrine way.

In an attempt to identify the major cadherin ectodomain-generating proteases, Reiss et al. (2005) used a panel of ADAM-deficient fibroblasts and neuronal cells to demonstrate that ADAM10 is the major protease responsible for constitutive and regulated N-cadherin ectodomain shedding in fibroblasts and neuronal cells. This finding was also confirmed for E-cadherin, by using again ADAM10-deficient fibroblasts and human HaCaT cells (Maretzky et al., 2005). ADAM10 mediated cadherin shedding appeared to be involved in regulation of cell adhesion, migration and also β-catenin nuclear signalling.

Cleavage of cadherins by proteases has been implicated in the process of apoptosis as well. Inhibitor studies with cells undergoing apoptosis suggested that cadherins are cleaved by a metalloprotease activity to release the extracellular domain and also by members of the caspase family of apoptosis effector proteases (predominantly caspase-3) to cleave the cytoplasmic domain (Herren et al., 1998; Steinhusen et al., 2001). This caused dissolution of adhesive junctions and loss of cell-cell contacts, which are typical morphological changes observed during apoptosis.

1.5.2 Cleavage of the cadherin cytoplasmic domain

The cadherin cytoplasmic domain can serve as substrate for a presenilin-1 (PS1)/γ -secretase mediated cleavage as well. Treatment of A431 cells undergoing apoptosis with specific protease inhibitors resulted in the production of three E-cadherin C-terminal fragments of 38, 33 and 29 kD, termed CTF1, CTF2 and CTF3, respectively (Marambaud et al., 2002) (Fig. 1.4). Production of CTF1 was blocked by a general MMP inhibitor, suggesting that this fragment represented the counterpart of the secreted ectodomain, whereas production of CTF3 was blocked by caspase-3 specific inhibitors. Generation of CTF2 was blocked by a γ-secretase specific inhibitor, indicating that this fragment is produced by a PS1/γ-secretase mediated cleavage. Edman sequencing identified the MMP cleavage site seven residues upstream of the transmembrane domain and the PS1/γ-secretase cleavage site at the interface of the transmembrane and the cytoplasmic domain, resulting in the solubilization of the fragments. It had previously been shown that PS1/γ-secretase can directly interact with the juxtamembrane domain of E-cadherin, competing with $p120^{ctn}$ for cadherin binding (Baki et al., 2001).

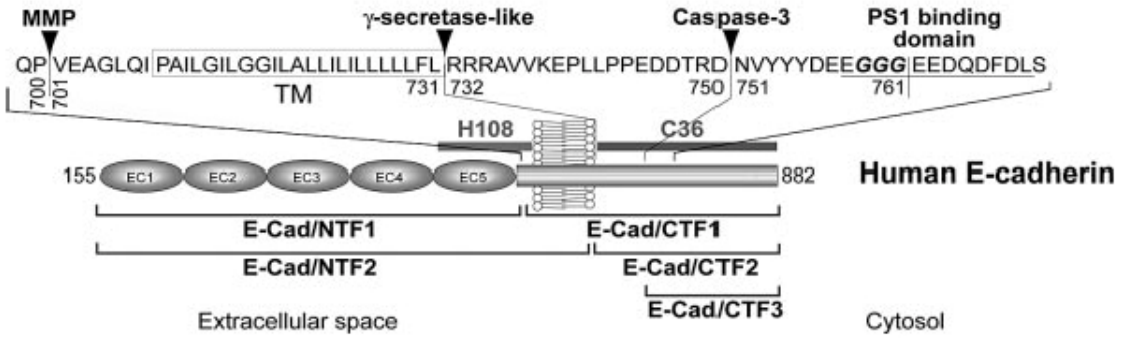


Figure 1.4: Human E-cadherin is processed by distinct proteolytic cleavage events. A metalloprotease activity (MMP) cleaves 7 amino acids upstream of the transmembrane domain (TM) to release the extracellular domain. PS1/γ-secretase mediated cleavage occurs at the interface of the transmembrane and cytoplasmic domain to release the cytoplasmic fragment. The cytoplasmic domain can also serve as substrate for caspase-3. CTF: C-terminal fragment, NTF: N-terminal fragment (taken from Marambaud et al., 2002).

Presenilins are multipass transmembrane proteins that localize predominantly to the ER and Golgi compartments, but also to the plasma membrane upon formation of cell-cell contacts. Accumulating evidence suggests that presenilins define a new class of intramembrane aspartyl proteases and constitute the catalytic core of a high-molecular weight γ-secretase complex (Fortini, 2002), which is responsible for the intramembrane cleavage of type I transmembrane proteins. Several substrates have been described for γ-secretase, including Notch, APP, and Nectin-1 (Cao and Sudhof, 2001; Kim et al., 2002; Struhl and Adachi, 1998). For some of these substrates it has been shown that upon cleavage by the γ-secretase complex the released cytoplasmic domain translocates to the nucleus and regulates gene expression. The example characterized best is Notch, a single-pass transmembrane receptor that transduces intercellular signals controlling cell fate (Weinmaster, 1997). In order to detect the presence of cytoplasmic portions of Notch in the nucleus, Struhl and Adachi used a very sensitive technique in which the chimeric transcription factor Gal4VP16 was inserted in the *Drosophila* Notch protein (Struhl and Adachi, 1998). This protein was expressed in *Drosophila* embryos that also carried a Gal4 responsive ß-galactosidase transgene, and the resulting ß-galactosidase-stained embryos served as indicator for the access of Gal4VP16 to the nucleus together with adjoining Notch sequences. Based on these results it was concluded that upon ligand binding Notch signals to the cell nucleus by γ-secretase mediated release of the intracellular domain, which translocates to the nucleus and directly activates transcription of target genes.

In analogy to the Notch pathway, the cadherin cytoplasmic domain might also be directly involved in mediating signal transduction. Indeed, a PS1/γ-secretase mediated cleavage of N-cadherin was shown to release the cytoplasmic domain into the cytoplasm, where it

participates in regulation of gene expression by promoting degradation of the transcriptional coactivator CBP (Marambaud et al., 2003). A direct role for the classical cadherin cytoplasmic domain in transcriptional regulation has not been described.
In recent studies members of the protocadherin subfamily (Haas et al., 2005; Hambsch et al., 2005) and cadherin-related proteins such as human Fat1 (Magg et al., 2005) have also been shown to be processed by metalloprotease and PS1/γ-secretase activities.

Taken together, ectodomain shedding and release of the cytoplasmic domain by distinct proteolytic cleavage events appears to be a general mechanism for regulated processing of cadherins. However, the functional significance of the soluble fragments is less clear.

1.6 Cadherins and their role in cancer

Approximately 80-90% of human cancers are of epithelial origin, termed carcinomas. In most, if not all, carcinomas E-cadherin mediated cell-cell adhesion is lost or downregulated, correlating with a progression towards tumor malignancy (Birchmeier and Behrens, 1994; Cavallaro and Christofori, 2004). A crucial role for E-cadherin in suppression of tumor invasion was demonstrated in cell culture systems. Transfection of E-cadherin cDNA into undifferentiated, invasive tumor cell lines resulted in a differentiated, non-invasive, epithelial phenotype, whereas interrupting E-cadherin mediated adhesion in these transfected cells reversed this effect by promoting invasive behavior (Frixen et al., 1991; Vleminckx et al., 1991). *In vivo* evidence for a causal role of E-cadherin loss in tumor progression came from a transgenic mouse model of pancreatic β-cell tumorigenesis, in which maintained expression of E-cadherin reduced transition from well differentiated adenoma to invasive carcinoma, whereas expression of a dominant negative E-cadherin increased the incidence of carcinoma formation (Perl et al., 1998). In a *Drosophila* genetic screen searching for mutations that could cause non-invasive tumors to invade the surrounding tissue, it was shown that inactivation of the cell polarity protein Scribble resulted in metastatic behavior of tumor cells, which could be suppressed by expression of E-cadherin (Pagliarini and Xu, 2003). These studies highlight the important role of E-cadherin in formation of malignant invasive carcinomas, a late stage of tumor progression.
During tumor progression E-cadherin can be functionally inactivated by various mechanisms, including somatic mutations, promoter hypermethylation and transcriptional repression (Peinado et al., 2004). Mutations affecting E-cadherin function have been observed in two particular subtypes of sporadic gastric and breast carcinomas, but are rarely observed in other types of tumors. Remarkably, E-cadherin germ-line mutations

were observed in cases of familial gastric cancers, indicating that E-cadherin inactivation is sufficient to predispose individuals to this disease (Guilford et al., 1998).

In most cases, E-cadherin downregulation occurs at the transcriptional level. Silencing of the human E-cadherin gene *(CDH1)* by hypermethylation of CpG islands in the promoter region resulted in the loss of E-cadherin expression in several different cancer cell lines as well as in primary hepatocellular and breast carcinomas. A direct role of hypermethylation in E-cadherin silencing was supported by the observation that E-cadherin expression in cancer cell lines can be reactivated by treatment with demethylating agents (Berx et al., 1998). In addition, it has been shown in many cancer cell lines as well as carcinomas of different tissues, such as human breast and hepatocellular carcinomas and mouse skin carcinomas, that E-cadherin expression and expression of its repressors snail, slug and E12/E47 are inversely correlated.

Post-translational mechanisms, for example changes in the phosphorylation status or proteolytic degradation of the adhesive complex, might also play a role in modulating cadherin function during tumor progression, although functional implications of these processes in tumor progression need to be further analysed. Several clinical studies detected a significant increase in soluble 80 kD E-cadherin levels in the serum of carcinoma patients compared to controls, suggesting that E-cadherin ectodomain shedding may contribute to cancer progression (Banks et al., 1995; Chan et al., 2003; Griffiths et al., 1996; Sundfeldt et al., 2001).

In several cancer types loss of E-cadherin expression is correlated with *de novo* expression of mesenchymal cadherins, such as N-cadherin and cadherin-11, similar to the cadherin switch occurring during EMT in embryonic development. Whereas E-cadherin is mainly expressed in epithelial cells, N-cadherin is normally found in cells of the surrounding stroma, for example fibroblasts. Thus, it is presumed that upregulation of N-cadherin in tumor cells might allow their interaction with mesenchymal cells, thereby promoting invasion of tumor cells into the stroma (Cavallaro and Christofori, 2004). Additionally, N-cadherin upregulation could provide cells with a migratory signal, since its expression caused induction of invasion without affecting E-cadherin levels (Nieman et al., 1999). Using chimeras between E- and N-cadherin, the migratory properties of N-cadherin were contributed to its EC4 domain and this might be important for interaction with certain growth factors (Kim et al., 2000).

Loss of E-cadherin and a switch to N-cadherin expression may also be a key step in the development of malignant melanoma. Under physiological conditions, E-cadherin is expressed on the surface of keratinocytes and melanocytes and is the major mediator of adhesion between these two cell types (Haass et al., 2004). Keratinocytes play an essential role in providing growth control over melanocytes, and it is thought that E-

cadherin is the critical molecule through which melanocyte control is mediated (Hsu et al., 2000; Tang et al., 1994). When E-cadherin expression is lost, melanocytes are able to escape keratinocyte mediated growth control.

1.7 *Xenopus laevis* early development

In early vertebrate development, gastrulation is the process in which coordinated cell movements and rearrangements underlie the formation of the distinct, elongated body shape with its three germ layers, ectoderm, mesoderm, and endoderm.

These morphogenetic movements have been extensively studied in the amphibian *Xenopus laevis* (Keller, 1991; Fig. 1.5). Continued cleavage events form a blastula stage *Xenopus* embryo that consists of the blastocoel, a liquid-filled cavity, the prospective ectoderm at the animal pole, and the prospective endoderm at the vegetal pole. The prospective mesodermal tissue is located in the marginal zone of the embryo, shaped as a ring. At the beginning of gastrulation, bottle cells are formed on the dorsal surface of the embryo by cells of the endodermal epithelium that begin to contract their apices and thus appear bottle shaped. Formation of bottle cells starts on the dorsal side, proceeds laterally and occurs ventrally at the late gastrula stage. The contracting cells cause the formation of an initial invagination and the marginal zone is rotated above it, thereby starting involution. The inflection point of the involuting tissue is called blastoporus and spreads laterally along with the bottle cells. The deep mesodermal cells that have involuted around the blastoporus are able to actively migrate along the roof of the blastocoel on an extracellular matrix predominantly consisting of fibronectin. This migrating mesoderm, which resembles the prospective head mesoderm, is followed immediately by the prospective notochordal and somitic mesoderm. As the latter moves inside the embryo, this tissue undergoes convergent and extension movements, which are responsible for the establishment of the elongated body axis. The overlying prospective neural tissue converges and extends in conjunction with the underlying mesoderm. Convergence of these tissues pushes the blastoporal lips together, until at the end of gastrulation the blastoporus is completely closed, and the ectoderm surrounds both mesodermal and endodermal tissue.

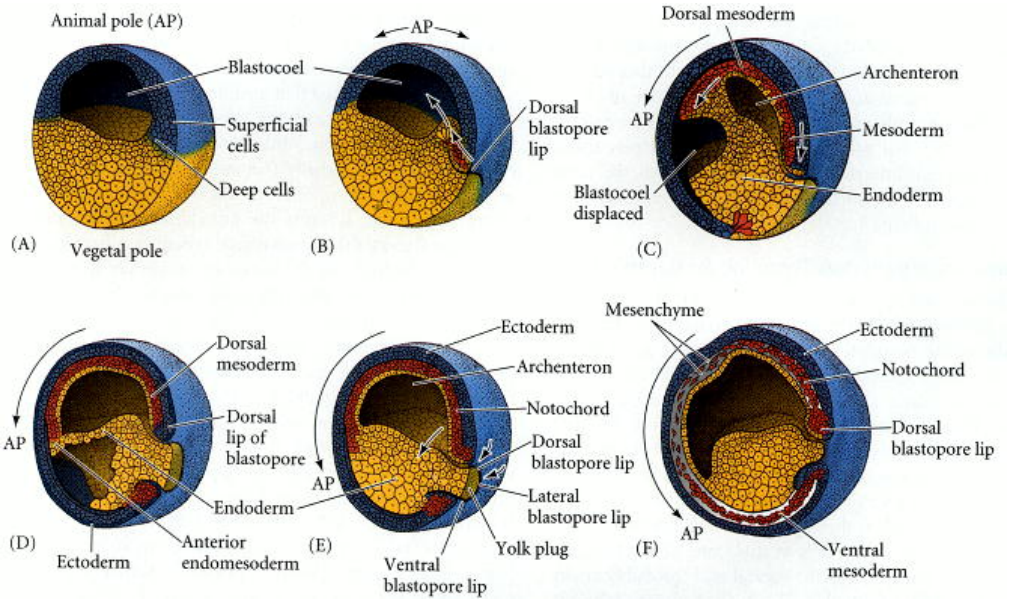


Figure 1.5: Cell movements during *Xenopus* gastrulation. During *Xenopus* gastrulation the prospective mesoderm (red) starts to involute around the dorsal blastopore lip and continues involution laterally and ventrally, thereby replacing the blastocoel and forming the archenteron. At the end of this process, the ectoderm (blue) surrounds the complete embryo, the endoderm (yellow) is placed inside, and the mesoderm located between ecto- and endoderm (taken from Gilbert, S., Developmental Biology).

Convergent extension behavior is a fundamental process in vertebrate gastrulation and neurulation (Keller, 2000). The driving force of convergent extension movements is generated by mediolateral cell intercalations, meaning that cells move between each other along the mediolateral axis and thereby elongate the embryo along the anterior-posterior axis (Fig. 1.6). Mesodermal cells undergoing intercalations exhibit a bipolar organization in *Xenopus*, with extensive lamelliform protrusions at the mediolateral ends of the cell. These protrusions are attached to neighbouring cells by focally localized adhesion contacts, thus generating traction on the anterior and posterior surfaces of the adjacent cell that pulls cells between each other.

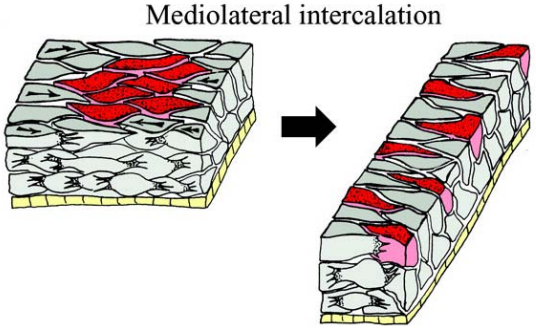


Figure 1.6: Convergent extension movements are driven by mediolateral intercalation of bipolarized cells, leading to an elongation of the anterior-posterior body axis (taken from Keller, 2002).

Recent findings suggest that the signals underlying convergent extension are mediated by members of the planar cell polarity (PCP) pathway that controls the planar polarity of various morphogenetic processes, such as polar hair growth on wing epidermal cells or polarity of ommatidia in the compound eye of Drosophila (Adler, 2002). Components of the PCP pathway include the seven-pass transmembrane receptor Frizzled, the cadherin-related protein Flamingo, the signalling molecule Dishevelled as well as the probable membrane protein Strabismus, but the exact mechanism of action is not completely understood yet (Keller, 2002). Members of the secreted Wnt molecules that activate the noncanonical Wnt pathway, such as Wnt11 and Wnt5a have been implicated upstream of Frizzled in vertebrate convergent extension movements by expressing dominant inhibitory forms of these proteins in *Xenopus* (Moon et al., 1993; Tada and Smith, 2000). Downstream of Dishevelled, the small GTPase Rho is activated, via interaction of Dishevelled with Daam1 (Dishevelled associated activator of mophogenesis), linking the PCP pathway with a cytoskeletal regulator (Habas et al., 2002).

Intercalating cells need to rearrange and at the same time stay in close contact with one another, suggesting that modulation of cadherin-mediated cell adhesion plays a central role in the control of convergent extension during *Xenopus* gastrulation. The classical *Xenopus* C-cadherin is the main mediator of cell-cell adhesion in blastula stage embryos (Haesman et al., 1994). It is maternally encoded and continues to be ubiquitously expressed through gastrula stages. In studies analysing the regulation of cell-cell adhesion during convergent extension, activin-induced animal cap tissue explants were used, which represent explants of presumptive ectoderm isolated from the animal hemisphere of a blastula stage embryo. If animal caps are treated with activin, a mesoderm inducing growth factor of the TGF-β family, the isolated tissue undergoes major cellular rearrangements with a strong elongation of the tissue, mimicking the convergent extension movements occurring during gastrulation (Symes et al., 1988). Thus, activin-treated animal cap tissue explants are widely used as a model for convergent extension movements. Brieher and Gumbiner performed aggregation assays with blastomeres isolated from activin induced and non-induced animal tissue explants, and they could show that activin induced blastomeres formed significantly smaller aggregates, caused by a specific decrease in the adhesive function of C-cadherin (Brieher and Gumbiner, 1994). However, steady state levels of C-cadherin were not affected by activin treatment. Similarly, treatment of activin-induced animal caps with a C-cadherin activating antibody inhibited elongation behavior, indicating that regulation of C-cadherin function is essential for convergent extension movements (Zhong et al., 1999). Interestingly, the *Xenopus* paraxial protocadherin PAPC has also been shown to be

involved in convergent extension movements by stimulating Rho and inhibiting Rac (Medina et al., 2004; Unterseher et al., 2004).

Cadherin function in *Xenopus* development has also been studied by using dominant negative cadherin mutants. Overexpression of a truncated, dominant negative form of C-cadherin consisting of the extracellular domain and the transmembrane domain into the dorsal involuting marginal zone caused severe gastrulation defects, demonstrated by a failure to close the blastoporus (Lee and Gumbiner, 1995). Expression of this mutant also inhibited elongation of activin-induced animal cap explants, indicating that the process of convergent extension is particularly sensitive to changes in cell adhesion. In contrast, disruption of *Xenopus* E-cadherin (first expressed at mid-gastrulation) (Levi et al., 1991) and N-cadherin (first expressed at beginning of neurulation) (Detrick et al., 1990) by corresponding truncated dominant negative mutants caused specific defects in ectoderm integrity and neural tube formation, respectively (Levine et al., 1994).

1.8 Aim of this thesis

Cadherins can be regulated by distinct proteolytic cleavage events, resulting in release of either the ectodomain or cytoplasmic domain. Although this directly downregulates the adhesiveness of full length cadherin, it is unclear if the released fragments have biological activity by themselves. The aim of this thesis is to address the functional significance of the generated cadherin fragments by asking three questions:

1. Does expression of cadherin extracellular domains interfere with early Xenopus development?
2. Is there a correlation between soluble cadherin levels and melanoma progression?
3. Does the cadherin cytoplasmic domain translocate to the nucleus and function as transcriptional activator?

1.

It was previously suggested that the released ectodomain may retain biological function upon cleavage by metalloproteases. The N-cadherin ectodomain was shown to serve as an adhesive substrate and thus promote neurite outgrowth (Paradies and Grunwald, 1993; Utton et al., 2001). Furthermore, the human E-cadherin ectodomain was suggested to induce invasion and inhibit cell-cell aggregation (Noe et al., 2001; Wheelock et al., 1987). However, these observations did not answer the question whether the cleaved cadherin extracellular domain does have a biological function *in vivo*, or whether it has the capacity to directly interfere with endogenous cadherin adhesive function. Therefore,

overexpression studies in *Xenopus laevis* embryos were done as an approach to address the function of the cadherin ectodomain. *Xenopus laevis* development was chosen since it provides a powerful *in vivo* model system to study cadherin regulation due to major cellular rearrangements that require constant remodelling of adhesive contacts. Deletion constructs were generated encoding the extracellular domain of *Xenopus* C-, E- or N-cadherin, which represent the most abundant classical cadherins in early *Xenopus* development, and expressed in early *Xenopus* embryos to study the *in vivo* consequences.

2.

In several studies a correlation between soluble E-cadherin levels in patient sera and carcinoma progression was observed, suggesting that cadherin ectodomain shedding may also contribute to tumor progression. Keratinocytes are thought to exert growth control over melanocytes by direct E-cadherin mediated interactions. A switch from E-cadherin to N-cadherin expression is common during melanoma progression. To examine if E-cadherin shedding may contribute to melanoma progression, a panel of melanoma cell lines as well as sera of melanoma patients was analysed for the extent of soluble E-cadherin in correlation with invasiveness or tumor progression.

3.

Release of the cadherin cytoplasmic domain by the PS1/γ-secretase complex may serve as a mechanism to communicate changes in adhesion to the interior of the cell. Cleavage by PS1/γ-secretase has been described for several other type I membrane proteins and for some of these substrates it was shown that the cleaved cytoplasmic domain can translocate to the nucleus and mediate transcriptional activation, such as the well-known example Notch (Struhl and Adachi, 1998). To examine the functional role of the released cadherin cytoplasmic domain, a similar approach was taken as has been described for the analysis of Notch nuclear access. The classical cadherins *Xenopus* C-cadherin and human E-cadherin were fused C-terminally to either Gal4 or Gal4VP16 transcription factor. These constructs were used in transient transfection experiments to analyse if the cadherin cytoplasmic domain has access to the nucleus and if it has intrinsic transactivation capacity.

2. Results

2.1 The role of cadherin ectodomain shedding

2.1.1 Cloning and expression of recombinant cadherin extracellular domains

Xenopus laevis early development was chosen as an *in vivo* model system to address the functional significance of the cadherin extracellular domain. Overexpression of cadherin extracellular domains into early *Xenopus* embryos should reveal if these fragments have the capacity to interfere with *in vivo* cell adhesion, cell movements and rearrangements, which occur extensively during early development. For this purpose, cDNAs encoding the extracellular domains of *Xenopus* C-, E-, and N-cadherin were cloned into the pCS2+6xmyc vector allowing subsequent efficient *in vitro* transcription and injection of RNA into embryos. *Xenopus* C-cadherin is maternally encoded and the most abundant type I classical cadherins present in early *Xenopus* stages, whereas E- and N-cadherin are first detected during mid-gastrulation and beginning of neurulation, respectively. All constructs were cloned such that they encode the complete extracellular domain repeats EC1 to EC5 and a myc tag fused C-terminally to EC5 (Fig. 2.1).

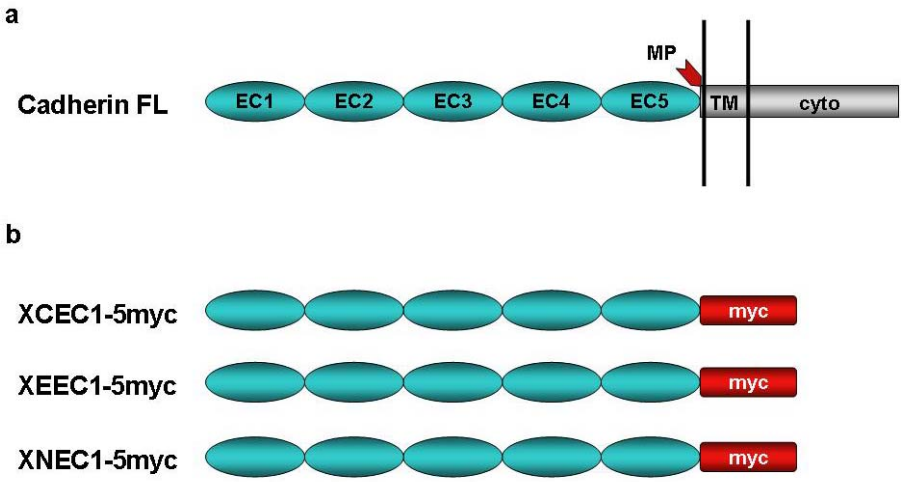


Figure 2.1: (a) Domain structure of classical cadherins. FL: full length, EC: extracellular domain, TM: transmembrane domain, cyto: cadherin cytoplasmic domain, MP: metalloprotease cleavage site. **(b)** Schematic representation of mature recombinant extracellular domains of *Xenopus* C-cadherin (XCEC1-5myc), *Xenopus* E-cadherin (XEEC1-5myc) and *Xenopus* N-cadherin (XNEC1-5myc) fused to a 6x myc tag (myc).

Western blot analysis of late gastrula embryos (stage 12) injected with *in vitro* transcribed RNA of the different cadherin extracellular domains at the four cell stage demonstrated that the injected RNA is efficiently translated into protein: using a myc specific antibody, positive signals were detected for XCEC1-5myc, XEEC1-5myc and XNEC1-5myc injected embryos, whereas embryos injected with the same amount of control RNA were negative (Fig. 2.2a). Two bands were detected for each of the extracellular domains that most likely

represent the precursor and the mature polypeptides generated by furin cleavage of the pro-domain. The apparent molecular mass of the lower bands is in agreement with the predicted molecular mass of a mature cadherin extracellular domain fused to the myc tag, which is ~95 kD for XCEC1-5myc and ~110 kD for XEEC1-5myc or XNEC1-5myc.

To analyse the localization of the expressed fragments, whole mount double immunofluorescence staining was performed on animal cap tissue explants of XEEC1-5myc injected blastula embryos (stage 8) using an anti-C-cadherin antibody to detect the endogenous cadherin and an anti-myc specific antibody to detect XEEC1-5myc. Similar to endogenous C-cadherin, XEEC1-5myc was mainly localized to cell borders, although some cytoplasmic staining was observed as well (Fig. 2.2b). A similar staining pattern was detected upon expression of either XCEC1-5myc or XNEC1-5myc (data not shown).

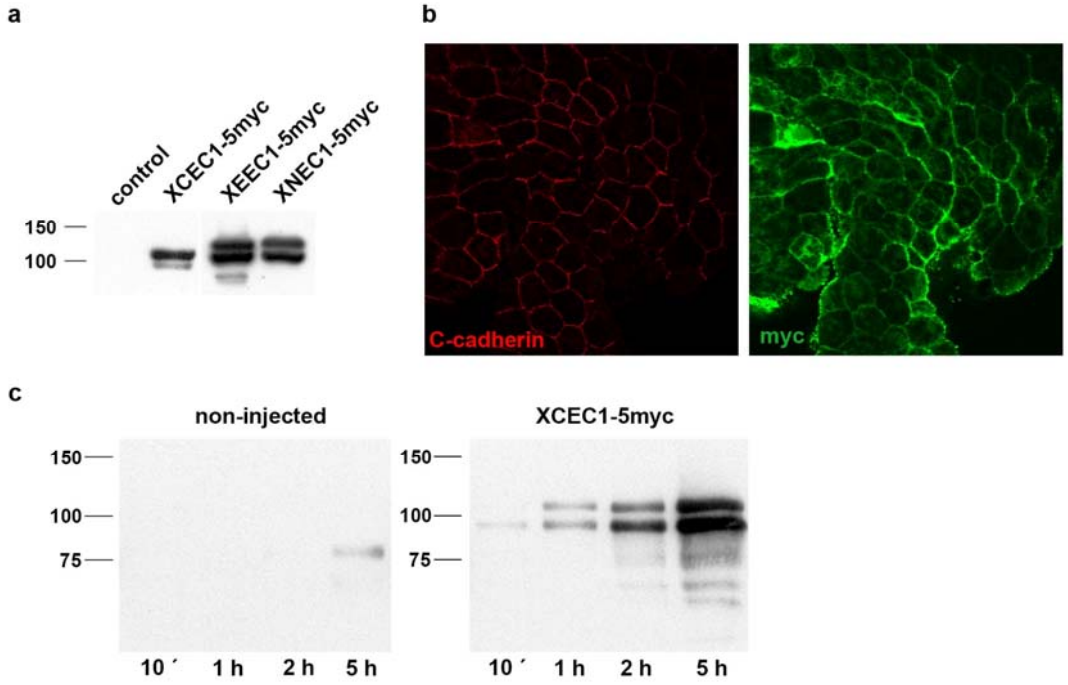


Figure 2.2: The recombinant cadherin extracellular domains are expressed and secreted. (a) Early *Xenopus* embryos were injected with 4 ng of the indicated RNA; total lysates were taken at stage 12 and analysed by western blot with an anti-myc specific antibody. **(b)** Whole mount immunofluorescence analysis of animal cap tissue explants from XEEC1-5myc injected embryos probed with an anti-C-cadherin and an anti-myc specific antibody. **(c)** Animal cap tissue explants were taken from stage 8 control and XCEC1-5myc injected embryos and transferred to a 50 µl drop of 1x MBS (five animal cap explants each). After the indicated time points, supernatant 1x MBS was loaded on a 7% SDS-gel and analysed by western blot using an anti C-cadherin antibody.

To confirm that the exogenously expressed cadherin extracellular domain was indeed secreted, supernatants of animal cap tissue explants of control and XCEC1-5myc injected embryos were taken at the indicated time points and analysed by western blot with an anti-C-cadherin antibody. Bands of ~95 kD and ~110 kD were detected in XCEC1-5myc injected tissue explants, similar to those in Fig. 2.2a (Fig. 2.2c). XCEC1-5myc was detected as early as 10 min after explanting the tissue and accumulated over time. From

this it can be concluded that the exogenous protein is secreted from the isolated blastomeres. As control supernatants of non-injected embryo explants were subjected to western blot analysis and no signals corresponding to XCEC1-5myc were observed (Fig. 2.2c, left panel). Nevertheless, a signal of ~80 kD was detected in these supernatants after 5h, which most likely represents the endogenous cleaved C-cadherin ectodomain.

2.1.2 *In vivo* occurance of the *Xenopus* C-cadherin ectodomain

Xenopus C-cadherin is maternally encoded and highly expressed during blastula and gastrula stages. Therefore, we focussed first on the role of the C-cadherin ectodomain on *Xenopus* development. To examine if the C-cadherin ectodomain does occur *in vivo* and if C-cadherin ectodomain shedding is regulated during gastrulation, western blot analysis was performed on embryos of stages covering gastrulation (stages 9 to 12). A signal of 120 kD corresponding to full length C-cadherin was present in all stages, and levels of C-cadherin increased slightly during gastrulation (Fig. 2.3a). In addition, a fragment of ~80 kD was present in all stages, showing increasing levels over time. The size of this fragment corresponds to the mature C-cadherin extracellular domain and could therefore represent the shed protein. To confirm that the appearance of an 80kD fragment was a result of shedding and not due to a post-lysis effect, blastocoel liquid was removed from stage 9 to stage 12 embryos and analysed by western blot. Again, a C-cadherin positive fragment of ~80 kD was present in all stages and the amount accumulated over time (Fig. 2.3b). This demonstrated that C-cadherin is cleaved in the early embryo and its extracellular domain is secreted into the blastocoel, presumably by cells of the blastocoel roof and floor.

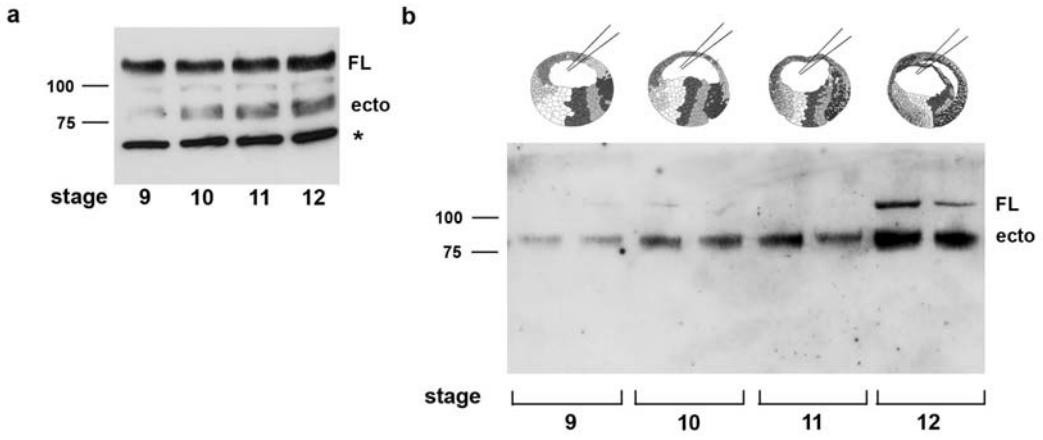


Figure 2.3: The *Xenopus* C-cadherin ectodomain does occur *in vivo*. (a) Total lysates of stage 9 to stage 12 embryos were analysed by western blot using an anti C-cadherin antibody directed against the extracellular domain of C-cadherin. **(b)** Blastocoel liquid was removed from stage 9 to stage 12 embryos using a glass capillary. Blastocoel liquid of 20 embryos (~2µl) was loaded per lane on a 7% gel, proteins were separated by SDS-Page and analysed by western blot with an anti-C-cadherin antibody directed against the extracellular domain of C-cadherin. Samples were loaded in duplicates. FL: full length C-cadherin, ecto: C-cadherin ectodomain, (*): unspecific band.

2.1.3 Overexpression of the C-cadherin extracellular domain causes gastrulation defects

To analyse the effect of cadherin extracellular domain overexpression on *Xenopus* early development, RNA injections were performed into the prospective dorsal involuting marginal zone, the region undergoing the most extensive cellular rearrangements during early development. Embryos injected with control RNA showed normal gastrulation similar to non-injected embryos with an almost complete closure of the blastoporus by the end of gastrulation (stage 12). In contrast, embryos injected with XCEC1-5myc failed to close the blastoporus, with major parts of the endodermal yolk plug still visible at the end of gastrulation (Fig. 2.4a). Western blot analysis showed that XCEC1-5myc was highly overexpressed compared to endogenous C-cadherin when using amounts of RNA that caused a phenotype (Fig. 2.4c). To exclude the possibility that the gastrulation defect was caused by the addition of a C-terminal myc tag, embryos were injected with XCEC1-5 alone, resulting in a similar phenotype as XCEC1-5myc (data not shown).

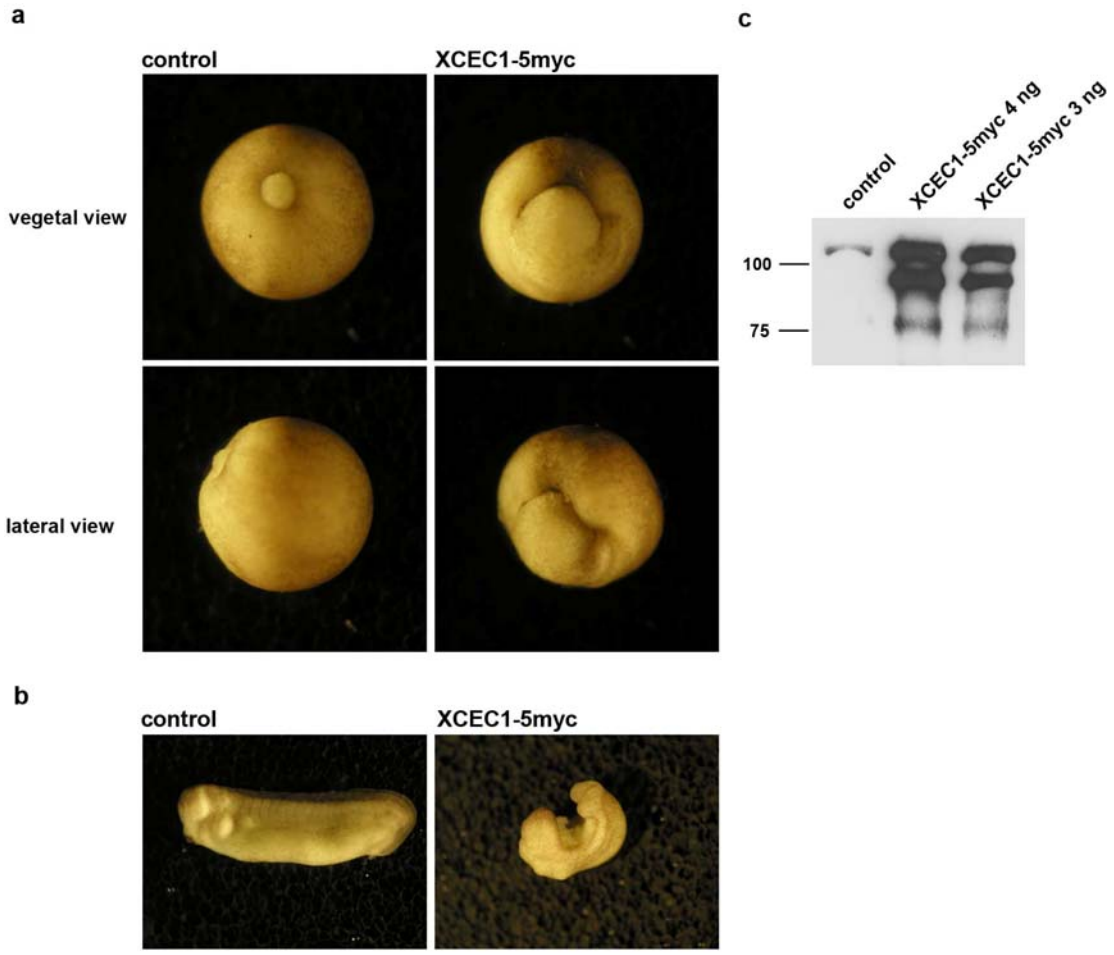


Figure 2.4: Dorsal injection of C-cadherin extracellular domain leads to gastrulation defects. Four cell stage embryos were injected with 4 ng of control or XCEC1-5myc RNA into the dorsal involuting marginal zone and allowed to develop until control embryos reached **(a)** stage 12 or **(b)** tadpole stage. (**c)** Lysates of stage 12 embryos injected at the four cell stage with 4 ng or 3 ng of XCEC1-5myc RNA were analysed by western blot with an anti C-cadherin antibody and compared to lysates of non injected embryos.

Approximately 77% of XCEC1-5myc injected embryos failed to complete blastopore closure compared to only 2% of controls (Table 2.1). The effect was dose dependent, since injecting less RNA lowered the percentage of defect embryos (for example 54% when 3 ng of XCEC1-5myc were injected; Table 2.1). Less than 1.5 ng of XCEC1-5myc RNA did not cause a visible phenotype.

When embryos were allowed to develop until tadpole stage, a large opening in the dorsal surface remained in XCEC1-5myc injected embryos with endodermal cells of the yolk plug still exposed (Fig. 2.4b). However, despite this defect XCEC1-5myc injected embryos exhibited normal head structures containing eyes and cement gland similar to control injected embryos.

Taken together, these results show that the C-cadherin extracellular domain has the capacity to interfere with morphogenetic movements *in vivo*.

RNA	total amount (ng)	number injected embryos	closure defect (%)
XCEC1-5myc	5,0	37	100
XCEC1-5myc	4,0	>200	77,4
XCEC1-5myc	3,0	13	53,8
XCEC1-5myc	1,5	18	5,5
XCΔtail	1,5	22	94,0
XCΔtail	0,7	19	61,0
XCΔtail	0,2	13	0
XEEC1-5myc	4,0	42	59,5
XNEC1-5myc	4,0	34	64,7
W2A XCEC1-5myc	4,0	54	96,3
XCEC1	4,0	35	74,3
XCEC1-3	4,0	34	82,4
Gal4	5,0	36	5,5
Gal4	4,0	131	2,3
PAPC DN	4,0	43	11,6
Xcad11EC1-5myc	4,0	37	16,2

Table 2.1: Overview of injections into the dorsal involuting marginal zone. Percentage of embryos exhibiting gastrulation defects scored by a failure to close the blastoporus is given.

2.1.4 The observed gastrulation phenotype is specific for type I classical cadherins

Surprisingly, overexpression of either recombinant E-cadherin or N-cadherin extracellular domain caused a similar gastrulation defect as observed for XCEC1-5myc. Expression of both XEEC1-5myc and XNEC1-5myc resulted in a failure to close the blastoporus (Fig. 2.5). Again, in tadpoles a large opening in the dorsal surface remained, but head structures developed normally (data not shown). Approximately 60% of XEEC1-5myc injected and 65% of XNEC1-5myc injected embryos showed this defect (Table 2.1), which is in the range of that detected for XCEC1-5myc (77%).

Figure 2.5: *Xenopus* E- and N-cadherin extracellular domain overexpression causes a similar phenotype as C-cadherin extracellular domain overexpression. Four cell stage embryos were injected with 4 ng of the indicated RNA into the dorsal involuting marginal zone and allowed to develop until control embryos reached stage 12.

To examine if expression of a type II cadherin extracellular domain can interfere with gastrulation movements, the extracellular domain of the type II cadherin *Xenopus* cadherin-11 was fused to a 6x myc tag (Xcad11EC1-5myc, Fig. 2.6a). Injection of Xcad11EC1-5myc RNA resulted in strong expression of this domain, as examined by western blot analysis using a myc-specific antibody (Fig. 2.6b). Embryos injected with 4 ng of Xcad11EC1-5myc RNA developed normally and did not show any gastrulation defects unlike embryos injected with the same amount of XCEC1-5myc RNA (Fig. 2.6c). Approximately 84% of Xcad11EC1-5myc injected embryos had completely closed their blastoporus at the beginning of neurulation and were indistinguishable from control embryos (Table 2.1).

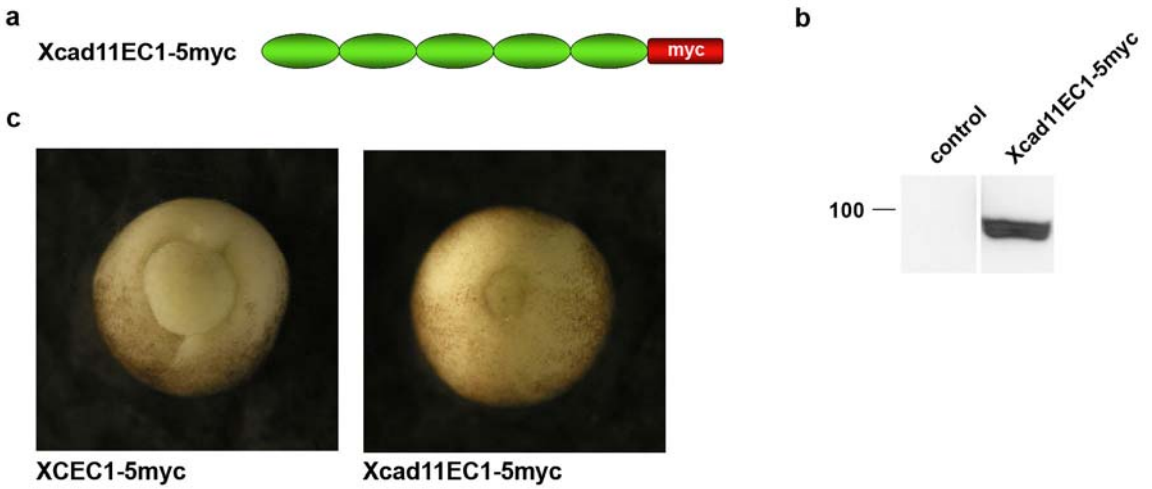


Figure 2.6: (a) Schematic representation of the recombinant extracellular domain of type II classical cadherin *Xenopus* cadherin-11 (Xcad11EC1-5myc). myc: 6x myc tag. **(b)** Western blot analysis of embryos injected with 4 ng of Xcad11EC1-5myc or control RNA using an anti myc specific antibody. **(c)** Four cell stage embryos were injected with 4 ng of the indicated RNA into the dorsal involuting marginal zone and allowed to develop until control embryos reached stage 12.

Similarly, overexpression of the extracellular domain of *Xenopus* paraxial protocadherin (Fig. 2.7a), a protocadherin expressed in the paraxial mesoderm of the gastrulating embryo and involved in convergent extension movements, did not result in a blastopore closure defect, even though the protein was expressed at high levels (Fig. 2.7b and c; Table 2.1). This indicates that the observed phenotype is specific for type I, classical cadherins.

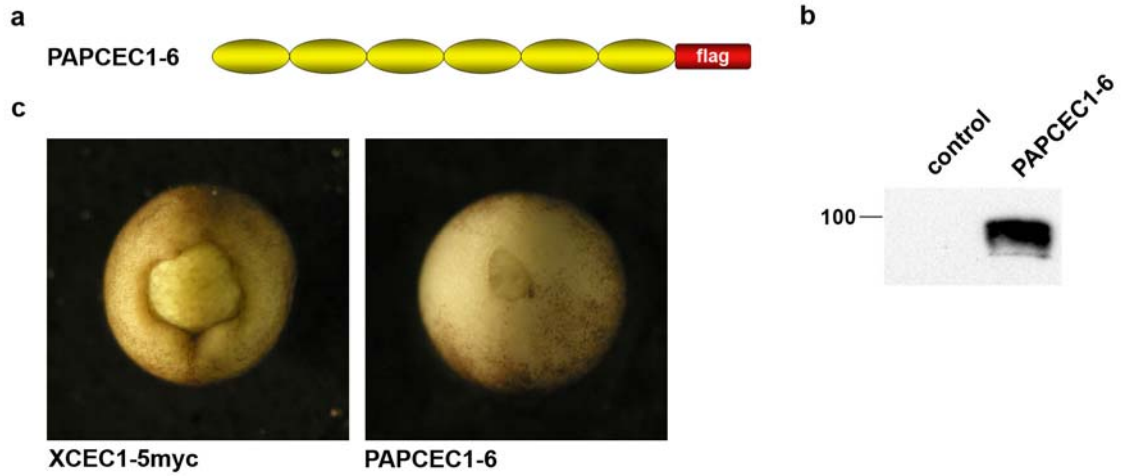


Figure 2.7: (a) Schematic representation of the recombinant extracellular domain of *Xenopus* paraxial protocadherin PAPC (PAPCEC1-6flag). flag: flag tag. **(b)** Western blot analysis of embryos injected with 4 ng of PAPC DN RNA using an anti flag specific antibody. **(c)** Four cell stage embryos were injected with 4 ng of the indicated RNA into the dorsal involuting marginal zone and allowed to develop until control embryos reached stage 12.

2.1.5 The conserved tryptophan 2 of the EC1 domain is not involved in causing gastrulation defects

The conserved tryptophan at position 2 of the EC1 domain is crucial for adhesive activity of classical cadherins (Patel et al., 2003). To address the question if adhesive activity is required for the extracellular domain to interfere with gastrulation movements, a mutant form of the recombinant C-cadherin extracellular domain was made in which the tryptophan at position 2 of the mature protein was substituted for alanine (W2A XCEC1-5myc, Fig. 2.8a). Western blot analysis detected two bands corresponding to the expected molecular weight of the precursor and processed protein for both the C-cadherin ectodomain and its mutant form (Fig. 2.8b). Injecting 4 ng of W2A mutant RNA caused a similar gastrulation defect as observed for wt XCEC1-5myc injections, whereas control embryos developed normally (Fig. 2.8c).

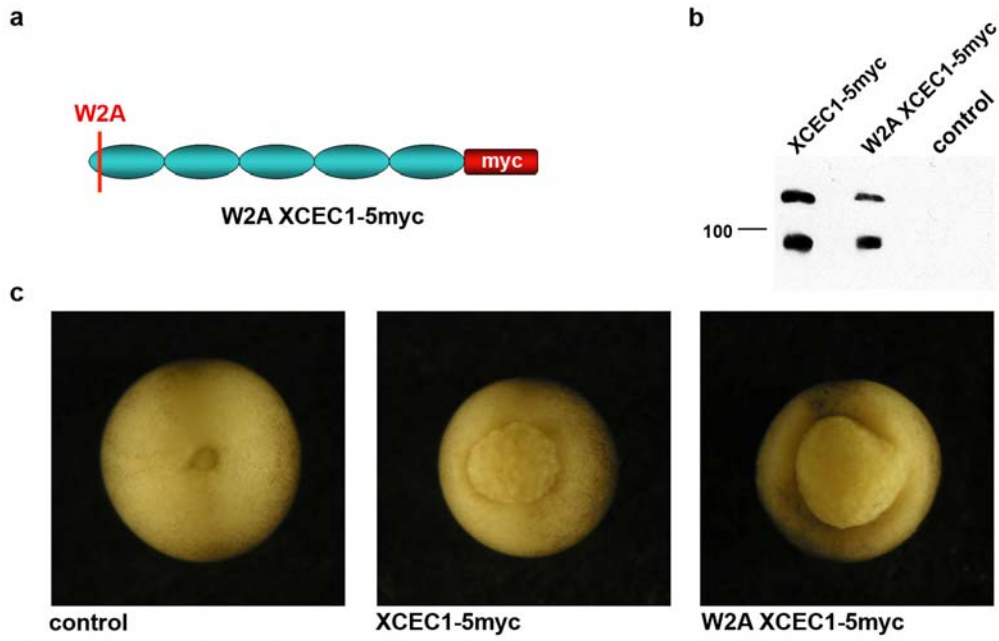


Figure 2.8: Adhesive activity is not required for the dominant negative effect of the extracellular domain. (a) Schematic representation of the W2A mutant of recombinant *Xenopus* C-cadherin extracellular domain. W2A: tryptophan to alanine mutation at position 2 of EC1, myc: 6x myc tag. **(b)** Western blot analysis of embryos injected with 4 ng of RNA into the dorsal involuting marginal zone at the four cell stage, extracted at stage 12 and blotted with an anti myc specific antibody. **(c)** Four cell stage embryos were injected with 4 ng of the indicated RNA into the dorsal involuting marginal zone and allowed to develop until control embryos reached stage 12.

2.1.6 The EC1 domain of *Xenopus* C-cadherin is sufficient for disruption of gastrulation movements

To further map the minimal domain of XCEC1-5myc necessary to interfere with gastrulation, constructs encoding either C-cadherin EC1 or C-cadherin EC1-3 were generated (Fig. 2.9a). EC1 was chosen because this domain was shown to mediate sorting specificity (Nose et al., 1990), whereas EC1-3 was the minimal domain necessary to confer cadherin-mediated adhesion in an adhesion flow assay (Chappuis-Flament et al., 2001). Western blot analysis of embryos injected with XCEC1 or XCEC1-3 RNA (Fig. 2.9b) showed signals corresponding to full length C-cadherin and the 80kD fragment. Two additional bands were detected in the lysate of XCEC1-3 injected embryos compared to controls that corresponded to the expected molecular weight of precursor and processed form of a peptide consisting of the extracellular domains 1 to 3. Expression of XCEC1 alone could not be detected by western blot with an anti C-cadherin antiserum, although the complete extracellular domain of C-cadherin was used as immunogen. However, the EC1 domain did cause gastrulation defects (see below), arguing for its expression. Lysate of XCEC1-5myc injected embryos was loaded for comparison. Injection of either XCEC1 or XCEC1-3 RNA resulted in a gastrulation phenotype indistinguishable from that of XCEC1-5myc injected embryos (Fig. 2.9c). XCad11EC1-5myc injection served as negative control. The percentage of embryos that failed to properly close the blastoporus

was in the same range as that of XCEC1-5myc injected embryos (74% for XCEC1 and 82% for EC1-3 versus 77% for XCEC1-5myc). This result indicates that the EC1 domain of C-cadherin contains the necessary information to disrupt gastrulation movements.

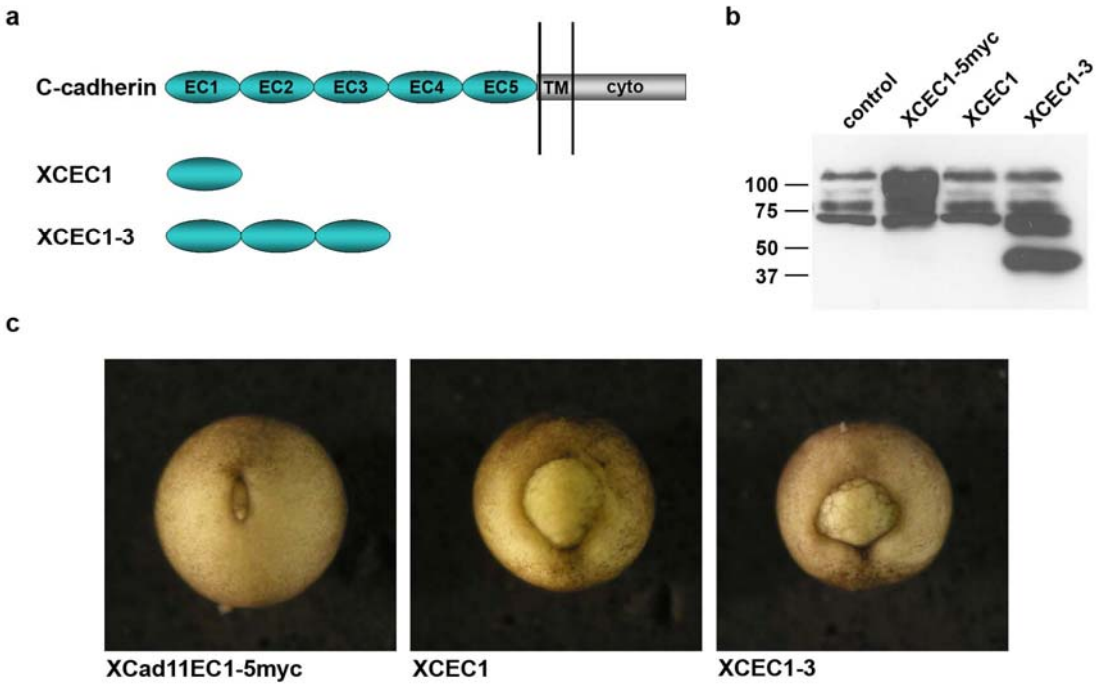


Figure 2.9: The C-cadherin EC1 domain is sufficient to interfere with gastrulation movements. **(a)** Schematic representation of the recombinant *Xenopus* C-cadherin deletion mutants XCEC1 and XCEC1-3 in comparison to full length cadherin type I domain structure. EC: extracellular domain, TM: transmembrane domain, cyto: cytoplasmic domain. **(b)** Early *Xenopus* embryos were injected with the indicated RNA; total lysates were taken at stage 12 and analysed by western blot with an anti C-cadherin antibody. **(c)** Four cell stage embryos were injected with 4 ng of the indicated RNA into the dorsal involuting marginal zone and allowed to develop until control embryos reached stage 12.

2.1.7 Overexpression of the C-cadherin extracellular domain does not strongly interfere with cell adhesion

To analyse the effect of C-cadherin extracellular domain on *Xenopus* development and cell-cell adhesion when injected into the animal hemisphere, embryos were injected animally with 4 ng of RNA encoding XCEC1-5myc and analysed for disintegrity of the ectoderm. A truncated form of C-cadherin lacking the cytoplasmic domain (XCΔtail) was used as positive control. As has been described before, all XCΔtail injected embryos had ruptures in the ectoderm resulting in the exposition of inner cell layers (Lee and Gumbiner, 1995). Such phenotypes were already visible at stage 8 to 9 (Fig. 2.10 and Table 2.2). At stage 10 to 11 the animal pole was completely disordered and embryos did not develop further. Similar results were obtained with a truncated form of *Xenopus* E-cadherin lacking the cytoplasmic domain (XEΔtail; Table 2.2). In contrast, no abnormalities could be detected in XCEC1-5myc injected embryos and ectoderm integrity appeared intact, suggesting that the extracellular domain did not strongly interfere with cell adhesion (Fig. 2.10 and Table 2.2).

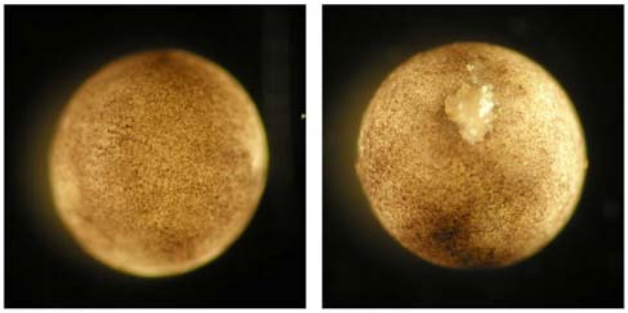


Figure 2.10: Overexpression of C-cadherin ectodomain in the animal hemisphere does not affect ectoderm integrity. Four cell stage embryos were injected into the animal hemisphere with 4 ng of XCEC1-5myc or XCΔtail RNA and images were taken at late blastula.

RNA	total amount (ng)	number injected embryos	ectoderm rupture (%)
XCEC1-5myc	4,0	15	0
XEEC1-5myc	4,0	13	0
XNEC1-5myc	4,0	13	0
XCΔtail	4,0	19	100
XEΔtail	4,0	35	100

Table 2.2: Overview of injections into the animal hemisphere. Percentage of embryos showing disruption of the ectoderm is given.

To test if the cadherin extracellular domain directly interferes with adhesive capacity of the endogenous cadherin, a blastomeres adhesion assay was performed (Zhong et al., 1999). In this assay blastomeres are allowed to bind directly to an extracellular cadherin domain substrate coated on a culture dish, thus excluding the influence of other cell-cell interactions. Animal cap explants of control or XCEC1-5myc injected embryos were dissociated to single cells and allowed to bind to various concentrations of recombinant C-cadherin extracellular domain (XCEC1-5FC). Adhesion was measured by counting blastomeres attached to the substrate before and after rotation of the substrate coated culture dish.

Most control (95%) and XCEC1-5myc (88%) injected blastomeres were still attached to the substrate when 100 µg/ml of cadherin substrate was used (Fig. 2.11), suggesting that both adhere similarly well to the substrate. Lowering the substrate concentration to 25 µg/ml or 10 µg/ml did not change this result. Only very low amounts of substrate (1 µg/ml) revealed differential binding between the control and XCEC1-5myc injected cells. Only 1.5% of XCEC1-5myc injected blastomeres adhered, whereas 28% of control blastomeres were still attached after rotation of the dish.

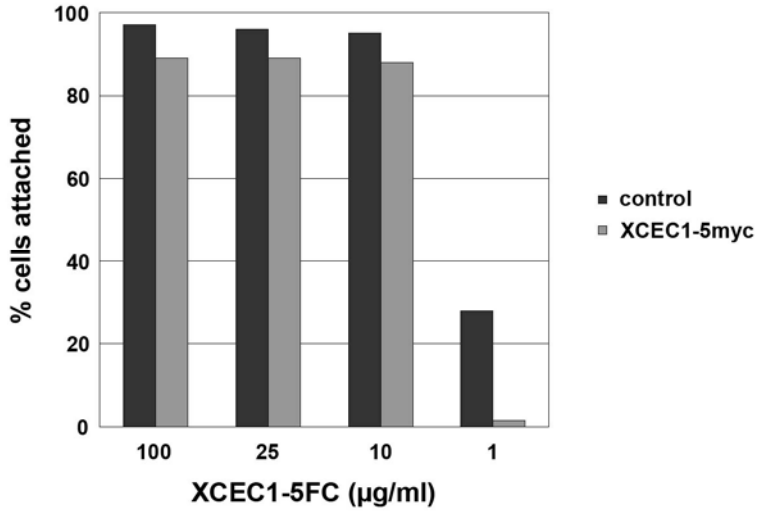


Figure 2.11: Blastomere adhesion assay. Blastomeres isolated from animal caps of embryos injected with 4 ng of either control or XCEC1-5myc RNA were dissociated in single cells and allowed to adhere to a dish coated with the indicated amounts of recombinant C-cadherin extracellular domain substrate XCEC1-5FC. Adhesion was measured by percent cells attached to the substrate after rotation of the substrate coated dish. Adherence of blastomeres to BSA alone (100 µg/ml) was measured to test for unspecific binding and to the substrate in the presence of EDTA (2 mM) to test for Ca^{2+}-dependency, which was negligible (not shown).

2.1.8 The C-cadherin extracellular domain interferes with convergent extension movements

The C-cadherin adhesive function is tightly regulated during convergent extension movements of the mesoderm (Brieher et al., 1994; Zhong et al., 1999).

When control embryos were coinjected with a ß-galactosidase encoding plasmid serving as lineage tracer, ß-galactosidase staining was restricted to the midline region of the embryo as a result of convergent extension of the prospective neuroectoderm that occurs in conjunction with mesodermal convergent extension (Fig. 2.12). In contrast, in XCEC1-5myc injected embryos expression of coinjected ß-galactosidase was extended laterally, indicating that convergent extension was impaired.

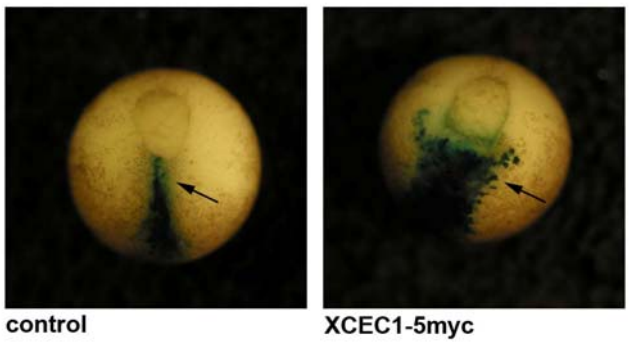


Figure 2.12: C-cadherin extracellular domain overexpression perturbed convergent extension of the neuroectoderm. Four cell stage embryos were injected into the dorsal involuting marginal zone with 4 ng of control or XCEC1-5myc RNA in combination with tracer amounts of a ß-galactosidase encoding plasmid; stage 11.5 embryos were fixed and stained for ß-galactosidase activity.

To examine if expression of the cadherin extracellular domain does indeed interfere with convergent extension movements, an animal cap elongation assay was performed. Treatment of animal cap tissue explants with the mesoderm inducing factor activin, a TGF-ß growth factor family member, will induce convergent extension movements resulting in elongation of the tissue (Fig. 2.13a). In contrast, animal cap explants cultured without activin round up but do not elongate. Thus, the activin elongation assay is often used as a model for convergent extension behavior. To test the behavior of XCEC1-5myc injected explants in this assay, animal cap explants of control or XCEC1-5myc injected embryos were cultured in the presence or absence of activin to induce mesoderm formation. Non-treated control explants rounded up, whereas those treated with activin elongated and showed typical protrusions (Fig. 2.13b). In contrast, elongation was strongly inhibited in explants injected with 1 ng of XCEC1-5myc RNA. The tissue formed small bulges which were not observed in non-treated animal caps, but failed to elongate properly. A similar appearance was observed in explants injected with 0.5 ng of XCΔtail RNA. Elongation behavior was significantly blocked to approximately 60% of that of controls in the presence of XCEC1-5myc as well as XCΔtail (Fig. 2.13c).

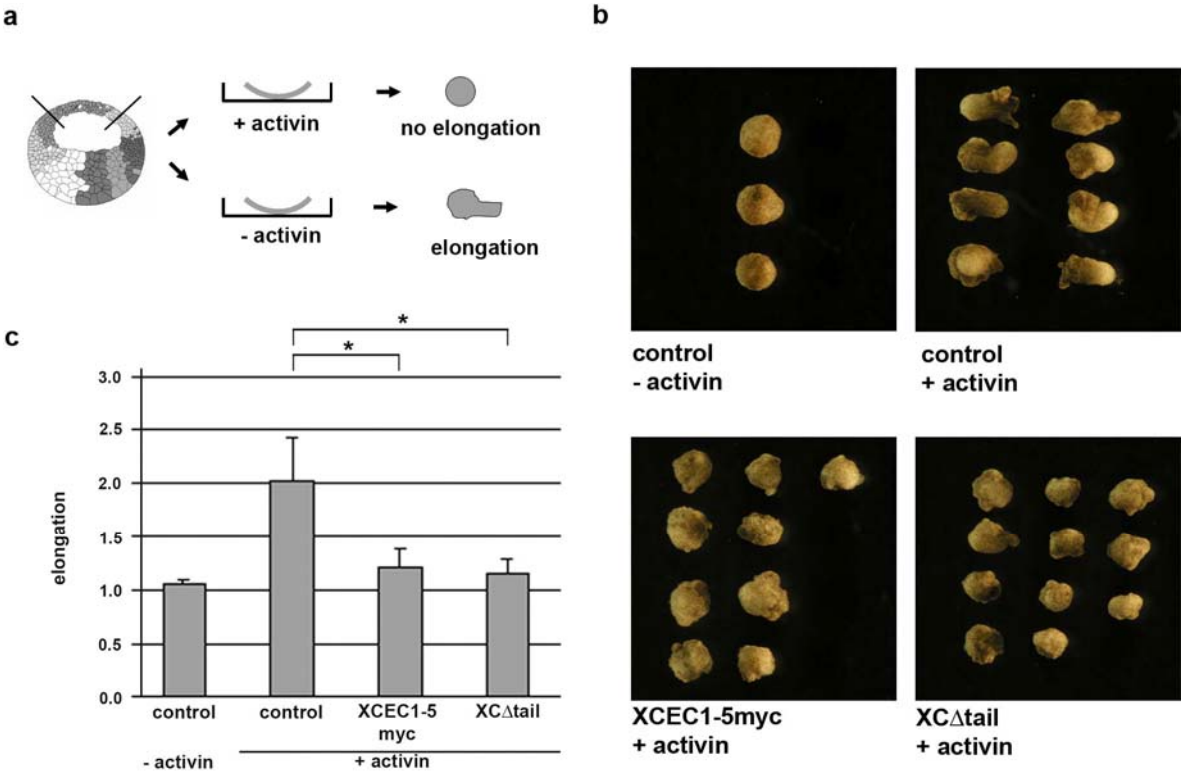


Figure 2.13: C-cadherin extracellular domain overexpression interferes with convergent extension movements. (a) Schematic representation of the activin elongation assay: animal cap tissue explants are treated with the mesoderm inducing growth factor activin resulting in elongation of the tissue due to convergent extension. **(b)** Four cell stage embryos were injected into the animal pole with 1 ng of control, 1 ng of XCEC1-5myc, or 0.5 ng of XCΔtail RNA; stage 8 animal caps were explanted, treated with or without activin for one hour and cultured over night to allow elongation of the tissue by convergent extension. **(c)** Tissue elongation was quantified by scoring length divided by width of animal cap explants. The results shown are representatives of three independent experiments. Statistically significant differences ($p<0.05$) are marked (*).

The C-cadherin extracellular domain could inhibit convergent extension by two different mechanisms, either by affecting morphogenetic movements underlying tissue elongation or by inhibiting activin-mediated mesoderm induction. To test the latter possibility, reverse transcription PCR was performed, and expression of the mesodermal marker Brachyury was analysed in non-treated and activin treated animal tissue explants (Fig. 2.14). Activin did induce expression of Brachyury in both non-injected and XCEC1-5myc injected embryos. These results indicate that XCEC1-5myc directly interferes with elongation but not with differentiation.

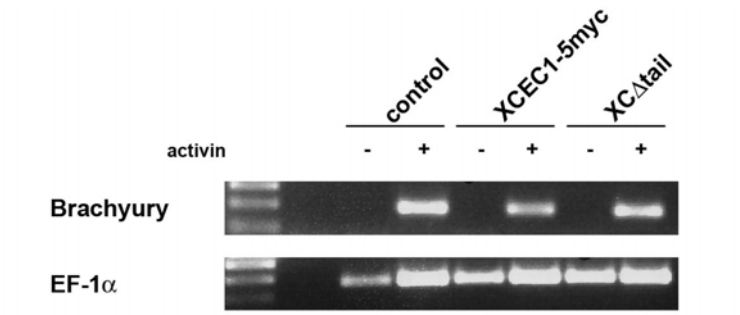


Figure 2.14: C-cadherin extracellular domain does not inhibit activin-induced expression of the mesodermal marker Brachyury. Four cell stage embryos were injected with 1 ng of control, 1 ng of XCEC1-5myc RNA, or 0.5 ng of XCΔtail RNA; stage 8 animal cap explants were treated with or without the mesoderm inducing growth factor activin for one hour and cultured over night; total RNA was isolated and expression of the mesodermal marker Brachyury was analysed by reverse transcriptase PCR using specific primers to Brachyury (a marker of general mesoderm) and EF-1 α (ubiquitously expressed).

2.2 The soluble E-cadherin ectodomain in melanoma progression

To determine whether E-cadherin ectodomain shedding correlates with the invasive potential of melanoma cells, a panel of human melanoma cell lines and one mouse cell line (B16F1) displaying a range from non- to high invasive behavior was characterized with respect to their amount of E-cadherin cell surface expression and soluble E-cadherin shed into the supernatant. Equal amounts of cell lysate and Con A bound supernatant proteins, normalized to the protein concentration of the cell lysate, were analysed by western blot using a polyclonal anti-human E-cadherin antibody. As described by others, E-cadherin was almost absent from high invasive cells, determined by western blot analysis of cell lysates (Fig. 2.15, upper panel). Variable amounts of surface E-cadherin were found in non- to low invasive cell lines. When comparing the amount of E-cadherin cell surface expression and soluble E-cadherin shed into the supernatant (Fig. 2.15, lower panels), no direct correlation could be observed between invasive potential and extend of E-cadherin shedding. For example, the non-invasive cell line IF6 shows the same amount of E-cadherin present in the supernatant as the high invasive MeWo cells, but has a much higher cell surface expression (Fig. 2.15, lanes 4 and 5). Nevertheless, the amount of cadherin ectodomain shed into the supernatant was not linear with surface levels of cadherin expression (for example Fig. 2.15, compare lanes 2 and 6), suggesting that E-cadherin ectodomain shedding may be a regulated process.

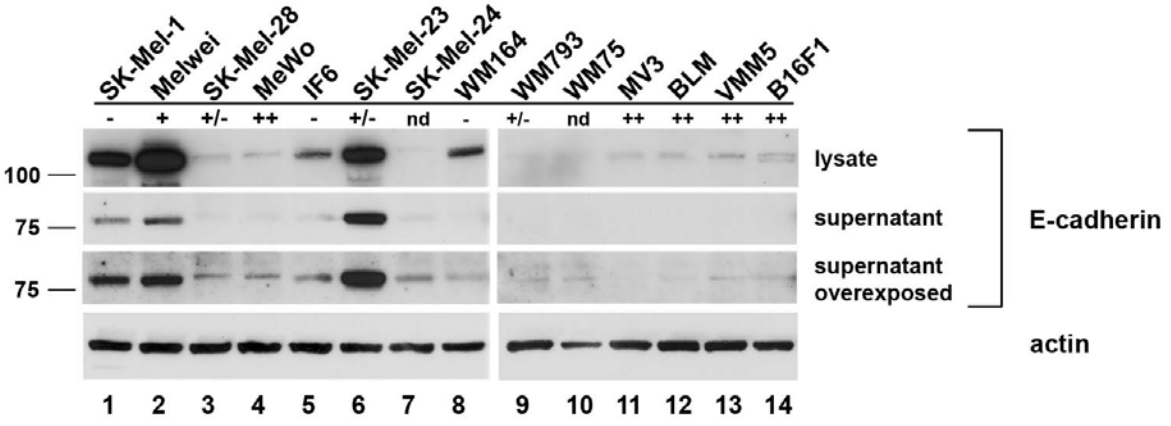


Figure 2.15: Shedding may be regulated. E-cadherin expression in lysates and supernatants of melanoma cell lines. Equal amounts of protein and normalized amounts of supernatants concentrated by Con A were separated on 4-12% gradient gels and analysed by western blot using a rabbit polyclonal anti E-cadherin antibody. To show small amounts of E-cadherin extracellular domain an overexposed image is included (third panel). Actin was used as loading control. (-) non, (+/-) non to low, (+) low, (++) high invasive potential, nd: not determined.

To examine the extend of E-cadherin ectodomain shedding in human melanoma progression, two to three serum samples of 24 melanoma patients were selected from a melanoma serum bank. These patients showed a normal level of serum S100, a diagnostic marker for disease progression, at the time of diagnosis and surgical removal of the primary tumor and increasing S100 values over time, associated with the appearance of multiple metastases in various organs including lung, liver and brain. As controls sera of six healthy subjects were analysed. Serum E-cadherin concentrations of controls were consistent with those of earlier reports (median ~3200 ng/ml, Fig. 2.16a). A significant increase in E-cadherin serum levels ($p < 0.05$) was detected in melanoma patients (median ~5000 ng/ml, Fig. 2.16a) when compared to controls. When sera were subdivided according to their S100 value, elevated E-cadherin levels were detected in correlation with higher S100 (Fig. 2.16b). Remarkably, in patients with low S100 levels (0-0.2 µg/ml) the median E-cadherin concentration was already increased compared to controls (~4500 ng/ml versus ~3200 ng/ml, Fig. 2.16b), although significant differences were only found in the group with either an S100 of 1-10 µg/l or above 10 µg/l. These results suggest that increased E-cadherin ectodomain shedding correlates with melanoma progression.

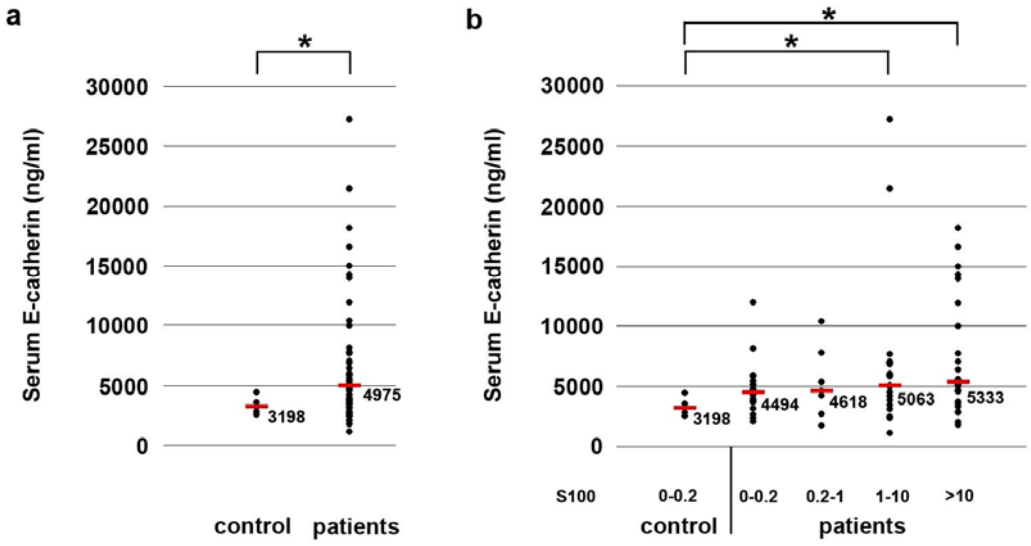


Figure 2.16: Increased soluble E-cadherin in serum of melanoma patients. (a) E-cadherin levels in all sera of patients and controls. **(b)** E-cadherin levels in sera of control and patients, grouped according to their S100 values (µg/l). Statistically significant differences ($p<0.05$) are marked (*). Single (•) and median (–) values are indicated.

2.3 The role of the cadherin cytoplasmic domain

2.3.1 Cadherin Gal4 and Gal4VP16 fusion proteins

The cadherin cytoplasmic domain can be released from the membrane by a PS1/γ-secrease mediated cleavage. For several substrates of the PS1/γ-secrease, such as Notch, it has been shown that the released cytoplasmic domain can translocate to the nucleus and is involved in regulation of gene expression (Struhl and Adachi, 1998). To address the question if the cadherin cytoplasmic domain has the capacity to enter the nucleus and activate transcription, a similar approach as described for Notch nuclear access was taken. In brief, this approach entails the insertion of the chimeric transcription factor Gal4VP16 into the *Drosophila* Notch protein. This fusion protein was expressed in *Drosophila* embryos that also carried a UAS-ß-galactosidase transgene and the resulting ß-galactosidase-stained embryos served as indicator for the access of Gal4VP16 into the nucleus together with adjoining Notch sequences.

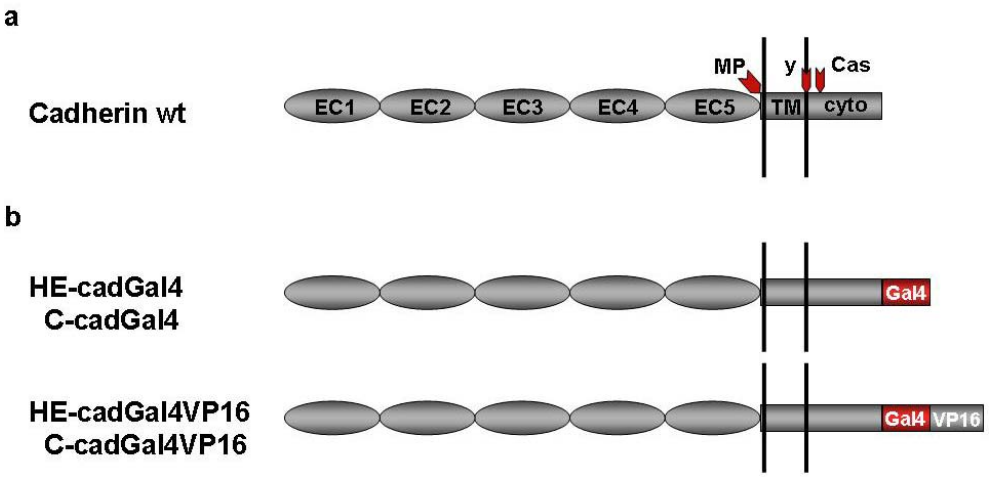


Figure 2.17: Schematic representation of cadherin Gal4 and Gal4VP16 fusion proteins. (a) General structural features of classical cadherins and cleavage sites (arrows) of proteases which have been shown to cleave human E-cadherin. EC: extracellular domain, TM: transmembrane domain, cyto: cytoplasmic domain, MP: metalloprotease, γ: γ–secretase, Cas: caspase-3. **(b)** Both human E-cadherin and *Xenopus* C-cadherin were fused C-terminally to either the DNA binding domain of the yeast Gal4 transcription factor (Gal4) or to Gal4 DNA binding domain and the transcriptional activating domain of the viral VP16 (Gal4VP16).

Cadherins of two different species, *Xenopus* C-cadherin and human E-cadherin, were fused at the C-terminus to the chimeric transcription factor Gal4VP16 (Fig. 2.17). Gal4VP16 consists of the DNA binding domain of the yeast Gal4 transcription factor fused to the transcription activating domain of the viral VP16 protein (Sadowski et al., 1988). It was hypothesized that transfection of constructs encoding these fusion proteins together with a Gal4 responsive luciferase reporter plasmid into cells would result in activation of the reporter, if the cadherin intracellular portion was released from the membrane and translocates to the nucleus. Similarly, *Xenopus* C-cadherin and human E-cadherin were

fused C-terminally to the Gal4 DNA binding domain alone. These constructs were used to examine whether the cadherin cytoplasmic domains possess intrinsic transactivation activity: if co-transfection of these constructs with a Gal4 responsive luciferase reporter resulted in luciferase activity, this would indicate transactivation by the cadherin cytodomain itself. Gal4 and Gal4VP16 encoding plasmids served as negative and positive control, respectively.

2.3.2 Cadherin fusion proteins are expressed and localized at the cell membrane

Expression of cadherin fusion proteins was verified using CHO-K1 cells, which express very little endogenous cadherin. CHO-K1 cells were transiently transfected with plasmids encoding the different cadherin fusion proteins and lysates of these cells were analysed by western blot using the PEP1 polyclonal antibody recognizing the cytoplasmic domain of all classical cadherins (Choi and Gumbiner, 1989). Cells transfected with a construct encoding wt C-cadherin served as positive control, and a protein was detected at the expected size of 120 kD; transfections of empty vector alone served as negative control. All fusion proteins were detected and shifted upwards compared to wt C-cadherin due to the Gal4 and Gal4VP16 attachments (Fig. 2.18, upper panel). Proteins of corresponding sizes were also detected with a Gal4 specific antibody (Fig. 2.18, second panel). CHO-K1 cells express very little endogenous β-catenin, but expression of β-catenin is upregulated upon transfection of its interaction partner cadherin. The presence of any of the cadherin Gal4 and Gal4VP16 fusion proteins led to increased β-catenin expression compared to negative control cells (Fig. 2.18, lower panel). This suggests that β-catenin binding to cadherin is not disturbed by the C-terminal fusions of Gal4 and Gal4VP16.

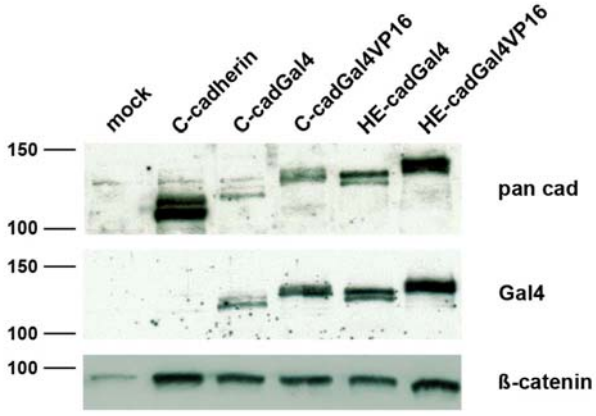


Figure 2.18: Expression of cadherin fusion proteins. CHO-K1 cells were transiently transfected with plasmids encoding the different cadherin fusion proteins and lysates of cells analysed by western blot using an anti pan cadherin antibody directed against the cytoplasmic domain of cadherins, an anti Gal4 antibody and an anti β-catenin antibody. CHO-K1 cells transfected with empty vector (mock) or wt C-cadherin were used as negative and positive control, respectively. Taken from the diploma thesis of Gilles Sequaris, which was performed under my supervision.

Correct localization of the cadherin-Gal4 and cadherin-Gal4VP16 fusion proteins was analysed by immunofluorescence staining of CHO-K1 cells transiently transfected with the different fusion constructs. Double labelling with HE-cadherin specific antibodies and Gal4 specific antibodies revealed the presence of both HE-cadGal4VP16 and HE-cadGal4 predominantly at sites of cell-cell contacts (Fig. 2.19a). A similar result was observed for C-cadGal4VP16 and C-cadGal4 when stained with C-cadherin and Gal4 specific antibodies (Fig. 2.19b).

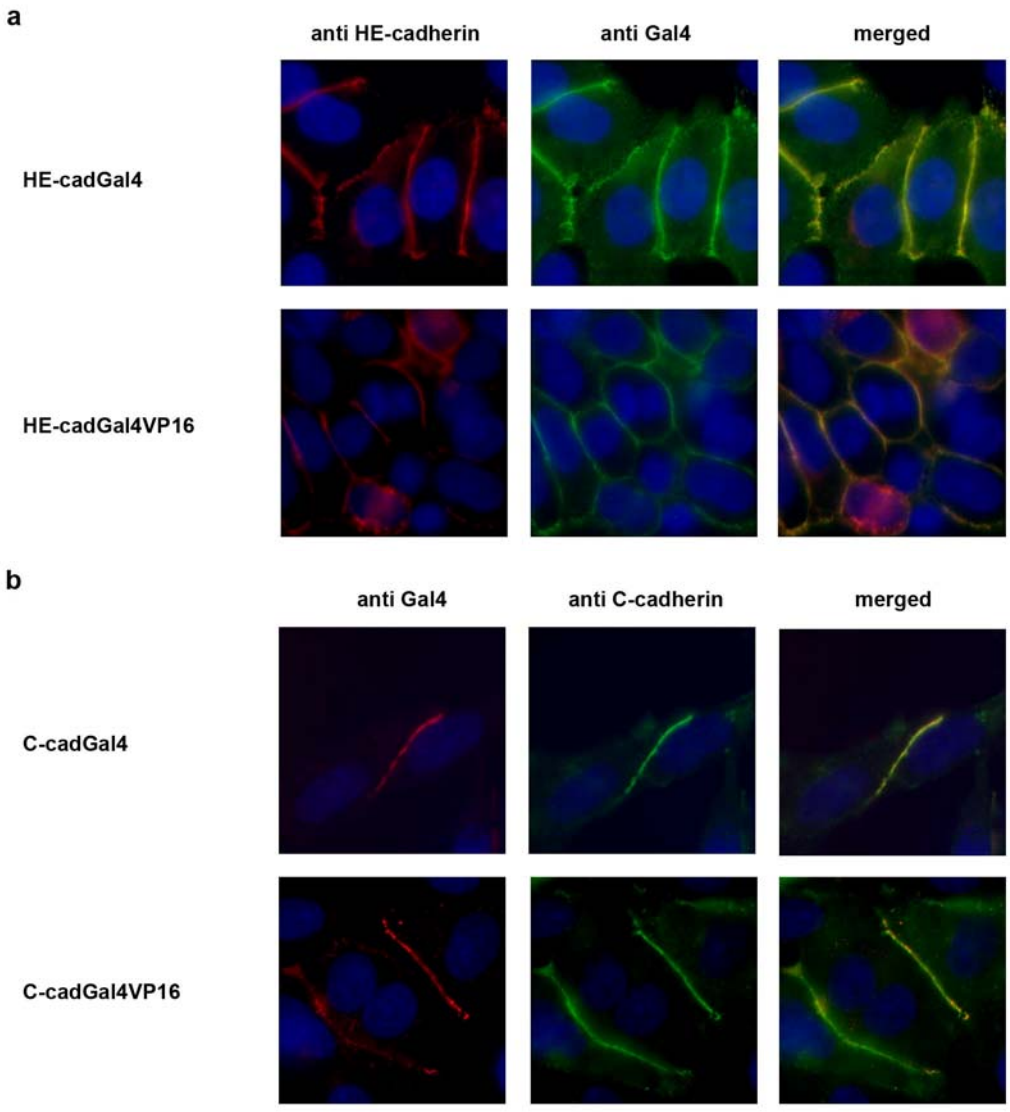


Figure 2.19: The cadherin Gal4 and Gal4VP16 fusion proteins are localized at the cell membrane. Immunofluorescence staining of CHO-K1 cells transiently transfected with the indicated constructs. Cells were stained with (**a**) a monoclonal anti HE-cadherin antibody in combination with a polyclonal anti Gal4 antibody to detect HE-cadherin fusions, or **(b)** a polyclonal anti C-cadherin antibody and a monoclonal anti Gal4 antibody to detect C-cadherin fusions. Nuclei were counterstained with DAPI. Taken from the diploma thesis of Gilles Sequaris, which was performed under my supervision.

To examine if the cadherin fusion proteins were able to bind their interaction partners p120^{ctn} and β-catenin, CHO-K1 cells were transfected with the HE-cadGal4VP16 construct and analysed by immunofluorescence staining. In CHO-K1 cells p120^{ctn} is localized in the cytosol, but is recruited to the membrane upon expression of cadherins, where it interacts with the juxtamembrane region of cadherins (Yap et al., 1998). Cells that were positive for HE-cadherin also expressed p120^{ctn} at sites of cell-cell contacts (Fig. 2.20a, large arrows), whereas in those cells negative for HE-cadherin the p120^{ctn} signal was diffusely present in the cytosol (Fig. 2.20b, small arrows). Cells expressing HE-cadGal4VP16 were also positive for ß-catenin, which was predominantly present at the cell membrane. In contrast, in cells negative for HE-cadherin ß-catenin was not detected (Fig. 2.20b). Taken together, these results show that the cadherin fusion proteins were correctly localized at the cell membrane and can interact with their binding partner p120^{ctn} and β-catenin, indicating that these molecules are functional in adhesion.

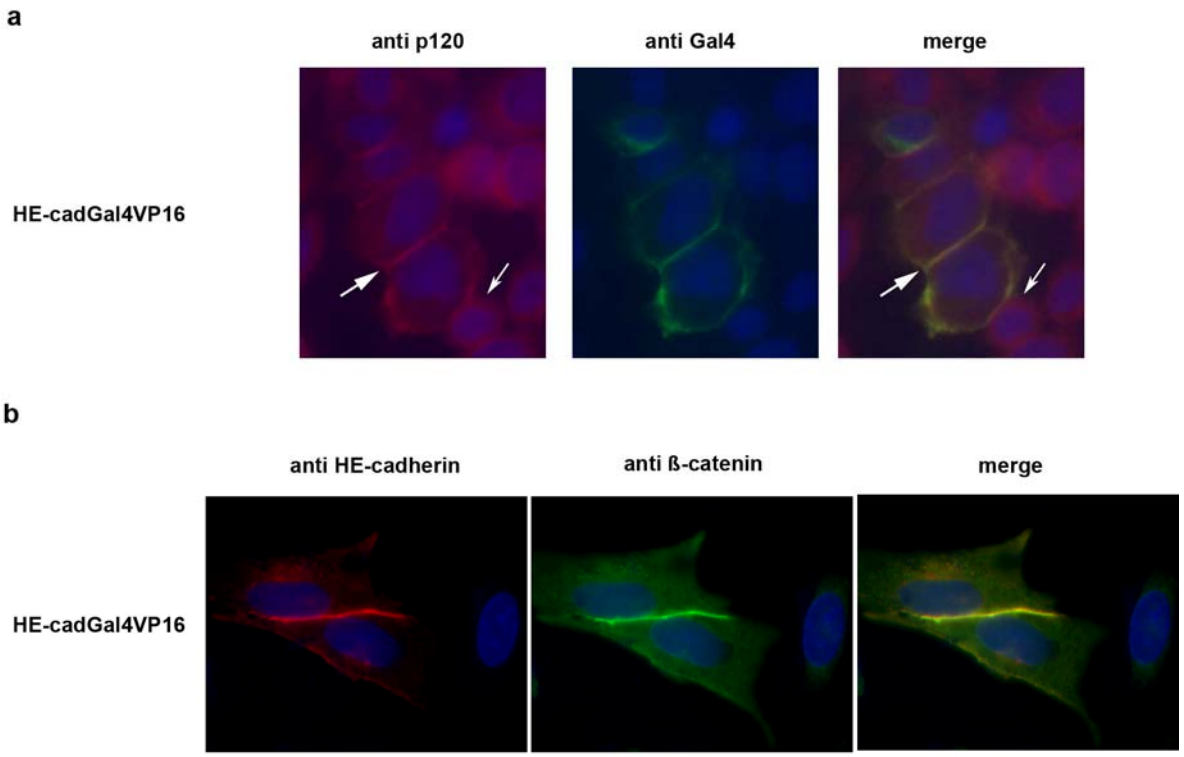


Figure 2.20: HE-cadGal4VP16 recruits p120^{ctn} and β-catenin to the membrane. CHO-K1 cells were transiently transfected with HE-cadGal4VP16 and stained for **(a)** Gal4 and p120^{ctn} using a monoclonal anti Gal4 antibody and a polyclonal anti p120^{ctn} antibody, or **(b)** HE-cadherin and ß-catenin using a monoclonal anti HE-cadherin antibody and a polyclonal anti ß-catenin antibody. Nuclei were counterstained with DAPI. Taken from the diploma thesis of Gilles Sequaris, which was performed under my supervision.

2.3.3 Cadherin Gal4VP16 fusion proteins can mediate transcriptional activity

Luciferase reporter assays were performed to examine if the cadherin cytodomain is released from the membrane and can enter the nucleus. For this purpose, cadherin Gal4VP16 fusion proteins were transiently transfected into CHO-K1 cells with a Gal4 responsive firefly luciferase reporter. A construct encoding the chimeric transcription factor Gal4VP16 was used as positive control, since Gal4VP16 can directly enter the nucleus and serve as a constitutive transcriptional activator of the firefly gene (Sadowski et al., 1988). Transfection of either HE-cadGal4VP16 or C-cadGal4VP16 caused a ~125 fold activation when compared to the negative control Gal4 (Fig. 2.21a and b). Similar levels of luciferase activity were also obtained when Gal4VP16 fusion proteins were expressed in the epithelial cell lines DLD-1 or MCF-7 (data not shown), indicating that transactivation also occured in epithelial cells. These experiments provided evidence that HE- and C-cadherin can enter the nucleus, possibly by cleavage and translocation of the cytoplasmic domain.

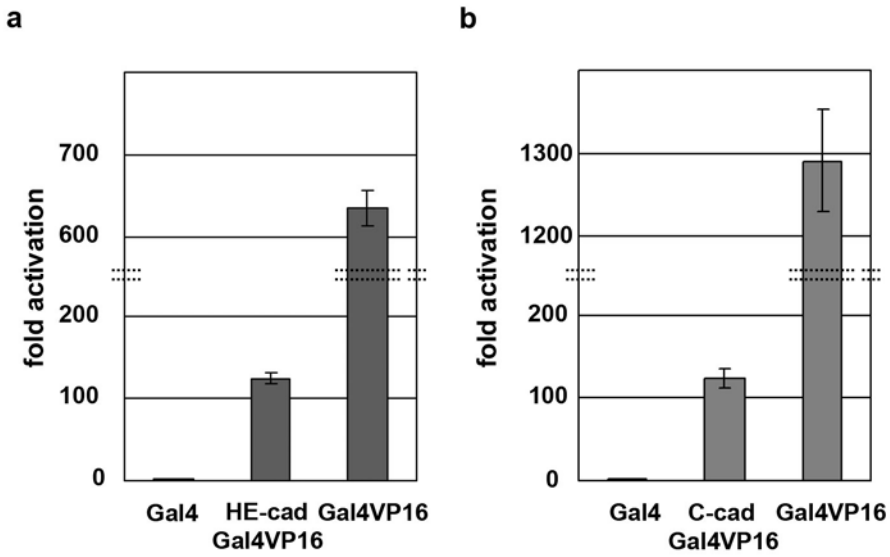


Figure 2.21: Cadherin Gal4VP16 fusions mediate transcriptional activation. A Gal4 responsive reporter plasmid was co-transfected with **(a)** HE-cadGal4VP16 or **(b)** C-cadGal4VP16 fusion constructs in CHO-K1 cells and tested for luciferase reporter activity. Gal4 and Gal4VP16 served as negative and positive control, respectively. Data is represented as fold activation of the negative control Gal4, which was set as 1. Transfections were performed in triplicates.

Access of C-cadherin to the nucleus does also occur *in vivo*, as shown by expression of C-cadGal4VP16 in *Xenopus* embryos (Fig. 2.22). Embryos injected with C-cadGal4VP16 and a Gal4 responsive luciferase reporter were lysed at stage 10 and 12 and assayed for luciferase activity. Transcriptional activation was detected in these embryos (two fold in stage 10 and 63 fold in stage 12 embryos when compared to controls), indicating that a C-cadherin fragment indeed translocates to the nucleus (Fig. 2.22). A high luminescent signal was also found in lysates of Gal4VP16 injected embryos, showing a ~250 fold activation in stage 10 embryos and a ~1,000 fold activation in stage 12 embryos.

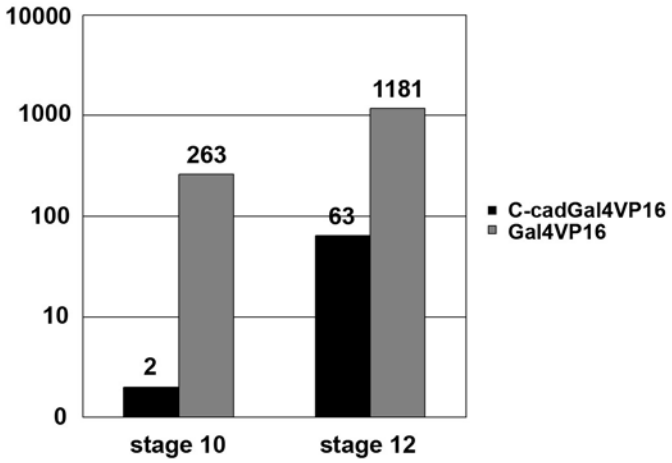


Figure 2.22: Nuclear access of C-cadherin cytodomain also occurs *in vivo*. Four cell stage *Xenopus* embryos were co-injected into the dorsal involuting marginal zone with 0.25 ng of a Gal4 responsive reporter plasmid and 1 ng of RNA of either Gal4, Gal4VP16 or C-cadGal4VP16. Lysates of stage 10 and stage 12 embryos were tested for luciferase reporter activity. Gal4 and Gal4VP16 served as negative and positive control, respectively. Data is represented as fold activation of the negative control Gal4, which was set as 1.

The observed difference in activity between stage 10 and 12 was approximately five fold for Gal4VP16 compared to 30 fold for C-cadGal4VP16 injected embryos. The five fold increase in Gal4VP16 activity indicates that overall transcriptional activity is not yet optimal in stage 10, which might be explained by the fact that in *Xenopus* transcription does not start before mid-blastula (stage 8-8.5). Nevertheless, the observation that C-cadGal4VP16 shows a 30 fold increase between stage 10 and 12 suggests a specific increase in nuclear access of the cadherin.

2.3.4 The cytoplasmic domains of *Xenopus* C-cadherin and human E-cadherin do not have intrinsic transactivation capacity

Constructs encoding HE-cadGal4 and C-cadGal4 fusion proteins were transfected into different cell lines and Gal4 dependent luciferase reporter assays were performed to address the question if the cytoplasmic domain of these cadherins can directly mediate transcriptional activation. HE-cadGal4VP16 was used as a positive control, resulting in high luciferase signals in all tested cell lines. No increase in luciferase activity was observed for HE-cadGal4 or C-cadGal4 in any of the cell lines compared to control Gal4. Instead, both HE-cadGal4 and C-cadGal4 showed a much lower luminescent signal of approximately 0.1, which is comparable to that of cells transfected with empty vector alone (Fig. 2.23). This is perhaps suggestive of an inhibitory effect of the cadherin cytoplasmic domain on transcription. In summary, these experiments suggest that *Xenopus* C-cadherin and human E-cadherin gain access to the nucleus, but do not have intrinsic transcriptional activation capacity.

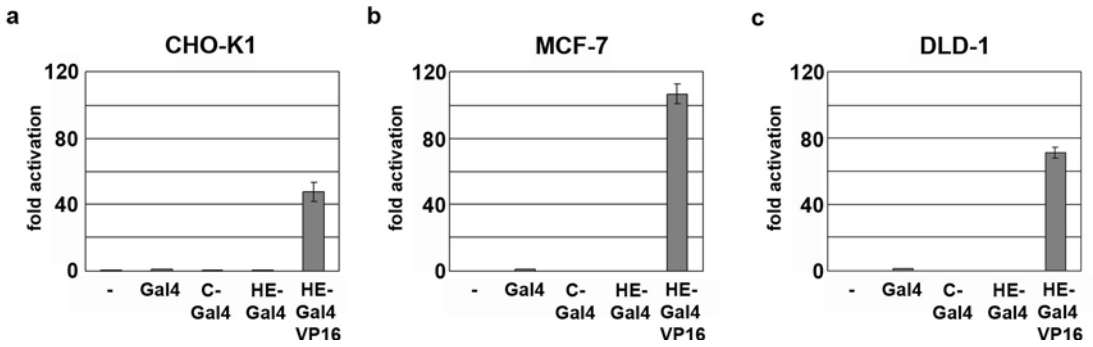


Figure 2.23: The HE- or C-cadherin cytoplasmic domain do not show intrinsic transcriptional activity. The cell lines **(a)** CHO-K1, **(b)** MCF-7 and **(c)** DLD-1 were transfected with a Gal4 responsive reporter plasmid in combination with either HE-cadGal4 or C-cadGal4 and tested for luciferase reporter activity. Gal4 and HE-cadGal4VP16 served as negative and positive control, respectively. Data is represented as fold activation of the negative control Gal4, which was set as 1. Transfections were performed in triplicates. mock: transfection of empty vector alone. Taken from the diploma thesis of Gilles Sequaris, which was performed under my supervision.

2.3.5 Transcriptional activation occurs in the absence of PS1 and 2

If PS1/γ-secretase mediated cleavage is essential for nuclear localization of cadherins, inhibition of presenilin activity should reduce the cadherin-Gal4VP16 mediated luciferase activity. To test this possibility MCF-7 cells were co-transfected with the Gal4 responsive luciferase reporter and C-cadGal4VP16 or HE-cadGal4VP16. High transcriptional activity was observed in cells transfected with HE-cadGal4VP16 or C-cadGal4VP16 in the presence of DMSO alone, but also in the presence of the γ-secretase inhibitor L-685,458 (Fig.2. 24a).

In addition to a γ-secretase cleavage site, a caspase-3 site has also been identified in the E-cadherin cytoplasmic domain (Fig. 1.4). However, incubation with a caspase-3 inhibitor alone or in combination with the γ-secretase inhibitor also did not significantly reduce luciferase activation (Fig. 2.24a). To confirm that the concentration of γ-secretase inhibitor used in the transactivation assay was indeed blocking γ-secretase mediated cleavage, CHO-K1 cells expressing HE- or C-cadherin were treated with the same concentration of this inhibitor. Cells were lysed and analysed by western blot using the PEP1 pan cadherin antibody recognizing the cytoplasmic domain of cadherins. If cleavage is blocked, a fragment of ~40 kD should be detected in addition to full length cadherin. This fragment corresponds to the cadherin peptide that remains located at the membrane upon ectodomain shedding and inhibition of γ-secretase mediated cleavage of the cytodomain. The fragment released by the γ-secretase is rapidly degraded in the proteasome and thus is not easily detected in western blot (Marambaud et al., 2002). Indeed, the expected HE-cadherin fragment was observed in both transiently and stably transfected CHO-K1 cells in the presence of L-685,458 (Fig. 2.24b). A similar fragment was also observed for C-cadherin. Treatment of cells with the caspase-3 inhibitor Z-DEVD-FMK led to small amounts of this 40 kD fragment.

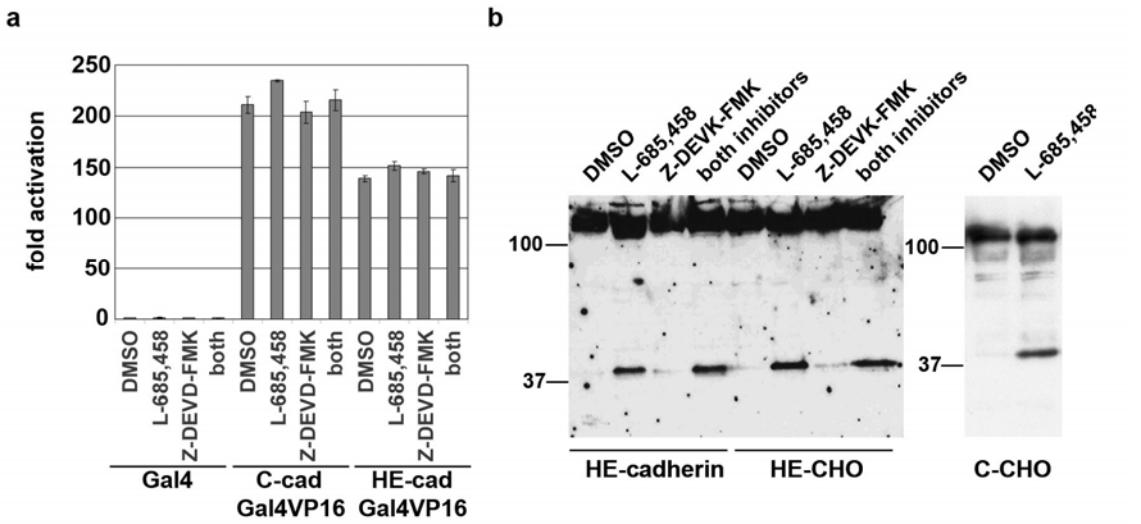


Figure 2.24: (a) Transcriptional activation of Gal4VP16 fusion proteins cannot be blocked by specific protease inhibitors. MCF-7 cells were transfected with a Gal4 responsive reporter plasmid in combination with either HE-cadGal4VP16 or C-cadGal4VP16. Cells were then treated with a specific inhibitor of γ-secretase (L-685,458), a specific inhibitor of caspase-3 (Z-DEVD-FKM), both inhibitors or DMSO alone and lysates were tested for luciferase reporter activity. Gal4 transfected cells were treated accordingly, and served as negative control. Data is represented as fold activation of the negative control Gal4, which was set as 1. Transfections were performed in triplicates. **(b) Both HE-cadherin and C-cadherin are substrates of the γ-secretase complex.** CHO-K1 cells transiently transfected with HE-cadherin and stably expressing HE-cadherin HE-CHO cells (left panel) or stably expressing C-cadherin C-CHO cells (right panel) were treated with the indicated protease inhibitors. Protein lysates were separated on a 4-12% gradient gel and analysed by western blot using an anti pan cadherin antibody directed against the cytoplasmic domain of cadherins. Taken from the diploma thesis of Gilles Sequaris, which was performed under my supervision.

As a further approach to analyse the role of PS1/γ-secretase mediated cleavage in transactivation, a mutant form of HE-cadGal4VP16 was generated, in which part of the presenilin binding site is mutated (EED762-764 to AAA; Fig 2.25a). The highly conserved amino acid sequence 760-771 of HE-cadherin was shown to be critical for binding of PS1 and $p120^{ctn}$, and both proteins compete with each other for binding to HE-cadherin (Baki et al., 2001; Thoreson et al., 2000). HE-cadGal4VP16 EED762-764AAA was transiently transfected into CHO-K1 cells in combination with a Gal4 dependent luciferase reporter. In agreement with the γ-secretase inhibitor results, no decrease in activation was observed for the HE-cadGal4VP16 EED762-764AAA mutant when compared to wt, but instead a significant 2-3 fold increase (Fig. 2.25b).

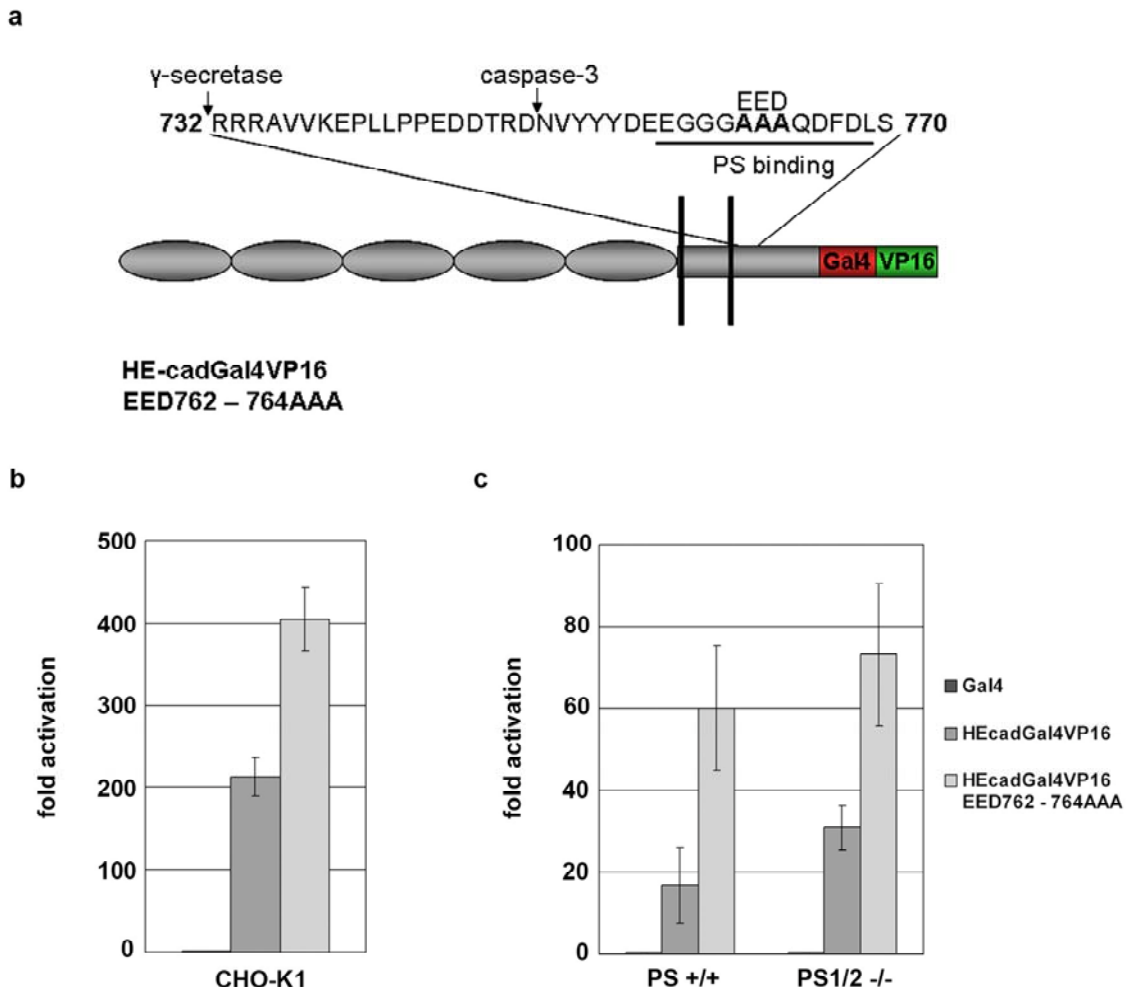


Figure 2.25: Transcriptional activation by cadherins occurs in the absence of PS1 and 2. (a) Schematic representation of the EED762-764AAA mutation in the presenilin binding site of HE-cadGal4VP16. The presenilin binding site is underlined; cleavage sites of γ-secretase and caspase-3 are indicated by arrows. **(b)** CHO-K1 cells and **(c)** mouse embryonic fibroblast cell lines of wt (PS +/+) and PS1 and 2 knock-out (PS1/2 -/) mice were transfected with HE-cadGal4VP16 or Gal4 and a Gal4 responsive luciferase reporter. Cells were lysed and tested for luciferase reporter activity. Gal4 served as negative control and was set as 1. Transfections were performed in triplicates.

To definitely rule out the involvement of presenilin cleavage in the translocation of C-cadherin or HE-cadherin to the nucleus, PS1 and 2 negative fibroblasts were used in luciferase reporter studies. Primary mouse embryonic fibroblasts of PS1 and 2 double knock-out (PS1/2 -/-) and wt (PS +/+) mice (Hartmann et al., 2002) were analysed for Gal4 dependent transcriptional activity upon expression of the HE-cadGal4VP16 fusion protein. No significant differences were observed in luciferase activity in wt and PS1/2 -/- fibroblasts upon expression of HE-cadGal4VP16 (Fig. 2.25c). Again, transfection of the mutant HE-cadGal4VP16 EED762-764AAA resulted in a significant 2-3 fold increase compared to HE-cadGal4VP16 in both wt and PS1/2 -/- fibroblasts.

Taken together, these results demonstrate that translocation of the cadherin to the nucleus is independent of presenilin/γ-secretase cleavage.

3. Discussion

Cadherin adhesive function can be altered by distinct proteolytic cleavage events, resulting in release of either the ectodomain or cytoplasmic domain from the membrane. However, the functional significance of the released extracellular and cytoplasmic domain is not clear yet. In this thesis it is demonstrated that the classical cadherin ectodomain interferes with gastrulation in *Xenopus* embryos by inhibiting convergent extension movements, indicating that this fragment can exert biological activity *in vivo*. Such a released extracellular fragment might actively contribute to cancer progression. Indeed, increased soluble E-cadherin was detected in serum of melanoma patients and this correlated with higher tumor mass in these patients.

It has been suggested that release of the ectodomain is connected to cleavage of the cytoplasmic domain, perhaps as a pathway to signal changes in intercellular adhesion to the interior of the cell. Using a sensitive reporter assay, it was found that cadherins translocate to the nucleus. This suggests that the cadherin cytoplasmic domain, similar to the Notch signalling pathway, may enter the nucleus to regulate transcription. However, no evidence was found for a transactivator function of cadherins.

3.1 Functional role of the cadherin ectodomain

3.1.1 The classical cadherin ectodomain has the capacity to interfere with morphogenetic movements *in vivo*

Overexpression studies were performed as an approach to address the function of the cadherin ectodomain *in vivo*. This approach was chosen, since it is not possible to specifically target the released fragment, for example by knockdown experiments, and thus study the consequences of the lack of only this peptide. Generation of a cadherin mutant that is insensitive to cleavage by metalloproteases is difficult as well, since these enzymes do not recognize a clearly defined consensus sequence. In addition, it has been reported that mutations in the primary sequence of substrates adjoining the metalloprotease cleavage site did not affect processing (Schlondorff and Blobel, 1999). Moreover, the latter approach would reveal if ectodomain shedding is important for cadherin function, but would not directly address the role of the cleaved fragment.

Overexpression of the C-cadherin extracellular domain in early *Xenopus* embryos led to a severe defect in gastrulation movements (Fig. 2.4), indicating that the C-cadherin extracellular domain has the capacity to interfere with morphogenetic processes *in vivo*. Previous *in vitro* experiments suggested that the cadherin ectodomain may retain biological function after cleavage by metalloproteases and release from the membrane.

Characterization of the purified chicken N-cadherin ectodomain indicated that it could serve as an adhesive substrate and thus function in promoting neurite outgrowth (Paradies and Grunwald, 1993). Wheelock et al. describe scattering of epithelial cell monolayers upon treatment with the purified soluble 80 kD E-cadherin fragment (Wheelock et al., 1987). However, no quantification of the presented data was given and own attempts to disrupt cell-cell aggregation of epithelial cell monolayers by adding recombinant human E-cadherin extracellular domain in varying concentrations were inconsistent. Noe et al. showed induced invasion and reduced aggregation of MDCK cells when these were cultured in conditioned medium containing increased amounts of soluble E-cadherin ectodomain (Noe et al., 2000). In a similar setup, conditioned medium containing increased ADAM10-released soluble E-cadherin promoted migration of keratinocytes in *in vitro* wound closure experiments (Maretzky et al., 2005). The observation that the cadherin extracellular domain disrupts *Xenopus* embryonic development therefore provides the first evidence that this fragment can interfere with morphogenetic processes *in vivo*.

3.1.2 How does the C-cadherin extracellular domain disturb gastrulation?

Several mechanisms could be envisioned by which cadherin extracellular domain expression disturbs gastrulation. The most obvious explanation is that it interferes with cell movement. Indeed, the cadherin extracellular domain did interfere with convergent extension movements in the animal cap assay (Fig. 2.13). It is possible that this effect is caused by a change in cell fate, thus altering migratory behavior. However, mesoderm induction was not blocked (Fig. 2.14), indicating that the cadherin extracellular domain directly affects elongation movements.

The extracellular domain seemed to only interfere with cadherin function when a tight regulation of cadherin activity is required, but did not strongly affect cell adhesion: First, overexpression of any of the C-, E-, or N-cadherin extracellular domains in the animal hemisphere did not cause any visible effects on intercellular adhesion (Table 2.2). This might be explained by the fact that tighter junctional complexes are present in the ectoderm undergoing epidermogenesis compared to mesodermal tissue. Second, in a blastomere adhesion assay no major differences were observed in XCEC1-5myc injected versus control blastomeres (Fig. 2.11). Only with low cadherin substrate concentrations XCEC1-5myc blastomeres adhered less well, although the significance of this result needs to be further tested. However, it has to be kept in mind that in the experimental setup of the blastomere adhesion assay, blastomeres are isolated in Ca^{2+}-free medium. This treatment disrupts endogenous cadherin mediated adhesion and therefore most likely also disrupts binding of XCEC1-5myc to endogenous cadherin. The incubation time

during which cells were allowed to bind to the substrate is probably not sufficient to secrete sufficient amounts of XCEC1-5myc to interfere with adhesion (Fig. 2.2). Therefore, differences in adhesion might be difficult to detect with the blastomere adhesion assay.

During convergent extension cadherin adhesive activity needs to be tightly regulated. C-cadherin activity is downregulated during elongation of activin-induced animal cap explants, without alterations in overall C-cadherin amounts on the cell surface (Brieher and Gumbiner, 1994). Importantly, downregulation of cadherin activity is necessary for convergent extension to proceed, since an activating antibody to C-cadherin that restores strong adhesion in animal cap explants caused inhibition of animal cap elongation (Zhong et al., 1999). Similarly, overexpression of wt C-cadherin disrupts gastrulation (Lee and Gumbiner, 1995), demonstrating that a proper balance in cell adhesion is required for convergent extension. Considering the observation that XCEC1-5myc interferes with convergent extension but does not affect ectoderm integrity, the results indicate that cells undergoing remodelling of intercellular adhesion during cellular rearrangements might be particularly sensitive to the presence of the C-cadherin extracellular domain. Under such circumstances, the soluble ectodomain might dysregulate appropriate levels of adhesive activity necessary for cell rearrangements. In addition, the presented data highlight and confirm the importance of cadherin regulation during motile processes.

Expression of a C-cadherin mutant lacking the cytoplasmic domain in early *Xenopus* embryos revealed similar defects as expression of the C-cadherin extracellular domain alone (Lee and Gumbiner, 1995). The most probable explanation for the observed dominant negative phenotype of these truncated C-cadherin fragments is that they directly bind to endogenous cadherin, thus interfering with its function. However, it has to be kept in mind that although the isolated cadherin extracellular fragment can serve as an adhesive substrate (Paradies and Grunwald, 1993; Utton et al., 2001; Niessen and Gumbiner, 2002), a direct binding of the exogenously expressed truncated C-cadherin proteins and endogenous C-cadherin has not been shown yet. Co-immunoprecipitation experiments would be necessary to confirm such an interaction. Our initial attempts to confirm such an interaction were unsuccessful, which might be explained by the low affinity of cadherin binding (Chappuis-Flament et al., 2001). It cannot be excluded that the cadherin ectodomain interferes with other cellular processes or signalling pathways, for example by interfering with other known binding partners such as receptor tyrosine kinases. In a study using culture medium supplemented with soluble recombinant N-cadherin extracellular domain, it was demonstrated that this fragment stimulated neurite outgrowth in an FGFR dependent manner (Utton et al, 2001). Nevertheless, the fact that

high amounts of cadherin ectodomain were necessary to cause developmental defects argues against a direct effect on signalling processes.

In general, the XCΔtail mutant exhibited a stronger effect on gastrulation than XCEC1-5myc when similar amounts of RNA were used (Table 2.1). In addition, half the amount of XCΔtail RNA compared to XCEC1-5myc was sufficient to block convergent extension (Fig. 2.13). Together these results implicate a stronger sensitivity of embryos towards this construct, which could be explained by the oriented integration of XCΔtail into the plasma membrane and therefore a stronger interference with endogenous cadherin. Indeed, whereas overexpression of XCEC1-5myc did not affect ectoderm integrity, this was disturbed upon XCΔtail expression in the animal hemisphere of the embryo (Fig. 2.10; Table 2.2).

A possible mechanism how these proteins might interfere with endogenous cadherin function is an alteration of cadherin cell surface expression. Such an effect was demonstrated upon overexpression of a membrane bound cadherin mutant lacking the extracellular domain, which led to downregulation of endogenous cadherin (Kintner, 1992). Western blot analysis using a pan cadherin antibody recognizing the cytoplasmic cadherin domain detected similar amounts of full length C-cadherin in control and XCEC1-5myc injected embryo lysates, suggesting that interference with endogenous cadherin is not due to an alteration in cell surface expression. Also, immunofluorescent analysis for endogenous C-cadherins in animal cap explants did not reveal any major changes in cell surface expression between control and XCEC1-5myc injected embryos (data not shown). It cannot be ruled out that the overexpressed proteins cause overall cell dysfunction, for example due to retention in the ER, and therefore perturbed normal cellular processes. This is unlikely because the endogenous cadherin appears to be expressed on the cell surface, as mentioned above. Second, secretion assays demonstrated efficient release of the expressed fragments from blastomeres, although some cytoplasmic staining was observed by immunofluorescent staining of animal caps as well (Fig. 2.2). Additionally, expression of other cadherin extracellular domains, such as that of the type II cadherin-11 or the paraxial protocadherin PAPC, did not cause blastopore closure defects (Fig. 2.6 and 2.7), indicating that the observed phenotype does not result from toxicity effects due to large amounts of exogenously expressed proteins secreted into the extracellular space.

How does the cadherin extracellular domain interfere with cadherin function? The critical involvement of the tryptophan at position 2 (W2) of mature classical cadherins in mediating adhesive activity by forming the strand dimer adhesive interface is well established (reviewed in Patel et al., 2003). Mutation of this site in full length cadherins results in non-functional molecules (Noe et al., 2000; Shan et al., 2000; Tamura et al.,

1998). Therefore, the dominant negative effect could be exerted by directly interfering with the strand dimer interface of endogenous cadherin. However, a mutant W2A extracellular domain of C-cadherin still retained the capacity to cause gastrulation defects (Fig. 2.8), indicating that this amino acid is dispensable to interfere with the endogenous cadherin. This result also shows that the extracellular domain itself does not need to be adhesive active to interfere with gastrulation. The C-cadherin EC1 domain alone caused similar defects (Fig 2.9), and thus must contain other sequences than W2 that can interfere with endogenous cadherin function. Indeed, the EC1 domain of cadherins appears to be particularly important for cadherin binding. First, it determines binding specificity, which was shown by exchanging EC1 domains of two cadherins resulting in concomitant exchanged specificity of cadherin expressing cell aggregates (Nose et al., 1990). Second, it contains several conserved motifs including the W2 strand dimer and the HAV motif. Third, crystal structures of different cadherin extracellular domains provided evidence that adhesive bonds were mediated by interactions between EC1 domains (Shapiro et al., 1995; Boggon et al., 2002). Cadherins form both trans (adhesive) and cis (lateral) dimers. Thus far it cannot be distinguished by functional analysis whether the W2 strand dimer is involved in formation of adhesive or lateral dimers. Some models suggest that the W2 interface forms lateral dimers, which in turn initiates formation of adhesive dimers, whereas other models contribute formation of adhesive dimers to the W2 interface, and it cannot be excluded that W2 is able to switch between both interactions (Fig. 1.3) (Gumbiner, 2005).

One possible mechanism by which the cadherin extracellular domain could interfere with endogenous cadherin function is via the HAV sequence. According to the ´linear zipper` model, based on the crystal structure of N-cadherin EC1, the adhesive bond forms via the HAV motif upon dimerization by the W2 interface. In this case, the cadherin EC1 domain could disrupt adhesive bonds between cadherin molecules by interaction via the HAV sequence. However, the putative adhesion interface surrounding the HAV sequence could not be confirmed by mutational analysis, arguing against a critical role of this motif in cadherin binding (Boggon et al., 2002).

Another possibility would be that the EC1 domain disturbs formation of lateral dimers, which are fundamental for cadherin adhesive function (Brieher et al., 1996; Yap et al., 1997). It has been suggested that lateral dimerization occurs at the Ca^{2+}-binding site between EC1 and EC2 (Pertz et al., 1999). Since the EC1 domain alone can efficiently cause gastrulation defects, interference of lateral dimers via the Ca^{2+}-binding site seems to be unlikely. A new putative interface for cadherin lateral dimerization was described by Boggon et al. in 2002. Here, the crystal structure of the complete C-cadherin extracellular domain revealed that the W2 interface mediates interactions between two antiparallel

ectodomains, each adopting a curved-like structure. In addition, an interaction in cis orientation was observed by binding of a W2 distal interface in EC1 with EC2 of a parallel molecule, at the interface of EC3. Thus, it can be hypothesized that the EC1 domain interferes with lateral dimer formation by binding the EC2 domain of endogenous cadherin via this W2 distal interface, resulting in severe impairment of adhesion. This possibility would combine the observation that EC1 alone can interfere with gastrulation and a previous report showing that a minimum of three of the EC domains is required for effective binding and adhesion (Chappuis-Flament et al., 2001): EC1 binds the cis interface present at the C-terminal end of EC2. However, other as yet unknown binding sites may also be involved.

In conclusion, the presented data suggest that the cadherin EC1 fragment interferes with endogenous cadherin function independently of the W2 adhesive interface to disrupt gastrulation movements, possibly by disturbing cadherin lateral dimerization.

3.1.3 Classical cadherins reveal heterophilic binding capacity

Surprisingly, both overexpression of N- and E-cadherin extracellular domains caused gastrulation defects similar to those observed for C-cadherin extracellular domain (Fig. 2.5). Endogenous E-cadherin expression only starts at mid-gastrulation and remains confined to the ectodomain (Levi et al., 1991). In addition, N-cadherin expression is not turned on before beginning of neural tube formation (Detrick et al., 1990). These observations can therefore not easily be explained by direct interference with endogenous N- or E-cadherin function. Instead, the results suggest that the dominant negative activity of the N- and E-cadherin extracellular domains is caused by direct interference with C-cadherin, thus indicating a heterophilic binding capacity. This was unexpected because cadherins are considered homophilic adhesion molecules and thought to only recognize the same type of cadherin. Furthermore, expression of XEΔtail in the animal hemisphere caused disruption of ectodermal tissue at blastula stage, before expression of endogenous E-cadherin starts (Table 2.2). Again, this is indicative of heterophilic cadherin interactions. The latter result is in contrast to a previous report, in which peptides consisting of the extracellular and transmembrane domain of E- and N-cadherin were expressed in *Xenopus* and caused a distinct disruption of the ectoderm and the neural tube, respectively (Levine et al., 1994). These observations were explained by selective inhibition of the endogenous cadherin function due to a homophilic mechanism. The observed phenotypic differences might be explained by variable quality of the *in vitro* transcribed RNA, causing severe defects at early stages.

On the other hand, it has become evident that cadherins are able to engage in heterophilic interactions and exhibit a much wider array of binding specificity than was

previously assumed. In cell mixing experiments it was shown that cells expressing N-cadherin can mix with those expressing R-cadherin (Shapiro et al., 1995). Similarly, cells expressing chick B-cadherin mixed with those expressing E-cadherin, thus suggesting heterophilic interactions between these different classical cadherins (Murphy-Erdosh et al., 1995). Importantly, when CHO-K1 cells expressing different cadherins were used in adhesion flow assays with recombinant extracellular cadherin substrates, thereby taking only cadherin-cadherin interactions into account, it was demonstrated that none of the tested classical cadherins (*Xenopus* E- and C-cadherin, human E- and N-cadherin) displayed any specificity in binding to the cadherin substrate (Niessen and Gumbiner, 2002). Using the *Xenopus laevis* model system the presented data indicate that the classical cadherin extracellular domains can interfere with a different member of the same subgroup, thus displaying heterophilic binding capacity *in vivo*.

The data also indicate that type II cadherins as well as protocadherins cannot directly bind type I cadherins, since overexpression of cadherin-11 (type II) or PAPC (protocadherin) extracellular domains did not cause any developmental defects comparable to those observed for classical cadherin extracellular domain (Fig 2.6 and 2.7). This is in agreement with cell aggregation assays, in which cells expressing different type II cadherins were able to mix with each other, but did not mix with type I cadherin expressing cells (Shimoyama et al., 2000).

3.1.4 Does cadherin shedding play a role in melanoma progression?

The E-cadherin ectodomain was found in the supernatant of a panel of melanoma cell lines, but no direct correlation was observed between extend of E-cadherin shedding and invasive potential of these cell lines (Fig. 2.15). As has been shown by others (Danen et al., 1996; Haass et al., 2004), E-cadherin was almost absent from high invasive cells. Nevertheless, the observation that the amount of cadherin ectodomain in the supernatant was not linear with surface levels of cadherin expression suggests that E-cadherin ectodomain shedding may be a regulated process. Regulation of cadherin ectodomain shedding is not fully understood yet. Recently, ADAM10 has been shown to be the major sheddase of E-, and N-cadherin on mouse fibroblasts, neuronal cell lines, and human HaCat cells (Reiss et al., 2005; Maretzky et al., 2005) although other metalloproteases such as Matrilysin and Stromelysin-1 as well as the serine protease plasmin have been implicated in cleavage of E-cadherin as well (Lochter et al., 1997; Noe et al., 2001; Ryniers et al., 2002). It would be interesting to see whether ADAM10 expression is indeed upregulated in correlation with increased soluble E-cadherin.

A previous study reported increasing levels of soluble E-cadherin in malignant melanoma compared to controls, but here only a very small patient group was analysed and S100

values were not taken into account (Shirahama et al., 1996). In this thesis additional evidence is provided that increased E-cadherin shedding occurs in melanoma and correlates with its progression. This was suggested by higher E-cadherin levels in correlation with an overall increase of the melanoma progression marker S100, although significant differences could only be found in patient groups with S100 values higher than 10 µg/l (Fig. 2.16). In various types of carcinomas it has been shown that increased soluble E-cadherin levels are detected in patient sera compared to controls, and in some cases a higher amount of soluble E-cadherin correlated with tumor stage, for example ovarian and gastric carcinoma (Chan et al., 2001; Gadducci et al., 1999). In some of these clinical studies, western blot analysis of the patient material detected an 80 kD E-cadherin fragment, indicating that the soluble E-cadherin does indeed represent the shed ectodomain (Banks et al., 1995; Soler et al., 2002). How could increased E-cadherin shedding contribute to melanoma progression: on the one hand shedding may result in an overall decrease in E-cadherin cell surface concentration; on the other hand, the released E-cadherin ectodomain might additionally decrease cadherin-mediated cell-cell adhesion by interfering with endogenous cadherin function.

Remarkably, in the patient group showing normal S100 values a subgroup existed that already showed elevated E-cadherin levels (Fig. 2.16b), suggesting that E-cadherin shedding represents an early event in melanoma progression, although patient numbers were too small to be statistically significant. This might be interesting in light of the fact that in the basal layer of the skin keratinocytes are thought to exert growth control over melanocytes via E-cadherin mediated adhesion (Tang et al., 1994). In melanoma cells, loss of keratinocyte-mediated growth control occurs in association with downregulated E-cadherin expression, whereas E-cadherin expression in melanoma cells restores adhesion to keratinocyte and growth control (Hsu et al., 2000). Thus, early inactivation of E-cadherin due to ectodomain shedding might contribute to deregulated growth of melanocytes and thus be an early step in melanoma development.

3.1.5 What is the biological significance of the released cadherin ectodomain?

An important question that remains is if the shed cadherin ectodomain also contributes to regulation of cadherin function under physiological or pathological conditions. Endogenous C-cadherin extracellular fragments were observed during development, indicating that cadherin shedding indeed occurs *in vivo*. However, only minimal changes in its amount at different stages of gastrulation were found, suggesting that the process is not strictly regulated during development. In addition, it was found that at least a ten times excess of the extracellular domain over endogenous full length protein was necessary to interfere with gastrulation (Fig. 2.4c). Such a high amount of cadherin ectodomain versus

corresponding full length protein is unlikely to occur under normal physiological conditions, raising the question whether the endogenously shed cadherin ectodomain might be able to affect the full length protein *in vivo*. In a report studying mechanotransduction pathways it was shown that mechanical stress in bronchial epithelial cells leads to an 80% reduction of the lateral intercellular space, thereby increasing the local concentration of EGF family ligands and initiating an autocrine EGFR signalling loop (Tschumperlin et al., 2004). This finding suggests that even with little amounts of cadherin ectodomain shed into the intercellular space, local concentrations of this fragment may be sufficiently high due to other mechanisms to affect the function of full length protein. Further, it might be possible that local differences in concentration of the shed cadherin ectodomain exist, for example in cells undergoing convergent extension, even though no major differences were found in whole embryo lysates. A careful analysis of the occurance of the shed ectodomain in mesodermal explants would be necessary to answer this question.

Thus, the cadherin ectodomain may have the capacity to disturb cellular processes the cadherin function is downregulated to critical levels. This may occur in development, for example during convergent extension of the mesoderm, or under pathological conditions, such as tumor progression. Most likely, the effect is caused by direct binding of the endogenous cadherin in a dominant negative manner, possibly by interference of the EC1 domain with lateral dimer formation. Our results provide the first evidence that release of the extracellular domain may actively regulate cellular movements (Fig 3.1).

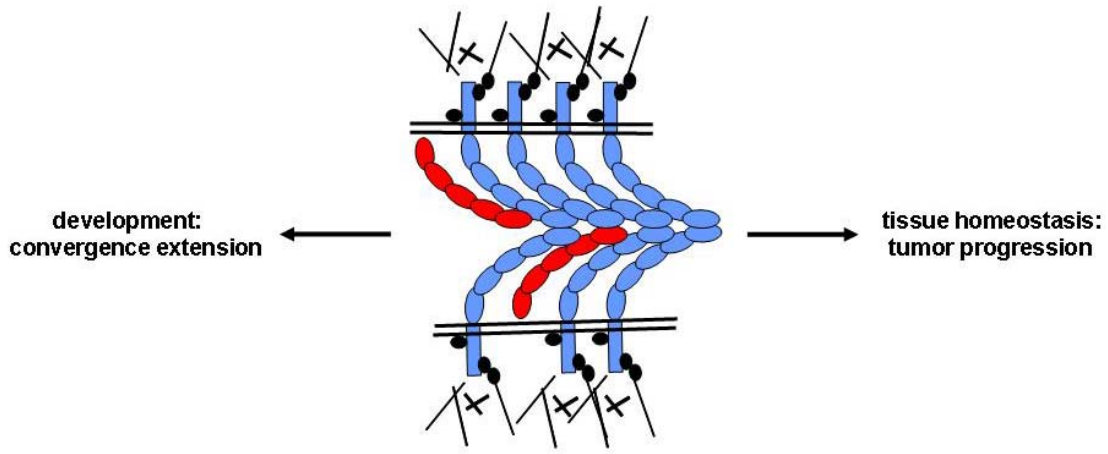


Fig. 3.1: Potential function of the cleaved cadherin ectodomain. Most likely the cadherin ectodomain affects gastrulation by direct binding of the endogenous cadherin in a dominant negative manner. The EC1 fragment contains all the necessary information to disrupt gastrulation movements, but this occurs independently of the W2 adhesive interface, possibly by interference with lateral dimers via binding to the EC2 domain of a parallel molecule. The cadherin ectodomain may have the capacity to disturb cellular processes that require a tight regulation of cadherin function. This may occur in development, for example during convergent extension of the mesoderm, or under pathological conditions, such as tumor progression.

3.2 Functional role of the released cadherin cytoplasmic domain

Next to the ectodomain, the cytoplasmic domain of cadherins can be proteolytically cleaved and released from the membrane as well. The relation between these two events is unclear. Some data suggest that ectodomain shedding is a prerequisite for cytodomain release (Reiss et al., 2005), whereas other data imply that the two cleavage events can occur independently of each other (Marambaud et al., 2002). Nevertheless, it is tempting to speculate that changes in intercellular adhesive activity are communicated to the interior of the cell by release of the cytoplasmic domain. Cytodomain cleavage is mediated by the PS1/γ-secrease complex and occurs at the interface of the transmembrane and cytoplasmic domain (Marambaud et al., 2002). Cleavage by PS1/γ-secretase has been described for several other type I membrane proteins and for some of these substrates it was shown that the cleaved cytoplasmic domain can translocate to the nucleus and mediate transcriptional activation, such as the well-known example Notch (Struhl and Adachi, 1998).

To analyse the functional role of the released cadherin cytoplasmic domain, a similar approach as described for Notch nuclear access was taken. *Xenopus* C-cadherin and human E-cadherin were fused to either Gal4 or Gal4VP16 and transcriptional activation assays were performed to reveal if the cadherin cytoplasmic domain can enter the nucleus, and if it has intrinsic transactivation activity. This sensitive method was chosen, since it had previously been reported that γ-secretase-cleaved cytoplasmic fragments are rapidly degraded by the proteasome and a detection of small amounts of fragment in the nucleus by conventional biochemical and immunological methods had failed (Struhl and Adachi, 1998).

3.2.1 The cadherin cytoplasmic domain can translocate to the nucleus, but does not have intrinsic transactivation capacity

Both expression of HE-cadGal4VP16 and C-cadGal4VP16 fusion resulted in high transcriptional activation of the luciferase reporter (Fig. 2.21), indicating that cadherins can enter the nucleus, possibly due to release and translocation of the cytoplasmic domain. This result was not only found in transient transfections of different cell lines, but was also confirmed using *Xenopus* embryonic development as *in vivo* model (Fig. 2.22).

It is possible that the observed transcriptional activation is due to improper cleavage of the Gal4VP16 domain and thus occurs independently of cadherin cleavage. Although there is no formal proof against this point yet, this possibility is not very likely because antibodies to Gal4 detected bands corresponding to full length cadherin Gal4VP16 fusion proteins in cadherin Gal4VP16 transfected cells, but no bands corresponding to Gal4VP16 alone (not shown). Second, the cadherin fusion proteins might be improperly localized and therefore

not functional. However, immunofluorescence analysis showed proper localization at sites of cell-cell contacts (Fig. 2.19). Furthermore, β-catenin and $p120^{ctn}$ were recruited to the cell membrane upon cadherin expression (Fig. 2.20), suggesting that the cadherin fusions are functional.

If the cadherin cytodomain is able to activate transcription, nuclear translocation would result in luciferase activation upon expression of either HE-cadGal4 or C-cadGal4. No transactivation of the luciferase was detected in HE-cadGal4 or C-cadGal4 expressing CHO-K1 cells (Fig. 2.23), indicating that the cytodomains of *Xenopus* C-cadherin and human E-cadherin do not have intrinsic transactivation capacity. These findings were confirmed in the epithelial cell lines DLD-1 and MCF-7, which express endogenous HE-cadherin in contrast to CHO-K1 cells. Instead, expression of HE-cadGal4 or C-cadGal4 showed even lower reporter transactivation than expression of the Gal4 domain alone. This is perhaps suggestive of a repressor function of the cadherin cytoplasmic domain, which could be analysed using constitutive reporter constructs with high basal activity. However, considering the observation that Gal4 shows some transcriptional activity compared to mock transfections, this result could also be explained by the fact that less amounts of the Gal4 domain enter the nucleus, when fused to cadherins compared to Gal4 alone, since Gal4-cadherin fusions need to be released from the membrane to enter the nucleus.

3.2.2 Access to the nucleus does not require PS1 and 2

Previous results suggested that presenilin cleavage is required for cadherin cytodomain cleavage and subsequent cadherin cytodomain activity (Marambaud et al., 2003). However, the results presented here strongly indicate that nuclear translocation of cadherins can occur independently of presenilin cleavage. This is based on the following three observations. First, a γ-secretase inhibitor did not block transcriptional activation in C-cadGal4VP16 or HE-cadGal4VP16 expressing MCF-7 cells (Fig. 2.24a). It is possible that the amount of inhibitor was insufficient, although a range of inhibitor concentrations was tested in combination with different time points of inhibitor treatment. In no case inhibition of transactivation was observed (not shown). Moreover, western blot analysis of HE-CHO cells using an antibody directed against the cadherin cytoplasmic domain demonstrated that inhibitor treatment efficiently blocked γ-secretase activity, similar to previous studies (Fig. 2.24b) (Marambaud et al., 2002). The presence of the 40 kD cytoplasmic fragment in the lysate of C-CHO cells upon γ-secretase inhibition demonstrated that also C-cadherin can serve as substrate for PS1/γ-secretase, which had not been shown before. Second, a HE-cadGal4VP16 protein that was mutated in the PS1 binding site did not result in decreased reporter activity (Fig. 2.25). This mutation is known

to inhibit p120^{ctn} binding to HE-cadherin (Thoreson et al., 2000). Since this binding site has been shown to completely overlap with the presenilin binding site, and presenilin and p120^{ctn} for cadherin binding (Baki et al., 2001), it was reasoned that this mutation would also block presenilin binding. Final proof that translocation to the nucleus does not require PS1 and 2 came from experiments using PS1 and 2 negative fibroblasts (Hartmann et al., 2002): activation of the reporter in HE-cadGal4VP16 expressing cells occurred in the absence of both PS1 and 2 (Fig. 2.25c).

How does transcriptional activation occur in the absence of PS1 and 2? A possible explanation would be cleavage of the cytoplasmic domain by another protease. In fact, ectodomain shedding in association with caspase-3 mediated cleavage of the cytoplasmic domain of HE-cadherin is increased upon induction of apoptosis, and results in disassembly of adherens junctions and loss of cell-cell contacts (Herren et al., 1998; Steinhusen et al., 2001). This might implicate that cadherin cleavage only occurs in cells undergoing apoptosis. However, transactivation did also occur in early *Xenopus* embryos (Fig. 2.22) and in living cells followed by time lapse microscopy (not shown), arguing against cleavage of cadherins solely under apoptotic conditions. In addition, treatment of C-cadGal4VP16 or HE-cadGal4VP16 transfected cells with a specific caspase-3 inhibitor (Fig. 2.24a) or a general caspase inhibitor cocktail (not shown) did not block activation. Alternatively, the cytoplasmic domain could be cleaved by other, as yet unknown proteases. The size of this fragment of approximately 40 kD would presumably allow entrance of the nucleus without requirement of a nuclear localization signal (NLS), since proteins smaller than 50 kD are able to pass the nuclear pore complex by diffusion (Cyert, 2001). Indeed, overexpression of the cadherin cytodomain resulted in nuclear localization of this fragment (Niessen, unpublished observations).

Another possibility which cannot be ruled out at the moment is that the cadherin is not cleaved, but translocated to the nucleus as a full length molecule. Such a mechanism is thought to exist for several other transmembrane proteins, including the EGF-like growth factor receptor c-erbB-3 (Offterdinger et al., 2002).

Interestingly, mutation of the HE-cadherin binding site to p120^{ctn} was shown to increase transcriptional activation. The possibility that a lack of p120^{ctn} results in recruitment of PS1 and thus increased cleavage is excluded by the finding that even in PS1 and 2 negative cells an increase in transactivation could be observed (Fig. 2.25c). Recent reports have shown that p120^{ctn} acts as a regulator of cadherin turnover. In the absence of p120^{ctn}, cadherins cannot be stably retained at the cell surface resulting in rapid internalization (Davis et al., 2003). Vesicles are then either recycled back to the plasma membrane or targeted for lysosomal and/or proteasomal degradation (Reynolds and Carnahan, 2004).

Increased internalization induced by the absence of p120ctn binding to cadherin may serve as a trigger for cytoplasmic domain cleavage by an as yet unidentified protease on internalized vesicles, thus leading to nuclear translocation.

3.2.3 Potential roles of the cadherin cytoplasmic domain

It is by now well established that cadherins serve as a substrate for the PS1/γ-secretase complex. Two important questions remain open. Does the cadherin cytoplasmic domain have functional activity, and what are the signals that drive proteolytic cleavage of cadherins? One potential role for cytodomain cleavage might be regulation of intercellular contacts. Marambaud et al. showed that E-cadherin cleavage resulted in disassembly of the cadherin junctions (Marambaud et al., 2002). A signalling function was indicated for the presenilin-cleaved N-cadherin domain. This fragment remains located in the cytosol upon release from the membrane, where it is indirectly involved in regulation of gene expression: binding to the transcriptional coactivator CBP leads to degradation of CBP through the ubiquitin-proteasome system. This in turn decreases amounts of nuclear CBP and suppresses CREB-mediated transcription (Marambaud et al., 2003). A direct role in gene expression is known for γ-protocadherin. The cytoplasmic domain of γ-protocadherin accumulates in the nucleus upon PS1/γ-secretase cleavage and activates transcription of the γ-protocadherin gene locus in an autoregulatory fashion (Hambsch et al., 2005).

Although no intrinsic transactivation capacity was detected for C-cadherin and HE-cadherin, the cytoplasmic domain might have a function in the nucleus, possibly by interaction with unknown binding partners involved in regulation of gene expression. Another intriguing possibility is that the released cadherin cytoplasmic domain remains associated with its binding partners, such as β-catenin or $p120^{ctn}$, to modulate their localization and function, for example by protection of β-catenin from degradation and conjunctional entrance into the nucleus. Both are nucleocytoplamic proteins with important roles in gene regulation. Interestingly, expression of the cadherin cytodomain in CHO-K1 cells upregulated β-catenin expression and together they were found in the nucleus. However, in this case no upregulation of β-catenin mediated signalling was observed (Niessen, unpublished data). Overexpresion of the N- or E-cadherin cytodomain resulted in colocalization of these fragment with β-catenin in the nucleus, where LEF-1 mediated transcription was blocked (Sadot et al., 1998).

ADAM10 was identified as the major sheddase for E- and N-cadherin, and deficiency of this enzyme results in activation of β-catenin signalling. This suggests that cadherin ectodomain shedding may serve as a trigger to communicate changes in cell adhesion to the nucleus, possibly via the cadherin cytodomain.

4. Materials and Methods

4.1 Molecular cloning

4.1.1 Bacterial cell culture

The *E.coli* strain DH5α was cultured as described Sambrook et al., 1989. For transformation and production of chemically competent DH5α cells the method of Hanahan (Hanahan, 1983) was applied. High efficiency transformation of ligated DNA molecules were performed with the *E.coli* strain XL10-Gold (Stratagene), which were transformed according to the manufacturer´s protocol. For cloning applications with *dam*- and *dcm*-sensitive restriction enzymes the One Shot® INV110 Competent Cells from Invitrogen were used.

4.1.2 Plasmid DNA preparation

For isolation of small amounts of plasmid DNA (20 µg) the alkaline extraction method (Birnboim, 1983) was applied. Large scale isolation of up to 500 µg plasmid DNA was performed using the Maxi-Prep kit (Qiagen) according to the manufacturer´s instructions. The DNA concentration was measured photometrically at 260 nm and calculated on the basis that an optical density of 1 equals a concentration of 50 µg/ml double stranded DNA. Purity was determined by measuring OD_{260nm}/OD_{280nm}.

4.1.3 Polymerase chain reaction

For several cloning strategies polymerase chain reactions were performed to amplify DNA fragments of up to 2.8 kb. The *Pfu* DNA polymerase (Stratagene), a proofreading DNA polymerase isolated from *Pyrococcus furiosus*, was used at a concentration of 0.1 U/µl in combination with 0.25 µM of both sense and antisense primer (MWG-Biotech, highly purified salt free quality), 0.25 mM dNTP mix (Roche), 1x reaction buffer (Stratagene) and 10 ng plasmid DNA as template in 50 µl reaction volume. The template DNA was denatured at 95°C for 2 min followed by 35 amplification cycles each consisting of 1 min at 95°C, 1 min at the appropriate annealing temperature, and 2 min per kb length of the amplified DNA fragment at 72°C for elongation. The following formula was used to estimate the melting temperature of primers: $T_m = 2(A + T) + 4(G + C)$.

4.1.4 Recombinant DNA techniques

The following recombinant and further standard DNA techniques were performed as described in Sambrook et al., 1989, or according to the manufacturer´s instructions: restriction digestion, T4 DNA ligation, dephosphorylation of DNA fragments, agarose gel

electrophoresis and elution of DNA fragments from agarose gels, phenol/chloroform extraction and ethanol precipitation. All DNA modifying enzymes (i.e. restriction enzymes, T4 DNA ligase, calf intestinal phosphatase) were purchased from New England Biolabs. Point mutations were introduced using the QuikChange™ Site-Directed Mutagenesis Kit obtained from Stratagene. DNA sequencing was performed by the service laboratory of the CMMC (Center for Molecular Medicine Cologne) using an ABI PrismTM 377 DNA Sequencer (Applied Biosystems) and sequences were analysed with the Wisconsin Sequence Analysis Package, Version 8.1 (Genetics Computer Group, Inc.).

4.2 Handling of *Xenopus* embryos and explants

4.2.1 *In vitro* transcription

Capped mRNA for injection into *Xenopus* embryos was generated *in vitro* using SP6 RNA polymerase. To obtain sense mRNA, plasmids containing the coding regions of the genes of interest were linearized by digestion with a restriction enzyme cutting 3´ of the coding sequence. The transcription reaction was performed by adding 1x transcription buffer, 10 mM DTT, 10 mg/ml BSA, 0.5 mM NTP mix (Roche), 0.5 mM Ribo m^7G Cap Analog, 1 U/µl RNasin ribonuclease inhibitor and 0.5 U/µl SP6 RNA polymerase to 3 µg of linearized template DNA in 100 µl of reaction volume and incubation for 1h at 40°C. Unless otherwise indicated, all components of the transcription reaction were obtained from Promega. Subsequently, the template DNA was degraded by adding 3 U of DNase I (Promega) and incubation for 15 min at 37°C. After phenol/chloroform extraction and ethanol precipitation, the RNA concentration was determined photometrically and size and homogeneity of the transcripts were examined by separation on an agarose gel. RNA was stored in 75% ethanol at -20°C.

4.2.2 Preparation of *Xenopus* embryos and animal cap tissue explants

Egg production was induced by injecting the dorsal lymph sac of *Xenopus laevis* females with 400 U of human chorionic gonadotropin (Sigma-Aldrich). The next day eggs were squeezed into a petri dish containing 1x MBS (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 10 mM HEPES (pH 7.4), 100 µg/ml streptomycin and 100 U/ml penicillin) and *in vitro* fertilized with macerated testis (Newport and Kirschner, 1982; Newport and Kirschner, 1982). The jelly coat was removed with a 2% cysteine solution (pH 8.0) and embryos were kept in 0.1x MBS at 15 to 20°C. Staging of embryos was carried out according to the normal table of *Xenopus laevis* development (Nieuwkoop and Faber, 1967).

Animal cap explants were isolated by removing the presumptive ectodermal tissue of blastocoel roofs of de-jellied stage 8-9 embryos in 1x MBS. First, the vitelline membrane surrounding the embryo was removed with a pair of curved forceps, after which the animal cap was separated from the vegetal side of the embryo by cutting with a pair of eye lashes fixed on top of injection needles.

4.2.3 Microinjection of RNA and plasmid DNA

Microinjection capillaries were prepared using a P-87 micropipette puller (Sutter Instruments) and fitted to the micropipette holder of a PL1-100 microinjector (Harvard Apparatus). To adjust the position of the capillary the micropipette holder was mounted on a three-dimensional micromanipulator (Science Products). Samples of RNA and plasmid DNA were diluted to the appropriate concentrations in Gurdon´s buffer (88 mM NaCl, 1 mM KCl, 15 mM Tris pH 7.4) and filled into the capillaries using the microinjector. Prior to injections, four cell stage embryos were placed in 4% Ficoll 400 (Sigma-Aldrich)/1x MBS for 20 min. Injections were performed either animally into the four blastomeres of the animal pole or dorsally into the marginal zone of the two dorsal blastomeres. Subsequently, embryos were allowed to recover in 4% Ficoll 400/1x MBS for 2h after which they were kept in 0.1x MBS until further analysis. RNA encoding the Gal4 DNA binding site served as negative control, if not otherwise indicated.

4.2.4 Activin elongation assay

Animal cap tissue explants were isolated (see 4.2.2) and incubated with 10 ng/ml human recombinant activin A (Sigma-Aldrich) in 0.1% BSA/1x MBS for 1h at RT to induce mesoderm formation. Controls were kept in the absence of recombinant activin A. Explants were transferred to 0.1% BSA/1x MBS until the tissue was rounded up and further incubated in 0.5x MBS overnight at 18°C. Animal caps were fixed in 4% PFA/1x PBS and photographed. Convergent extension movements were determined by dividing the length of animal caps by their width. Statistical analysis was carried out using the Mann-Whitney *U*-test.

4.2.5 ß-galactosidase staining of embryos

To detect ß-galactosidase activity embryos were fixed in 4% PFA/1x PBS on ice for 1h and rinsed two times with 1x PBS. Staining of embryos was performed at 37°C in a staining solution using X-gal (Peqlab) as a substrate for ß-galactosidase (1 mg/ml X-gal, 10 mM $K_3Fe(CN)_6$, 10 mM $K_4(CN)_6$, 1 mM $MgCl_2$, 0.1% Triton X-100 in 1x PBS) (Detrick et al., 1990). The reaction was stopped when blue stain marking the injected sites was clearly visible.

4.2.6 Isolation of RNA from explants and reverse transcription PCR

Total RNA was extracted from animal caps using the RNeasy kit (Qiagen). Five to ten animal caps were lysed in 350 µl RLT buffer containing guanidine isothiocyanate and homogenized using a syringe and needle. Lysates were then loaded on RNeasy spin columns and RNA was isolated according to the manufacturer´s instructions. The concentration of RNA samples was determined photometrically. First strand cDNA was synthesized using 0.4 µg total RNA in combination with 0.5 mM dNTP mix, 25 ng/µl oligo (dT) primers, 5 mM DTT, 2 U/µl RNase OUT, 1x reaction buffer and 10 U/µl SuperScript™ II reverse transcriptase (Invitrogen), according to the manufacturer´s instructions. PCR reactions were set up by adding 1 µl of cDNA and 0.25 µM of gene specific primers to 12.5 µl REDTaq™ (Sigma-Aldrich) in a 25 µl reaction volume. REDTaq™ is a PCR ready made, 2x concentrated reaction mix containing dNTPs, Taq DNA polymerase and $MgCl_2$. The template DNA was denatured at 95°C for 2 min followed by 30 amplification cycles each consisting of 1 min incubation at 95°C, 1 min incubation at 55°C for annealing of the primers and 1 min at 72°C for elongation.

Primers were obtained from MWG Biotech. The following primer sets were used: Brachyury, sense: GGATCGTTATCACCTCTG and antisense: GTGTAGTCTGTAGCAGCA ; EF-1α, sense: CAGATTGGTGCTGGATATGC and antisense: ACTGCCTTGATGACTCCTAG (Fagotto et al., 1997).

4.3 Cell culture

Unless otherwise indicated, all cell culture components were purchased from Invitrogen.

4.3.1 CHO-K1 cells and CHO-K1 cells stably expressing full length cadherins

CHO-K1 is a subclone of the parental CHO cell line, which was derived from Chinese hamster ovarian tissue. CHO-K1 cells were cultured in HAM´S F12 (PAA) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml each of penicillin and streptomycin at 37°C in a humidified incubator with a 5% CO_2 atmosphere.

HE-CHO cells stably transfected with cDNA encoding full length human E-cadherin in pcDNA3 were cultured in the same medium as CHO-K1 cells, with the addition of 500 µg/ml G418 to retain the plasmid (Niessen and Gumbiner, 2002). The C-CHO cell line was produced by stable transfection of CHO-K1 with cDNA encoding full length *Xenopus* C-cadherin in the pEE14 expression vector (Brieher et al., 1996), which contains the glutamine synthase minigene as selectable marker. CHO-K1 cells are able to grow in the absence of glutamine, but growth in the absence of glutamine and in the presence of

methionine sulfoximine requires expression of the glutamine synthase minigene. C-CHO cells were therefore cultured in glutamine free Glasgow MEM (Biochrom) supplemented with the following components: 10% dialyzed FCS (PAA), 100 U/ml each of penicillin and streptomycin, 10 mM HEPES pH 7.4, 30 mM L-glutamic acid and L-asparagine each, 1% (v/v) MEM nonessential amino acids, 1 mM sodium-pyruvate and 25 µM methionine sulfoximine (Sigma-Aldrich). Cells were grown in a humidified incubator with a 5% CO_2 atmosphere.

4.3.2 Culture of human cancer cell lines

The human melanoma cell lines SK-Mel-1, Melwei, SK-Mel-28, MeWo, IF6, SK-Mel-23, SK-Mel-24, WM164, WM793, WM75, MV3, BLM, VMM5 and B16F1 were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine and 100 U/ml each of penicillin and streptomycin at 37°C in a humidified incubator with a 5% CO_2 atmosphere.
The human colon cancer cell line DLD-1 and human breast cancer cell line MCF-7 were cultured in DMEM containing GlutaMax supplemented with 10% FCS, 100 U/ml each of penicillin and streptomycin at 37°C in a humidified incubator with a 5% CO_2 atmosphere.

4.3.3 Culture of mouse embryonic fibroblast cell lines

Primary mouse embryonic fibroblast cell lines of wt (PS +/+) as well as PS1 and 2 knock-out (PS1/2 -/-) mice (Hartmann et al., 2002) were cultured in DMEM containing GlutaMax supplemented with 10% FCS, 100 U/ml each of penicillin and streptomycin at 37°C in a humidified incubator with a 5% CO_2 atmosphere.

4.3.4 Serial passaging, freezing and thawing of cell lines

Serial passages were done twice a week by trypsinization in Trypsin-EDTA until all cells were detached from the culture dish. Subsequently, medium was added to dilute Trypsin-EDTA and stop trypsinization, and cells were pelleted by centrifugation at 250xg for 5 min at RT, resuspended and plated on new culture dishes in appropriate dilutions. To store cells, confluent cells were trypsinized, pelleted and resuspended in FCS supplemented with 10% DMSO (Sigma-Aldrich), after which they were transferred to cryogenic vials. Cells were allowed to slowly cool down to -80°C. After overnight incubation at -80°C, cells were transferred to liquid nitrogen for long term storage. Cells were thawed as rapidly as possible in a 37°C water bath, washed once with medium to remove DMSO and plated on culture dishes.

4.3.5 Transient transfections

CHO-K1 cells were transiently transfected with Lipofectamine™ reagent (Invitrogen), all other cells with Lipofectamine™ 2000 (Invitrogen) according to the provided protocol. For 24 well plates, 1.5x 10^5 cells were plated per well 24h before transfection. For each well in a transfection, 3 µl of Lipofectamine™ or 1.5 µl of Lipofectamine™ 2000 in combination with 0.5 µg total amount of plasmid DNA were diluted in 60 µl of Opti-MEM (Invitrogen) and incubated for 45 min at RT to allow DNA-liposome complexes to form. Cells were washed with 1x PBS and covered with 340 µl of Opti-MEM per well, after which the DNA-liposome complex was added. After incubation for 5h at RT, 400 µl of medium containing 20% FCS were added. Cells were harvested and analysed 24 to 48h after transfection. Cell number, amount of DNA, transfection reagent, and Opti-MEM were scaled up accordingly when transfections were performed in culture dishes of larger size.

4.3.6 Dual luciferase assay

To measure luciferase reporter activity the Dual-Luciferase® Reporter Assay kit (Promega) was used. Cells were co-transfected with a DNA construct encoding a Gal4 DNA binding domain fused to the cadherin cDNA (250 ng), a firefly luciferase reporter plasmid containing the Gal4 five prime upstream activating sequence (5´UAS) (250 ng) and a constitutive *Renilla* luciferase reporter plasmid (20 ng). The latter was used to control for transfection efficiency. For a 24 well plate, cells were lysed in 100 µl 1x passive lysis buffer (Promega) 24h after transfection. 10 µl of each lysate was transferred to a 96 well luminometer plate and luciferase activities were measured using a luminometer (LB 96V MicroLumat PLUS, Berthold) according to the manufacturer´s protocol. The assay was normalized for transfection efficiency by dividing the firefly luminescence by the *Renilla* luminescence. All data were represented as fold activation of the negative control value (construct encoding the Gal4 DNA binding domain alone). All assays were performed in triplicates.

4.3.7 Treatment of cell lines with protease inhibitors

The selective γ-secretase inhibitor L-685,458 and the caspase-3 specific inhibitor Z-DEVD-FMK were purchased from Calbiochem as stock solutions in DMSO and diluted in culture medium to appropriate concentrations (0.5 µM L-685,458; 2.5 µM Z-DEVD-FMK). HE-CHO and C-CHO cells were covered with culture medium containing L-685,458, Z-DEVD-FMK, both inhibitors, or DMSO and lysed after 24h for further analysis. When transient transfections were performed, culture medium containing inhibitors was added after the transfection reaction and cells were lysed after 24h.

4.4 Protein analysis

4.4.1 Immunoblot analysis of cells, *Xenopus* embryos and explants

Lysates of cells, *Xenopus* embryos or tissue explants were prepared in either NP40 lysis buffer (1% NP40, 150 mM NaCl, 4 mM EDTA, 50 mM Tris pH 7.4) or RIPA lysis buffer (1% NP40, 0.1% SDS, 0.5% deoxycholate, 150 mM NaCl, 4 mM EDTA, 50 mM Tris pH 7.4). Both lysis buffers were supplemented with a mammalian protease inhibitor cocktail (Sigma) plus 1 mM PMSF. Lysates were cleared by centrifugation at 16,000xg for 10 min at 4°C. Protein concentrations were determined using the Bradford assay (Biorad) for NP40 lysates and the DC (Detergent Compatible) protein assay (Biorad) for RIPA lysates. Protein samples were diluted 1:1 (v/v) in Laemmli sample buffer (1970), separated by SDS-PAGE on either a 7% gel or a 4-12% precast gradient gel (NuPage system, Invitrogen) and transferred to nitrocellulose according to standard procedures. The membrane was blocked with 5% non fat dry milk (Haerschle) in 1x TBST (0.1% Tween 20, 137 mM NaCl, 20 mM Tris pH 7.56) and incubated with the primary antibody diluted in blocking solution overnight at 4°C. After 3 times 10 min wash in 1x TBST, the membrane was incubated for 1h at RT with the appropriate horseradish peroxidase coupled secondary antibody diluted in 1x TBST and washed again 3 times 10 min in 1x TBST. Immunoreactive proteins were detected by enhanced chemiluminescence using the SuperSignal West Pico- and the SuperSignal West Femto kit from Pierce.

4.4.2 Immunoblot analysis of cell supernatants

Cell lines were grown to near confluency after which new medium was added for three days until cells were fully confluent. Cells were lysed in NP40 buffer as described in 4.4.1 and protein concentration of lysates determined using the Bradford assay (Biorad). Supernatants were collected and centrifuged at 250xg for 5 min at RT to remove cell debris. The amount of supernatant was normalized to the protein concentration of the corresponding cell lysate and incubated with Concanavalin A Sepharose 4B (Con A, Sigma-Aldrich) for 1h at 4°C to enrich for glycosylated proteins. Con A beads were washed twice with NP40 buffer and resuspended in Laemmli sample buffer. After incubation for 5 min at 95°C to release proteins from the beads, protein samples were separated by SDS-PAGE on a 4-12% precast gradient gel (NuPage system, Invitrogen), transferred to nitrocellulose and analysed by immunoblotting as described in 4.4.1.

4.4.3 Immunofluorescence staining of cell lines

Cells were plated on coverslips and cultured until they reached the appropriate density. Cells were fixed either with 100% ice cold methanol for 5 min or with 4% PFA/1x PBS for

10 min at RT followed by a 3 times 5 min wash with 1x PBS and 5 min incubation in 0.5% Triton X-100/1x PBS. After 3 rinses in 1x PBS, unspecific binding sites were blocked with 1% BSA/1x PBS. All antibodies were diluted to the appropriate concentration in blocking solution. Incubation with the primary antibody was performed for 1h at RT, followed by 3 times 5 min wash in 1x PBS. After incubation with the appropriate secondary antibody conjugated to either Alexa488 or Cy3 for 1h at RT, cells were washed again 3 times 5 min with 1x PBS. Coverslips were mounted in Vectashield (Vector Laboratories) on microscope slides (VWR). For analysis a fluorescence microscope (Eclipse E800, Nikon) equipped with a Nikon DXM1200-F digital camera was used.

4.4.4 Immunofluorescence staining of animal cap tissue explants

Animal cap tissue explants were fixed in 4% PFA/1x TBS (100 mM Tris pH 7.4, 0.9% NaCl) for 1h at RT. Unspecific binding sites were blocked with 2% BSA in 0.3% Triton X-100/1x TBS. All antibodies were diluted in 0.3% Triton X-100/1x TBS to the appropriate concentration. Animal caps were incubated with the primary antibody overnight at 4°C, followed by 3 times washing for 30 min in 0.3% Triton X-100/1x TBS. After incubation with the appropriate secondary antibody conjugated to either Alexa488 or Cy3 for 1h at RT, animal caps were washed again 3 times for 30 min in 0.3% Triton X-100/1x TBS, and mounted in Vectashield (Vector Laboratories) on microscope slides (VWR). For analysis a laser scanning confocal microscope (TCS SP2, Leica) was used.

4.5 Blastomere adhesion assay

Recombinant C-cadherin extracellular domain fused to the Fc part of human IgG (XCEC1-5FC) served as substrate for the blastomeres adhesion assay. It was isolated from conditioned medium of CHO-K1 cells stably expressing and secreting this domain using a protein-A column (Amersham) as described in Niessen and Gumbiner, 2002. Adhesive activity of the isolated protein was tested in a laminar flow adhesion assay.

XCEC1-5FC was diluted in 1x CMFM (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 7.5 mM Tris pH 7.6) containing 1 mM $CaCl_2$. Different concentrations of XCEC1-5FC (1 µg/ml to 100 µg/ml) were coated on a 6 cm dish in a humidified chamber for 2h at RT, using 10 µl of each concentration and marking the coated area. Unspecific sites were blocked with 0.5% BSA in 1x CMFM, 1 mM $CaCl_2$ overnight at 4°C. Shortly before start of the assay, the medium was exchanged to 1x CMFM, 1 mM $CaCl_2$.

Isolated animal cap tissue explants were incubated in 1x CMFM without Ca^{2+} and Mg^{2+} until blastomeres were dissociated in single cells. Blastomeres of 5 animal caps were resuspended in 1ml of 1x CMFM containing 1 mM $CaCl_2$, and 50 µl of blastomere suspension was spotted on substrate coated areas of the dish. Blastomeres were allowed

to adhere to the substrate for 10 min, after which an image was taken. Subsequently, the dish was rotated at 80 rpm for 1 min and another image was taken. Adhesion was quantified by counting the cells before and after rotation. Adherence of blastomeres to BSA alone (100 µg/ml) was measured to test for unspecific binding, and to the substrate in the presence of EDTA (2 mM) to test for Ca^{2+}-dependency.

4.6 Determination of serum E-cadherin concentration in melanoma patients

24 melanoma patients were selected of which serum was collected at different time points of disease showing normal S100 values (< 0.12 µg/l) at the time of removal of the primary tumor and increasing S100 values during tumor progression with metastases in different organs (Jury et al., 2000). Sera of 6 healthy subjects were taken as control. Serum E-cadherin levels were determined using an enzyme immunoassay (EIA) kit (Takara, Japan) according to the manufacturer´s instructions. Samples were measured in duplicates. Statistical analysis was carried out using the Mann-Whitney *U*-test.

4.7 Antibodies and antisera

4.7.1 Primary antibodies

- mouse monoclonal anti Gal4, ZYMED LABORATORIES (working dilution 1:1000 in WB, 1:200 in IF)
- mouse monoclonal anti human E-cadherin (HECD1), ZYMED LABORATORIES (working dilution 1:5000 in WB, 1:500 in IF)
- mouse monoclonal anti ß-catenin, BD Transduction Laboratories (working dilution 1:1000 in WB, 1:200 in IF)
- mouse monoclonal anti p120, BD Transduction Laboratories (working dilution 1:200 in IF)
- mouse monoclonal anti actin, ICN Biochemicals (working dilution 1:10000 in WB)
- mouse monoclonal anti myc (9E11), Cell signalling technology, (working dilution 1:2000 in WB)
- mouse monoclonal anti flag (M2), Sigma-Aldrich, (working dilution 1:1000 in WB)
- rabbit polyclonal anti pan cadherin antibody (PEP1) against the cytoplasmic domain of cadherins, kindly provided by B. Gumbiner (Choi and Gumbiner, 1989) (working dilution 1:1000 in WB)
- rabbit polyclonal anti β-catenin, Sigma-Aldrich (working dilution 1:1000 in WB, 1:200 in IF)

- rabbit polyclonal anti Gal4, Santa Cruz Biotechnology (working dilution 1:1000 in WB, 1:200 in IF)
- rabbit polyclonal anti *Xenopus* C-cadherin directed against the extracellular domain, kindly provided by B. Gumbiner (Yap et al., 1997) (working dilution 1:40000 in WB, 1:1000 in IF)
- rabbit polyclonal anti human E-cadherin (Boussadia et al., 2002) (working dilution 1:5000 in WB)

4.7.2 Secondary antibodies for immunofluorescence analysis

- Alexa 488 goat anti-rabbit, Molecular Probes (working dilution 1:700)
- Cy3-conjugated AffiniPure goat anti-mouse, Jackson ImmunoResearch Europe (working dilution 1:700)

4.7.3 Secondary antibodies for western blot analysis

- goat anti-mouse IgG horseradish-peroxidase conjugated, Bio-Rad Laboratories (working dilution 1:5000)
- goat anti-rabbit IgG horseradish-peroxidase conjugated, Bio-Rad Laboratories (working dilution 1:5000)

4.8 Constructs and vectors

4.8.1 Cadherin extracellular domain and deletion constructs

XCEC1-5 in pCS2+MT

The extracellular domain of *Xenopus* C-cadherin (GenBank # U04707) was cloned into pCS2+MT by restriction digestion of a plasmid containing full length *Xenopus* C-cadherin in pcDNA3 with HindIII and isolation of a ~2.1 kb fragment containing C-cadherin coding region from position 1 to 2094, beginning with the start codon at the N-terminus and ending at amino acid 697, the published transition to the transmembrane domain (Levine et al., 1994). The 5´ overhang of the restriction sites were filled in using Klenow enzyme, and the fragment was ligated into the EcoRV restriction site of plasmid 1479, which is a modification of pCS2+MT containing several additional restriction sites in the polylinker (see 4.8.4).

XCEC1-5 in pCS2+

This construct was generated by ligation of the insert described for XCEC1-5 in pCS2+6MT into the StuI site of pCS2+.

XEEC1-5 in pCS2+MT

The extracellular domain of *Xenopus* E-cadherin (GenBank # U04708) was cloned by PCR using the plasmid XEpE12cCMV encoding full length *Xenopus* E-cadherin as template, T7 (GTAATACGACTCACTATA) as sense and XEtmClaI (CTATCGATGAGC CTTTTCCTCACATTG; position 2057-2074 of U04708) as antisense primer, which contains a ClaI site. The amplified product includes part of the XEpE12cCMV polylinker upstream of *Xenopus* E-cadherin containing a ClaI restriction site and the coding region of E-cadherin from position 1 to 2074, beginning with the start codon at the N-terminus and ending at amino acid 691, the published transition to the transmembrane domain (Levine et al., 1994). After digestion of the PCR product (~2.2 kb) with ClaI the fragment was ligated into the ClaI site of pCS2+MT. The correctness of the amplified region was confirmed by sequencing.

XEEC1-5 in pCS2+

This construct was generated by ligation of the insert described for XEEC1-5 in pCS2+6MT into the StuI site of pCS2+.

XNEC1-5 in pCS2+MT

The extracellular domain of *Xenopus* N-cadherin (GenBank # X57675) was cloned by PCR using the plasmid XNp64T86 encoding full length *Xenopus* N-cadherin as template, XNstart (CCGATATCCGCCACCATGTGCCGGAAAGAG; position 85-102 of X57675; contains EcoRV site) as sense and XNtmClaI (CTATCGATGAATGGGAGCTGTAGTGCT GCA; position 2212-2233 of X57675; contains ClaI site,) as antisense primer. After digestion of the PCR product (~2.2 kb) with EcoRV and ClaI the fragment was ligated into the EcoRV/ClaI site of plasmid 1479. The correctness of the amplified region was confirmed by sequencing.

XCΔtail

The construct XCΔtail encodes a truncated form of *Xenopus* C-cadherin consisting of the extracellular and transmembrane domain cloned into pSP64T, as described (Lee and Gumbiner, 1995) (kindly provided by B. Gumbiner).

XEΔtail

The construct XΔtail encodes a truncated form of *Xenopus* E-cadherin consisting of the extracellular and transmembrane domain cloned into pSP64T as described in (Levine et al., 1994) (kindly provided by B. Gumbiner).

W2A XCEC1-5 in pCS2+MT

The construct W2A XCEC1-5 encodes a mutant form of the *Xenopus* C-cadherin extracellular domain. The amino acid W at position 2 of the mature protein was changed to alanine using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) with the XCEC1-5 construct as template and WA1ccad (GGAAGAAGAGAGACGCGGTCATCC CTCCTATAAAG) and WA2ccad (CTTTATAGGAGGGATGACCGCGTCTCTCTTCTTCC) as primers. Introduction of the mutation was verified by sequencing.

XCEC1 and XCEC1-3 in pCS2+MT

The EC1 repeat of *Xenopus* C-cadherin (GenBank # U04707) was cloned into plasmid 1479 by PCR using a plasmid containing full length *Xenopus* C-cadherin in pcDNA3 as template and Start XCcad NcoI (CCGACCATGGGGGGCACCAGGCTT, contains NcoI site) as sense and EC1endEv (CGGATATCCCGAATTTGGGACGGTTATCATT, contains EcoRV site) as antisense primer. After digestion of the PCR product with NcoI and EcoRV the fragment was ligated into the NcoI/EcoRV site of 1479. The EC1-3 repeats of *Xenopus* C-cadherin were cloned accordingly, using EC3endEv (CGGATATCCCAAA

GAAGGGGGCTTCATTGAC, contains EcoRV site) as antisense primer. The correctness of the amplified region was confirmed by sequencing.

PAPCEC1-6 in pCS2+

The constructs PAPCEC1-6 consists of the complete extracellular domain of *Xenopus* paraxial protocadherin and was cloned into pCS2+ as described (Kim et al., 1998) (kindly provided by K. Robakis).

XCad11EC1-5 in pCS2+MT

The extracellular domain of *Xenopus* cadherin11 (GenBank # AF002983) was cloned by PCR using a plasmid encoding full length *Xenopus* cadherin11 in pcDNA3 (kindly provided by D. Wedlich) as template, Xcad11 EC1-5for (GAAGGCCTATGAAGAAAGA CTTTTGCTTA, contains StuI site) as sense and Xcad11 EC1-5rev (GAAGGCCT GCGTTAAGGGATTGGGGTTC, contains StuI site) as antisense primer. After digestion of the PCR product (~1.8 kb) with StuI the fragment was ligated into the EcoRV site of plasmid 1479. The correctness of the amplified region was confirmed by sequencing.

4.8.2 Cadherin Gal4 and Gal4VP16 fusion constructs

HE-cadGal4 in pcDNA3

This construct was generated by a two-step cloning strategy. First, the coding sequence of HE-cadherin was amplified by PCR on HE-cadherin in pcDNA3 using sense and antisense primers containing HindIII and EcoRV sites, respectively. After digestion of the PCR product, it was ligated into C-cadGal4/pcDNA3 that was cut before with HindIII and EcoRV. Second, a plasmid encoding full length HE-cadherin was cut with HindIII and BlpI and the isolated insert ligated into the corresponding sites of the construct generated in step one. The correctness of the amplified region was confirmed by sequencing.

HE-cadGal4VP16 in pcDNA3

The coding sequence of HE-cadherin was cut out of the construct HE-cadGal4 in pcDNA3 by EcoRV restriction digestion and ligated into the EcoRV site of the construct Gal4VP16 in pcDNA3.

HE-cadGal4VP16 EED762-764AAA

The same cloning strategy was used as described for HE-cadGal4 in pcDNA3, with the exception that PCR was performed using a construct encoding HE-cadherin EED762-

764AAA as template, and the amplified and digested fragment was ligated into the HindIII/EcoRV site of Gal4VP16 in pcDNA3.

C-cadGal4 in pcDNA3

This construct was generated by a three-step cloning strategy. First, PCR was performed on a full length C-cadherin plasmid and the complete transmembrane and cytoplasmic domain encoding sequence was amplified using sense and antisense primers containing HindIII and EcoRV restriction sites, respectively. The PCR product was cut with the according enzymes and ligated into the HindIII/EcoRV sites of pcDNA3. Second, PCR was performed on Gal4 in pCS2+ using sense and antisense primers containing EcoRV and XbaI restriction sites, respectively. The PCR product was cut with EcoRV and XbaI and ligated into the according restriction sites of the plasmid of step one. Third, the sequence encoding the C-cadherin extracellular domain was isolated from a full length C-cadherin plasmid by restriction digestion with HindIII and ligated into the HindIII site of the plasmid generated by step two. The correctness of the amplified region was confirmed by sequencing.

C-cadGal4 in pCS2+

This construct was generated by restriction digestion of C-cadGal4 in pcDNA3 with EcoRI/XbaI to isolate the complete coding sequence of the C-cadherin Gal4 fusion protein and ligation into the EcoRI/XbaI site of pCS2+.

C-cadGal4VP16 in pCS2+

This construct was generated by PCR using the plasmid Gal4VP16 in pcDNA3 as template to amplify the Gal4VP16 coding sequence with sense and antisense primer containing EcoRV and XbaI restriction sites, respectively. After digestion of the PCR product with EcoRV and XbaI the fragment was ligated into the EcoRV/XbaI site of a previously generated C-cadGal4VP16/pCS2+ construct containing a base pair deletion. The correctness of the amplified region was confirmed by sequencing.

Gal4 in pCS2+

The Gal4-DNA binding domain was cloned into pCS2+ as described (Kim et al., 2002) (kindly provided by P. McCrea).

Gal4VP16 in pCS2+

The Gal4-DNA binding domain and VP16 transactivation domain was cloned into pCS2+ as (Kim et al., 2002) (kindly provided by P. McCrea).

Gal4 in pcDNA3

The Gal4-DNA binding domain was cut out of Gal4 in pCS2+ by EcoRI/XbaI restriction digestion and ligated into the corresponding sites of pcDNA3.

Gal4VP16 in pcDNA3

The Gal4-DNA binding domain and VP16 transactivation domain were cut out of C-cadGal4VP16 in pcDNA3 by EcoRV/XbaI restriction digestion and ligated into the corresponding sites of pcDNA3.

4.8.3 Reporter plasmids

5`UAS-luc

The firefly luciferase is encoded downstream of the five times repeated Gal4-DNA binding upstream activating sequence (UAS) (kindly provided by J. Brüning).

pRL-TK

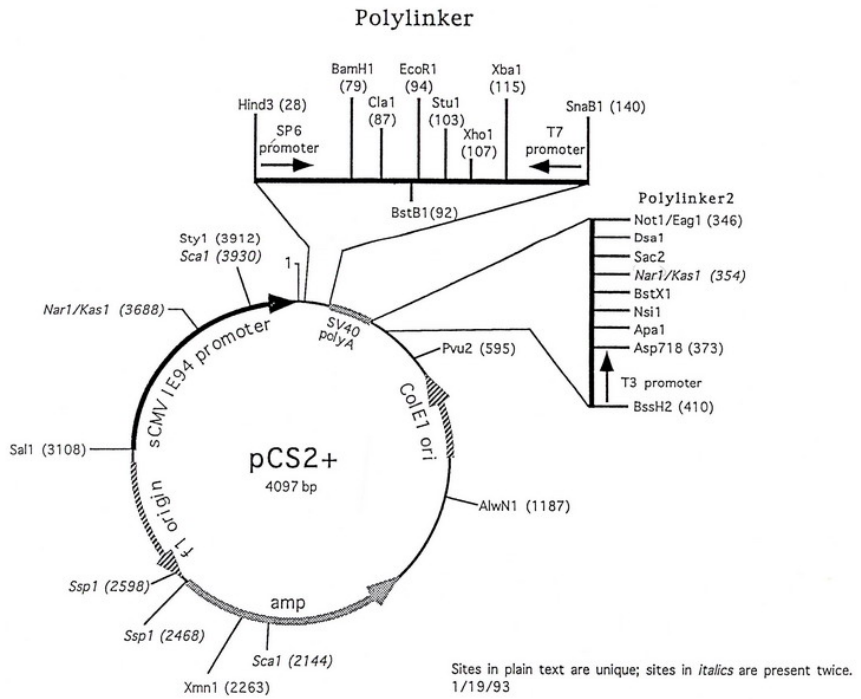
This plasmid encodes the *Renilla* luciferase under the herpes simplex thymidine kinase promoter and is used as an internal control reporter (Promega).

pSV- β**Gal**

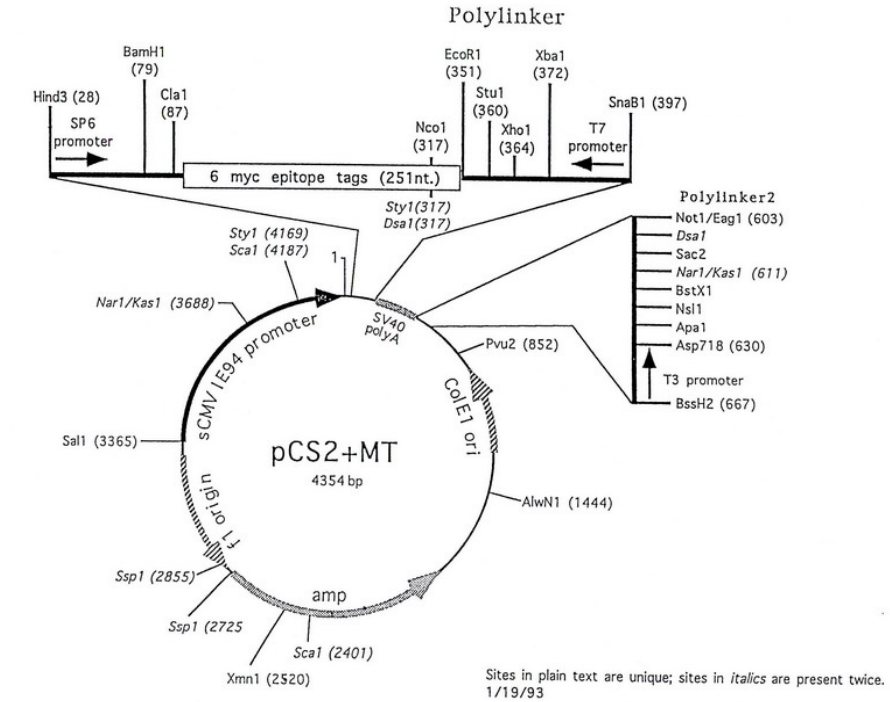
This reporter plasmid encodes the β-galactosidase under the SV40 promoter (Promega).

4.8.4 Vector maps

pCS2+



pCS2+MT



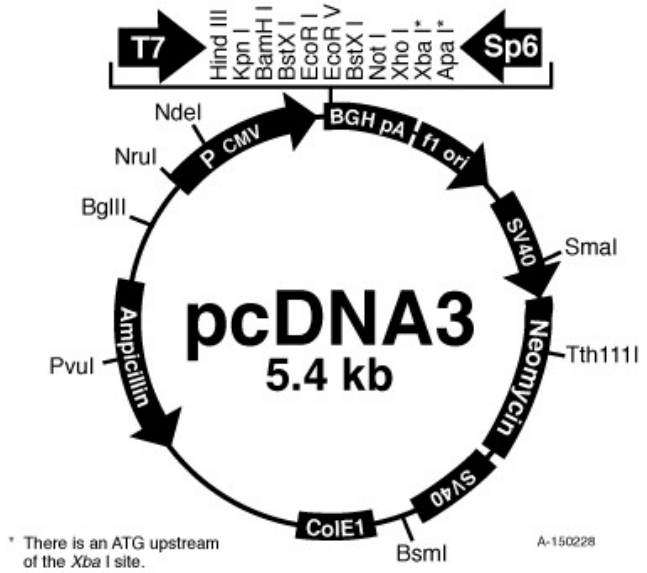
Polylinker pCS2+6XMYCextra 1479

This plasmid is a modification of pCS2+MT containing several additional restriction sites in the multiple cloning site.

```
                                                    BamHI        EcoRV
CTA TAG ATA CAA GCT ACT TGT TCT TTT TGC AGG ATC CCA TGG AGA TCC
                                                    NcoI

                 DraI
CAT CGA TTT AAA GCT | ATG GAG CAA AAG
  C1aI               M   E   Q   K
                     → 6x myc reading frame
```

pcDNA3



5. Abbreviations

A	alanine
aa	amino acid
ADAM	a disintegrin and metalloprotease
bp	base pair
BSA	bovine serum albumin
CMFM	calcium and magnesium free medium
Con A	concanavalin A
CTF	Carboxy-terminal fragment
cytodomain	cytoplasmic domain
D	aspartic acid
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleoside triphosphate
DTT	dithiothreitol
E	glutamic acid
EC	extracellular domain
ectodomain	extracellular domain
EDTA	ethylene diamine tetraacetate
EF1	elongation factor 1
EGFR	epidermal growth factor receptor
EMT	epithelial to mesenchymal transition
ER	endoplasmatic reticulum
FCS	fetal calf serum
FGFR	fibroblast growth factor receptor
g	gravitational accelaration
Gal4	Gal4 DNA binding domain
GEF	guanine nucleotide exchange factor
GTP	guanosine triphosphate
HBSS	Hank´s balanced salt solution
HGFR	hepathocyte growth factor receptor
IF	immunofluorescence
IgFc	immunoglobulin constant fragment
kb	kilobases
kD	kilodalton
MAPK	mitogene activated phosphate kinase

MCS	multiple cloning site
MDCK	madine darby canine kidney
MMP	matrix metalloprotease
mRNA	messenger RNA
NP40	Nonidet P40
NTP	ribonucleoside triphosphate
OD	optical density
O/N	over night
PAGE	polyacrylamide gel electrophoresis
PAPC	paraxial protocadherin
PBS	phosphate buffered saline
PFA	paraformaldehyde
PCP	planar cell polarity
PCR	polymerase chain reaction
PMSF	phenylmethanesulfonylfluoride
PS1	presenilin1
RIP	regulated intramembrane proteolysis
RT	room temperature
RNA	ribonucleic acid
rpm	rounds per minute
SDS	sodium dodecyle sulphate
TBS	tris buffered saline
TBS-T	tris buffered saline + Tween20
TGF-ß	transforming growth factor-ß
T_m	melting temperature
TM	transmembrane
U	unit
UAS	upstream activating sequence
VEGFR	vascular endothelial growth factor receptor
VH	vinculin homology
VP16	VP16 transcription activation domain
v/v	volume per volume
W	tryptophan
WB	western blot
wt	wild type
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactosidase

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