An adhesion-independent, aPKC-dependent function for cadherins in morphogenetic movements

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Summary

Cadherin shedding affects migration and occurs in development and cancer progression. By examining the in vivo biological function of the extracellular cadherin domain (CEC1-5) independently of the shedding process itself, we identified a novel function for cadherins in convergent extension (CE) movements in *Xenopus*. CEC1-5 interfered with CE movements during gastrulation. Unexpectedly, CEC1-5 did not alter cell aggregation or adhesion to cadherin substrates. Instead, gastrulation defects were rescued by a membrane-anchored cadherin cytoplasmic domain, the polarity protein atypical PKC (aPKC) or constitutive active Rac, indicating that CEC1-5 modulates a cadherin-dependent signalling pathway. We found that the cadherin interacts with aPKC and, more importantly, that the extracellular domain alters this association as well as the phosphorylation status of aPKC. This suggests that CE movements require a dynamic regulation of cadherin-aPKC interaction. Our results show that cadherins play a dual role in CE movements: a previously identified adhesive activity and an adhesion-independent function that requires aPKC and Rac, thereby directly connecting cadherins with polarity. Our results also suggest that increased cadherin shedding, often observed in cancer progression, can regulate migration and invasion by modulating polarity protein activity.

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Key words: Cadherin, Atypical PKC, Vertebrate morphogenesis, Convergent extension movement, Cancer migration

Introduction

Regulation of the cadherin family of intercellular adhesion proteins is crucial for morphogenetic movements during early embryonic development (Gumbiner, 2005). Control of cadherin function can occur on many levels ranging from promoter methylation to post-translational modification. An example of the latter is cadherin ectodomain shedding, a process in which the extracellular domain is released by proteolytic cleavage, resulting in a rapid and local reduction of functional cadherins at the cell surface. Since the first purification of a soluble E-cadherin fragment from the supernatant of MCF-7 cells (Wheelock et al., 1987), cadherin ectodomain shedding has been described in many different systems, including chick retinal development (Marambaud et al., 2002; Maretzky et al., 2005; Reiss et al., 2005; Roark et al., 1992). Recent reports identified ADAM10 as the major sheddase for N- and E-cadherin (Maretzky et al., 2005; Reiss et al., 2005), although other proteases have been implicated as well (Johnson et al., 2007; Ryniers et al., 2002). Cadherin shedding was found to be associated with decreased cell adhesion, increased migration and alterations in β-catenin signalling (Maretzky et al., 2005). In addition, several studies have implicated a role for the released cadherin extracellular domain in the regulation of intercellular adhesion, migration and invasion. For example, the soluble E-cadherin extracellular domain caused scattering of cells in culture (Symowicz et al., 2007; Wheelock et al., 1987), whereas the N-cadherin ectodomain promoted cell adhesion and neurite outgrowth (Paradies and Grunwald, 1993). Furthermore, the presence of conditioned medium containing increased amounts of E-cadherin ectodomain resulted in induced cell migration, invasion and reduced aggregation (Johnson et al., 2007; Maretzky et al., 2005; Noe et al., 2000). Together, these in vitro results have led to a model in which the released cadherin extracellular domain functions in a paracrine way to modulate cadherin-mediated adhesion.

During vertebrate gastrulation, the three germ layers are established while coordinated cell rearrangements form a distinct, elongated body axis. In *Xenopus laevis*, tissue elongation is mediated by convergent extension (CE) movements. These movements are driven by mediolateral intercalation of bipolarized cells, leading to an elongation of the anterior-posterior body axis (Keller, 2002). The classic *Xenopus* C-cadherin is maternally inherited and the main mediator of cell-cell adhesion in blastula embryos (Heasman et al., 1994). Proper execution of gastrulation movements requires a tight regulation of C-cadherin adhesive activity. A decrease in the adhesive function of C-cadherin is crucial to drive CE movements of activin-induced animal cap tissue explants, as antibodies that keep C-cadherin in its active adhesive state block elongation (Brieher and Gumbiner, 1994; Zhong et al., 1999). In addition, overexpression of full-length or dominant-negative forms of C-cadherin also strongly impaired elongation (Lee and Gumbiner, 1995; Zhong et al., 1999), further underlining the importance of properly balanced C-cadherin activity.

The planar cell polarity (PCP) pathway coordinates the bipolarization of cells across the tissue, which is necessary to drive CE, via activation of the seven-pass transmembrane receptors Frizzled and Dishevelled. The small GTPases Rac and Rho are activated by Frizzled-Dishevelled signalling (Habas et al., 2003; Habas et al., 2001) and control cell intercalation by mediating distinct cytoskeletal changes (Tahinci and Symes, 2003). Rac can

also be directly activated by cadherins (Kovacs et al., 2002; Noren et al., 2001), and constitutively active Rac could rescue the *Xenopus* gastrulation defects caused by cadherin instability caused by $p120^{ctn}$ knockdown (Fang et al., 2004).

Recently, several studies have indicated a role for cell polarity pathways during gastrulation. The apical determinant atypical PKC (aPKC) was found to have a polarized localization in cells undergoing CE movements and to control Par1 localization in a phosphorylation-dependent manner (Hyodo-Miura et al., 2006; Kusakabe and Nishida, 2004). This was confirmed by overexpression of dominant negative aPKC or downregulation of Par1, which did indeed interfere with CE movements during gastrulation (Kusakabe and Nishida, 2004). A recent study showed that aPKC regulated Frizzled-dependent PCP in *Drosophila* (Djiane et al., 2005), thus providing a molecular link between cell and planar polarity pathways.

In the present study we investigated whether ectodomain shedding occurs during early vertebrate development and found increased release of the cadherin extracellular domain during gastrulation. This prompted us to ask whether the cadherin extracellular domain has biological activity in itself in an in vivo system. At present it is technically not possible to specifically target only the released endogenous extracellular cadherin fragment, e.g. by knockdown experiments, to study the consequences of the lack of only the shed extracellular domain in an in vivo context. We therefore chose to address the role of the released cadherin ectodomain by expressing this domain in early *Xenopus* embryos, since this allowed us to separate a potential biological function of the ectodomain from the shedding process itself.

Expression of C-cadherin extracellular domain (CEC1-5) affected early *Xenopus* development by interfering with convergent extension movements. Surprisingly, CEC1-5 did not obviously alter the adhesive capacity of the endogenous C-cadherin or its regulation, which is necessary to drive CE (Zhong et al., 1999). Instead, CEC1-5 alters an aPKC-dependent cadherin signalling pathway since the CEC1-5-induced phenotype can be rescued by coexpression of a membrane-anchored cadherin cytoplasmic tail, aPKC or constitutive activate Rac. Further experiments showed that aPKC can interact with the cadherin. In addition, expression of the ectodomain enhanced aPKC phosphorylation, indicative of changes in activity and increased association of full-length cadherin and aPKC. Overall, our data unravel a novel mechanism by which type I classical cadherins can regulate convergent extension movements.

Results

Shedding of cadherins occurs during vertebrate gastrulation

To analyse whether cadherin shedding occurs during early vertebrate development we examined early, middle and late gastrulation stages of the frog *Xenopus laevis* for the presence of C-cadherin extracellular domain fragments. Western blot analysis of different gastrulation stages showed an 80 kDa fragment (Fig. 1A) corresponding to the size of the cadherin extracellular domain (Wheelock et al., 1987). Interestingly, later stages contained increasing amounts of this fragment. To show that this fragment is not a post-lysis artefact but is indeed released from the cell surface, we examined blastocoel liquid and again found a 80 kDa C-cadherin-positive fragment (Fig. 1B). As in whole embryo lysates, its amounts increased at later stages of development. Thus, C-cadherin shedding occurs during early development.

The relative increase in the released extracellular domain compared with full-length cadherin at later stages of development suggested that cadherin shedding is not directly coupled to total expression levels. This was further tested using a panel of human cancer cell lines. Whereas some cell lines, such as the colon carcinoma cell line WiDr, showed high total E-cadherin levels but virtually no shed fragment, others, such as the colon carcinoma cell line DLD1, showed substantial shedding (Fig. 1C). Together, these results indicate that shedding is not a constitutive process directly coupled to the amount of full-length cadherin but is subject to regulation.

Expression of C-cadherin extracellular domain interferes with gastrulation

Ectodomain shedding can have at least two functional consequences: (1) downregulation of the full-length active protein at the cell surface, and (2) generation of protein fragments with a separate biological function. We wanted to examine whether the shed extracellular domain has itself in vivo biological activity. To separate this from the shedding process itself, which is accompanied by a downregulation of cell surface cadherin, we injected RNA encoding the extracellular C-cadherin domain C-terminally fused to a Myc tag, for detection purposes (CEC1-5, Fig. 2A), into four-cell stage *Xenopus* embryos. The CEC1-5 protein was expressed at the expected size as detected by an anti-C-cadherin antibody (Fig. 2B). In addition, a higher molecular mass fragment corresponding to the size of proCEC1-5 was also stained by the C-cadherin antibody (Chappuis-Flament et al., 2001; Niessen and Gumbiner, 2002). More importantly, CEC1-5 was secreted (Fig. 2C) and associated with the cell surface (Fig. 2D), suggesting that it can interact with the endogenous cadherin.

Next we investigated whether CEC1-5 expression interferes with morphogenetic movements. CEC1-5 injections into the prospective dorsal involuting marginal zone (DIMZ), the region undergoing the most extensive cell rearrangements, interfered with gastrulation by blocking blastopore closure (Fig. 2E) when compared with control Gal4 injections (77% versus 2% phenotype; Table 1). This is in agreement with a previous study which showed that overexpression of a cadherin mutant lacking the complete cytoplasmic domain (CΔtail) also caused blastopore closure defects (Lee and Gumbiner, 1995) (Table 1). Expression of the first three C-cadherin repeats (CEC1-3) or the first cadherin repeat only (EC1) was sufficient to

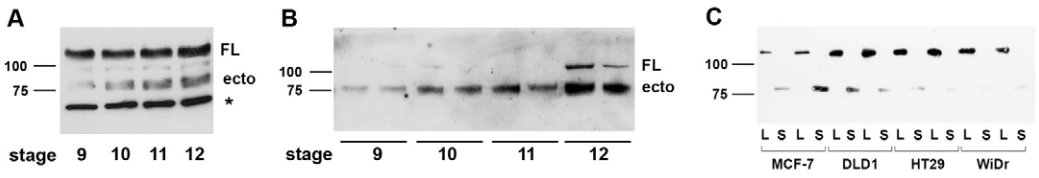

Fig. 1. Shedding of cadherins occurs in vivo and in vitro. (A,B) Western blot analysis of (A) total lysates and (B) blastocoel liquid of stage 9-12 embryos using an anti-C-cadherin extracellular domain antibody. FL, full length; ecto, ectodomain; *, non-specific bands. (C) Shedding of human E-cadherin is regulated. E-cadherin expression in lysates (L) and supernatants (S) of human cancer cell lines was analysed by western blotting using an anti-E-cadherin extracellular domain antibody.

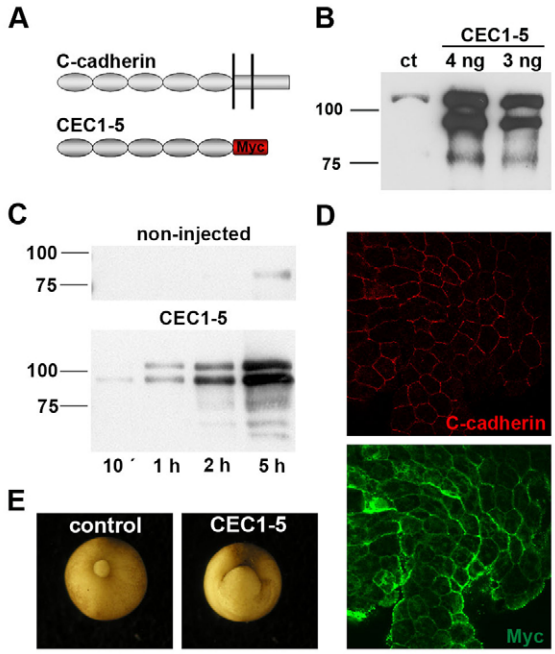

Fig. 2. The C-cadherin extracellular domain interferes with gastrulation. (A) Schematic representation of the full-length and the extracellular domain of *Xenopus* C-cadherin fused to a 6× Myc tag (CEC1-5). (B) Western blot analysis of extracts of stage 12 embryos injected with 4 ng of control Gal4 (Ct) or the indicated amounts of CEC1-5 RNA using an anti-C-cadherin antibody. (C) Secretion of CEC1-5 by animal cap explants. Animal caps injected with control Gal4 or CEC1-5 RNA were transferred to a 50 µl drop of 1× MBS (five each). Supernatants taken at the indicated time points were analysed by western blotting using an antibody against the extracellular domain. (D) Whole-mount immunofluorescence analysis of animal caps from CEC1-5-injected embryos with a C-cadherin- and a Myc-specific antibody. (E) Phenotypes of stage 12 embryos injected into the DIMZ with 4 ng of control Gal4 or CEC1-5 RNA.

interfere with gastrulation movements (Fig. 3A,B, Table 1). In addition, mutation of the tryptophan in position 2 to alanine (CEC1-5W2A) in EC1 of CEC1-5 (Fig. 3A), rendering the protein adhesion incompetent, did not alter the ability of the extracellular domain to be secreted (supplementary material Fig. S1A) and to block gastrulation (Fig. 3B, Table 1), suggesting that CEC1-5 works in a dominant-negative fashion.

Interestingly, RNAs encoding the *Xenopus* N-cadherin (NEC1-5) or *Xenopus* E-cadherin (EEC1-5) extracellular domains also interfered with gastrulation (Fig. 3B, Table 1), even though the endogenous cadherin is either not expressed or not present in the cells undergoing convergent extension. Like CEC1-5, NEC1-5 and EEC1-5 were secreted and associated with the cell surface (supplementary material Fig. S1A,C). However, gastrulation defects were not simply the result of overexpression of cadherin repeats since exogenous expression of the extracellular domains of either *Xenopus* type II cadherin 11 (Cad11EC1-5) or of the protocadherin PAPC (PAPC DN) did not interfere with gastrulation movements (Fig. 3B, Table 1), showing the specificity of the phenotype for type I classic cadherin extracellular domains. Thus, the type I extracellular domains have the capacity to interfere with morphogenetic movements in vivo.

CEC1-5 blocks gastrulation movements

Regulation of C-cadherin is crucial for CE movements of the mesoderm (Zhong et al., 1999). To examine if CEC1-5 overexpression interferes with CE movements during gastrulation, embryos were co-injected with a β-galactosidase expression plasmid. Control Gal4 RNA-injected embryos had restricted β-galactosidase activity to the midline region of the embryo as a result of CE of the prospective neuroectoderm that occurs in parallel with mesodermal CE (Fig. 4A). However, in CEC1-5-injected embryos β-galactosidase activity extended laterally, indicating impairment of CE movements. To test whether expression of CEC1-5 does interrupt CE, activin-induced animal cap elongation assays were performed, which serve as a model for the induction of CE movements. Animal cap tissue explants treated with activin elongated in a typical fashion, but elongation was strongly inhibited in explants expressing either CEC1-5, or, as reported previously (Lee and Gumbiner, 1995), CΔtail (Fig. 4B,C). CEC1-5 or CΔtail did not affect the induction of the mesodermal marker Brachyury (Fig. 4D), indicating that CEC1-5 interferes with gastrulation movements but not mesoderm induction. A similar observation was made when CE movements were blocked by addition of a C-cadherin antibody, which locks C-cadherin in its most active state (Zhong et al., 1999).

CEC1-5 and CΔtail do not affect adhesion or its regulation

We predicted that CΔtail and CEC1-5 blocked gastrulation by interfering with intercellular adhesion. Injection of CEC1-5 into the animal region did not cause any obvious loosening or dissociation of cells either in the blastocoel roof or in isolated animal caps (not shown), in contrast to what has been observed for other proteins that interfere with cadherin-mediated intercellular adhesion (Ogata et al., 2007). To test intercellular adhesive capacity, aggregation assays were performed with blastomeres injected with control Gal4 or CEC1-5. Surprisingly, CEC1-5 did not affect blastomere aggregation when compared with control Gal4 (Fig. 5A). However, CEC1-5 itself is not membrane bound and may diffuse away during dissociation of blastomeres. This may affect its ability to efficiently interfere with aggregation. By contrast, CΔtail only lacks the cytoplasmic domain and thus remains incorporated in the membrane. Similar to CEC1-5, no difference in the extent of aggregation could be observed (Fig. 5B), excluding diffusion as a possible explanation for their lack of effect on adhesion.

To examine if CEC1-5 or CΔtail interfered directly with cadherin-cadherin binding, we performed blastomere adhesion assays using cadherin-extracellular-domain-coated surfaces (Brieher et al., 1996; Niessen and Gumbiner, 2002). Surprisingly, blastomeres adhered to a similar extent in response to a range of concentration of cadherin substrates irrespective of whether control Gal4, CEC1-5 (Fig. 5C) or CΔtail (Fig. 5D) was injected. More importantly, cadherin binding was still reduced upon activin treatment in the presence of CΔtail

Table 1. Summary of injections

mRNA	Total amount (ng)	Embryos (*n*)	Closure defect (%)
Control Gal4	4.0	>200	2.3
CEC1-5	4.0	>200	77.4
CEC1-5	3.0	13	53.8
CΔtail	1.5	22	94.0
CΔtail	0.7	19	61.0
CΔtail	0.2	13	0
EC1	4.0	35	74.3
CEC1-5W2A	4.0	54	96.3
EEC1-5	4.0	42	59.5
NEC1-5	4.0	34	64.7
PAPC DN	4.0	43	11.6
Cad11EC1-5	4.0	37	16.2

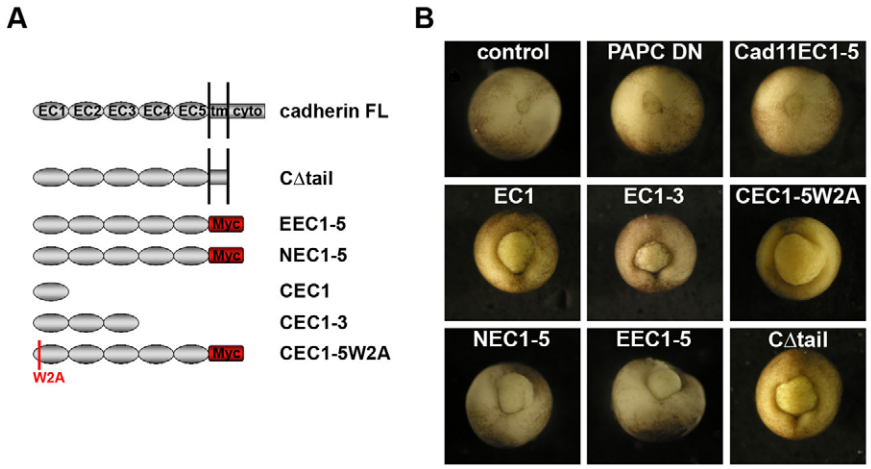

Fig. 3. Gastrulation defect is specific for type I classic cadherin extracellular domains. (A) Schematic representation of full-length and extracellular domain cadherin mutants; EC, extracellular domain repeat; cyto, cytoplasmic domain; tm, transmembrane domain; W2A, tryptophan to alanine mutation in position 2 of EC1. (B) Phenotypes of stage 12 embryos injected into the DIMZ with 4 ng of the indicated RNA.

(Fig. 5D). As was reported, a decrease in C-cadherin adhesive function is necessary to drive CE (Zhong et al., 1999). The fact that modulation of adhesion, which is crucial to drive CE, still occurs in the presence of the extracellular domain demonstrates that CΔtail or CEC1-5 do not function by keeping endogenous C-cadherin in its most active conformation, thereby preventing CE.

Thus, modulation of adhesion still occurs in the presence of the extracellular domain. Overall, these results show that CEC1-5 does not obviously interfere with the dynamic adhesive function of endogenous C-cadherin.

Human E-cadherin extracellular domain does not directly interfere with adhesion

Previous in vitro results using mammalian cell lines indicated that the shed ectodomain interfered with cell aggregation (Johnson et al., 2007; Noe et al., 2000; Ryniers et al., 2002). We therefore investigated whether the inability of cadherin extracellular domains to interfere with cadherin-mediated cell adhesion also applied to other cadherins. We thus generated MDCK and MCF-7 cell lines that stably expressed the extracellular domain of human E-cadherin (HEEC1-5) under a doxycycline-inducible promoter (Fig. 6A,B). Induced expression of HEEC1-5 did not interfere with the total level of cadherin (not shown) or its expression on the cell surface (supplementary material Fig. S2). More importantly, cadherin-mediated adhesion of HEEC1-5-expressing cells to a cadherin substrate in adhesion flow assays was similar (Fig. 6A) and the addition of conditioned medium containing HEEC1-5 did not reduce the extent of cell-cell aggregation (Fig. 6B). Thus, neither a human E-cadherin extracellular domain in cells in vitro nor *Xenopus* C-cadherin extracellular domain in developing frog embryos interfered directly with cadherin-mediated adhesion.

CEC1-5 interferes with endogenous C-cadherin function

The observation that neither CEC1-5 nor CΔtail impeded regulation of intercellular adhesion raised the question of whether these proteins interfere with cadherin-independent processes or block an

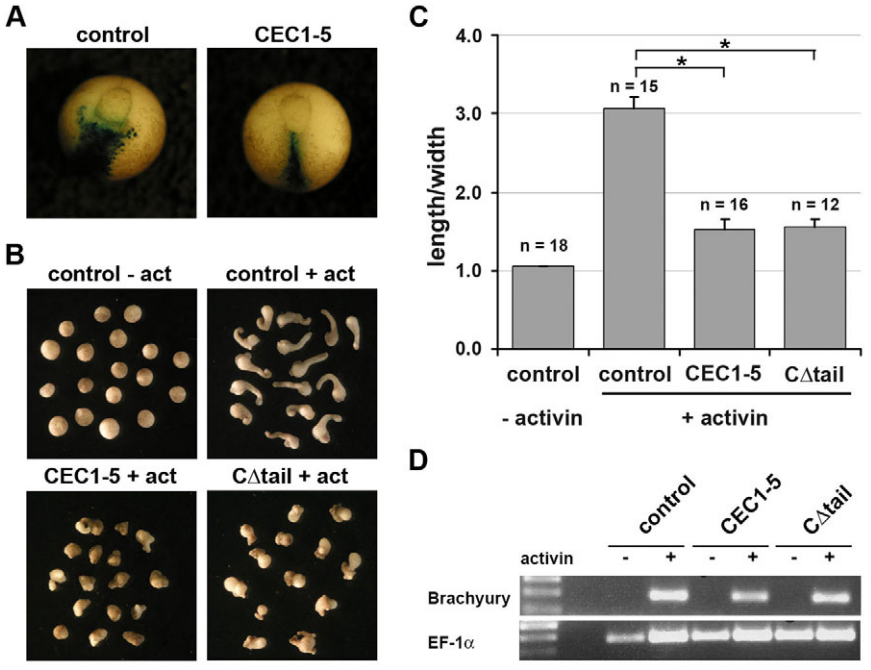

Fig. 4. The C-cadherin extracellular domain interferes with CE. (A) β-galactosidase activity at stage 11.5 in embryos co-injected into the DIMZ with 4 ng of control Gal4 or CEC1-5 RNA and a β-galactosidase encoding plasmid. (B,C) Animal cap elongation assay of explants isolated from embryos injected into the animal hemisphere with 1 ng of control Gal4, 1 ng of CEC1-5 or 0.5 ng of CΔtail RNA. In C, error bars indicate the s.e.m. *P<0.001; n, number of animal cap explants; act, activin. (D) RT-PCR analysis of the indicated transcripts from 10 animal caps of each group in B.

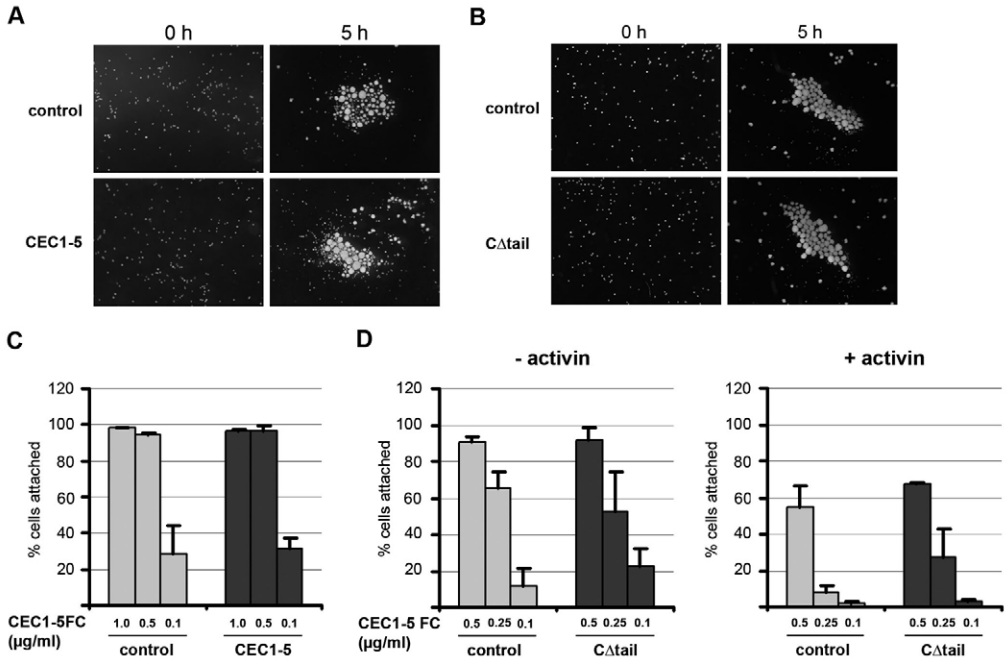

Fig. 5. The C-cadherin extracellular domain does not alter aggregation or adhesive activity of endogenous cadherin. (A,B) Blastomere aggregation with dissociated blastomeres from stage 8-9 embryos injected in the animal hemisphere with 1 ng of control Gal4 or CEC1-5 RNA (A), and 0.5 ng of control Gal4 or CΔtail RNA (B). (C,D) Blastomere adhesion to a recombinant C-cadherin extracellular domain substrate (CEC1-5FC) with dissociated blastomeres from stage 8-9 embryos injected in the animal hemisphere with 1 ng of control Gal4 or CEC1-5 RNA (C), and 0.5 ng control Gal4 or CΔtail RNA in the absence (D) or presence (E) of activin. Experiments were performed in triplicates and error bars indicate s.d.

endogenous C-cadherin function that is independent of adhesion. It was previously shown that full-length C-cadherin restored normal development in CΔtail-injected embryos (Lee and Gumbiner, 1995), indicating a cadherin-dependent scenario. If CEC1-5 hinders a cadherin-dependent but adhesion-independent function, we speculated that the CEC1-5-induced inhibition of CE movements might be restored by expression of a membrane-tethered C-cadherin cytodomain that is unable to mediate adhesion. Co-injection of a C-cadherin cytoplasmic domain fused to the transmembrane and extracellular domain of the IL2 receptor (IL2Rctail; Fig. 7A) with CEC1-5 resulted in a shift from severe CEC1-5-induced gastrulation defects to a mild phenotype (Fig. 7B,C), even though, as expected, it caused partial loosening of superficial epithelial layers (Fig. 7C) (Kintner, 1992; Lee and Gumbiner, 1995). This also indicates that CEC1-5 does not lower cadherin adhesive activity. If so, IL2RCtail would have caused a more severe blastopore closure defect instead of amelioration of the phenotype. Thus, CEC1-5 alters an endogenous C-cadherin function associated with its cytoplasmic domain.

The polarity protein aPKC rescues CEC1-5-induced gastrulation defects

We have recently found that E-cadherin regulates aPKC localization in the epidermis of mice (Tunggal et al., 2005). Since aPKC is crucial for *Xenopus* gastrulation (Kusakabe and Nishida, 2004), we hypothesized that the adhesion-independent regulation of CE movements by C-cadherin is mediated by aPKC. We therefore co-injected four-cell stage embryos with CEC1-5 and aPKC and found that aPKC but not control Gal4 injections shifted CEC1-5-induced gastrulation defects from a severe to a mild phenotype (Fig. 8A). This was specific for aPKC since Dsh, another polarity protein that regulates convergent extension, was unable to rescue the phenotype (not shown). These results suggested that cadherin extracellular domains inhibit CE by interfering with an aPKC-dependent cadherin signalling pathway.

Cadherins can stimulate the small GTPase Rac (Kovacs et al., 2002; Noren et al., 2001), which itself is an upstream regulator of aPKC (Joberty et al., 2000; Lin et al., 2000). Moreover, knockdown of $p120^{ctn}$ in *Xenopus* affects cadherin stability and interferes with gastrulation, which could be rescued by coexpression of dominant active Rac (Fang et al., 2004). We therefore co-injected a constitutive active form of Rac (RacDA) with CEC1-5. This was able to partially rescue CEC1-5-induced gastrulation defects (Fig. 8B), showing that adhesion-independent cadherin-mediated regulation of Rac GTPase activity is important for early morphogenetic movements.

The extracellular domain regulates aPKC phosphorylation and cadherin/aPKC interactions

To examine whether aPKC can interact with C-cadherin we performed GST pull down experiments. GST–C-cad cytodomain specifically precipitated aPKC when compared with GST alone (Fig. 9A). In addition, both the $p120^{ctn}$ binding domain (JMD) and the β-catenin binding domain (CBD) were able to precipitate aPKC, although to a lesser extent than the GST–C-cad cytodomain. Indeed, the β-catenin armadillo repeats fused to GST were sufficient to interact with aPKC. Thus, the cadherin cytoplasmic domain can interact with aPKC and this is at least partially mediated by β-catenin.

To examine whether the extracellular domain affects the phosphorylation status of aPKC, which would be indicative of a functional change, MDCK cells were grown in the absence or presence of HEEC1-5. The presence of HEEC1-5 increased phosphorylation of aPKC, although to a variable extent (Fig. 9B,C; supplementary material Fig. S3). We next asked whether expression of HEEC1-5 was able to alter the interaction between endogenous cadherin and aPKC. Surprisingly, expression of HEEC1-5 induced increased association of aPKC with the full-length cadherin (Fig. 9D). These results suggest that the cadherin extracellular domain is able to regulate the activation status of aPKC, perhaps by directly influencing the interaction between aPKC and full-length cadherin.

Discussion

Cleavage and release of the cadherin extracellular domain, a process known as shedding, has been implicated in the regulation

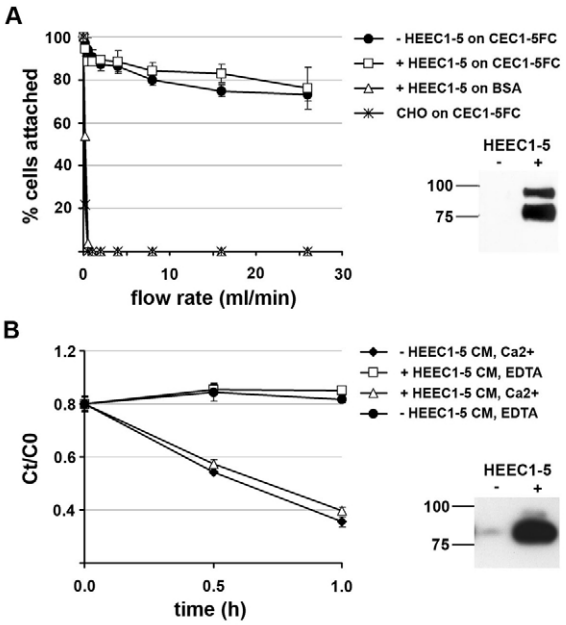

Fig. 6. The human E-cadherin extracellular domain does not alter aggregation or adhesive activity of endogenous cadherin. (A) Adhesion flow assay using MCF-7 cells with (+) or without (–) doxycycline-regulated expression of the human E-cadherin extracellular domain (HEEC1-5). Recombinant C-cadherin extracellular domain (CEC1-5FC, 100 µg/ml) served as the adhesive substrate, BSA coating or CHO cells were used as negative controls. (B) MDCK cell aggregation in conditioned medium (CM) of MDCK cells with (+) or without (–) doxycycline-regulated expression of HEEC1-5. EDTA served as a negative control. Inducible expression of HEEC1-5 in conditioned medium of MCF-7 (A) or MDCK (B) cells was analysed by western blot using HECD1 mAb. Experiments were performed in triplicates and error bars represent s.d.

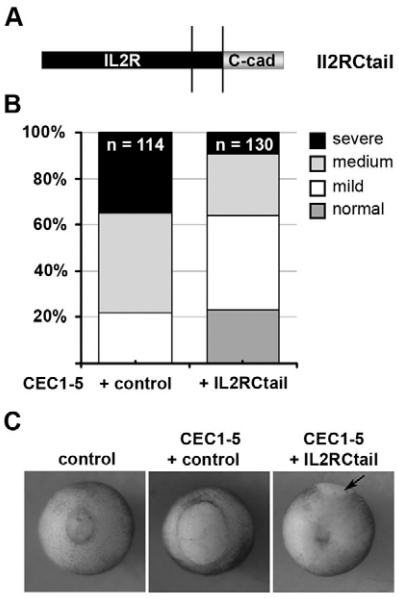

Fig. 7. CEC1-5 interferes with endogenous C-cadherin function. (A) Representation of the C-cadherin cytoplasmic domain fused to the extracellular and transmembrane domain of the IL2 receptor (IL2RCtail). (B,C) Phenotype of embryos injected in the DIMZ with 3 ng of CEC1-5 RNA in combination with either 1 ng of control or IL2RCtail RNA. The percentage of embryos showing normal blastopore closure, and a mild, medium or severe closure defect is shown in B; *n*, total number of animal cap explants from at least three independent experiments. Arrow in C indicates partial dissociation of superficial epithelial layers.

of migratory behaviour of cells. However, it is unclear whether the effects were the result of a reduction in full-length cadherin or a direct effect of the extracellular domain. We identify a novel mechanism by which the cadherin type I extracellular domain modulates migratory properties of cells, independent of the shedding process itself. In vivo expression of the cadherin extracellular domain during early *Xenopus laevis* development interfered with an aPKC-dependent function of the endogenous cadherin, thereby blocking convergent extension movements necessary for proper gastrulation. Surprisingly, no alterations in either adhesive activity of the endogenous cadherin or in its regulation were observed. Our results show that the shed extracellular domain can regulate migratory movements independently of adhesion, and provide the first evidence that convergent extension movements require two different cadherin functions: one associated with the regulation of cadherin adhesive activity and another signalling related, aPKC-associated function.

Several different studies have previously indicated a role for the shed cadherin extracellular domain of cadherins in enhanced migration and invasion (Johnson et al., 2007; Maretzky et al., 2005; Noe et al., 2000; Reiss et al., 2005; Ryniers et al., 2002; Symowicz et al., 2007; Wheelock et al., 1987). The most direct evidence was obtained using purified extracellular cadherin domains, which induced scattering of cells (Johnson et al., 2007). However, none of these studies addressed either the role of the cadherin extracellular domain in vivo or the mechanisms by which the extracellular domain regulates migratory behaviour. By using early development of *Xenopus laevis* in combination with expression of only the extracellular domain, we were able to address the in vivo biological function of this domain independent of the shedding process and thus of effects on the full-length cadherin. Surprisingly, our results indicate that the extracellular cadherin domain does not directly interfere with intercellular adhesion itself, but, instead, regulates a cadherin function associated with signalling. This conclusion is based on several observations.

First, aggregation and cadherin-dependent adhesion assays revealed that neither the human E-cadherin extracellular domain in cells in culture nor the *Xenopus* C-cadherin extracellular domain in developing frog embryos directly interfered with cadherin-mediated adhesion. This is in apparent contrast with findings of other groups that have reported that supernatants containing the shed extracellular domain interfered with aggregation of cells and this could be reversed by immuno-depleting the shed E-cadherin fragment (Johnson et al., 2007; Noe et al., 2000; Ryniers et al., 2002). There are two important differences between those studies and ours: the other studies did not separate the shedding process from the extracellular fragment since they used supernatants of cells with increased amounts of shed E-cadherin, but in our study only the extracellular domain was expressed. In addition, direct binding to a cadherin substrate was not tested in the other studies.

Second, downregulation of C-cadherin adhesive function, which is crucial to drive CE (Zhong et al., 1999) still occurs in the presence of the extracellular domain. This demonstrates that CΔtail or CEC1-5 do not function by keeping endogenous C-cadherin in its most active conformation, thereby preventing CE.

Third, expression of a membrane-anchored cytoplasmic domain was sufficient to rescue gastrulation defects caused by

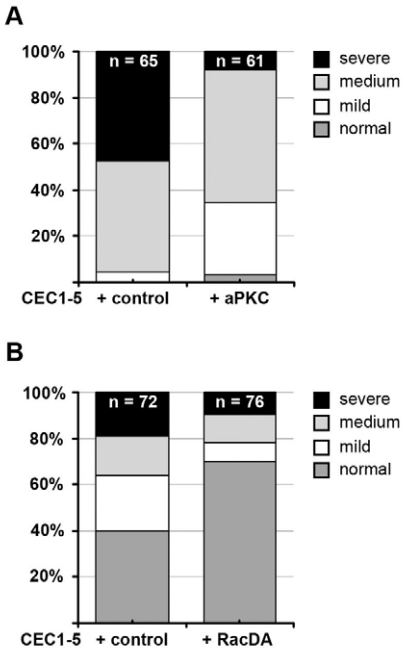

Fig. 8. CEC1-5 interferes with an aPKC/Rac-dependent function of the cadherin. (A,B) Phenotype of embryos injected in the DIMZ with 3 ng of CEC1-5 RNA in combination with either 1 ng of control Gal4 or aPKC RNA (A), or 1 pg of control Gal4 or RacDA RNA (B). The percentage of embryos showing normal blastopore closure, and a mild, medium or severe closure defect is given; *n*, total number of animal cap explants from at least three independent experiments.

overexpression of CEC1-5. Together with the observation that the full-length cadherin rescued gastrulation defects caused by CΔtail (Lee and Gumbiner, 1995), this result indicates that CEC1-5 interferes with a cadherin-dependent function. The cadherin cytodomain was shown to function as a dominant negative towards cadherin adhesiveness. Both the C-cadherin cytodomain and N-cadherin cytodomain caused animal cap explants to dissociate into single cells (Kintner, 1992; Lee and Gumbiner, 1995). In agreement with these reports, whole embryos injected with the C-cadherin cytodomain displayed dissociation of the epithelial layer during gastrulation, visible by the appearance of white lesions at injection sites. Despite inhibiting adhesion, the cytodomain efficiently rescued blastopore closure defects of the C-cadherin ectodomain. If CEC1-5 had interfered with cadherin adhesive activity, IL2RCtail would probably have caused a more severe blastopore closure defect instead of ameliorating the phenotype.

Dimerization of the cadherin ectodomain is required for homophilic adhesive activity of C-cadherin (Brieher et al., 1996). It can therefore not be excluded that the lack of effect on adhesion may be due to the fact that the secreted CEC1-5 is not present as a dimer. It is not known if the endogenous cadherin is shed as a dimer, but initial cross-linking results suggest that the exogenously expressed CEC1-5 is secreted as a dimer when expressed in animal caps (not shown). These experiments require further optimization to confirm that CEC1-5 is secreted as a functional adhesive dimer. Nevertheless, a mutated form of the cadherin extracellular domain, CEC1-5W2A, also results in gastrulation defects. Since W2 is crucial for the adhesive function of type I classical cadherins (Shan et al., 2000; Tamura et al., 1998), this result indicates that the adhesive function of CEC1-5 is not required for its effect on C-cadherin.

We cannot formally exclude that the observed gastrulation defects were caused by overexpression artefacts. However, the results strongly indicate that the phenotype is a specific effect of the cadherin type I ectodomain. Many previous studies have indicated an important role for C-cadherin in the regulation of convergent extension (Gumbiner, 2005). More importantly, gastrulation defects are not a result of non-specific overexpression of cadherin extracellular repeats, since the extracellular domains of either the type II classic cadherin or the protocadherin PAPC did not interfere with gastrulation upon injection of similar amount of their RNA as used for the CEC1-5 injections. Moreover, rescue experiments resulted in improved gastrulation, even though more RNA was introduced, thus excluding the possibility of non-specificity and RNA toxicity.

Interestingly, the extracellular domain of *Xenopus* N-cadherin (NEC1-5) or E-cadherin (EEC1-5) also interfered with blastopore closure, even though these type I classic cadherins are either not expressed at this stage (N-cadherin) or not present on cells undergoing cell rearrangements (E-cadherin). This might suggest that NEC1-5 or EEC1-5 use different mechanisms from CEC1-5 to interfere with gastrulation. However, several recent papers

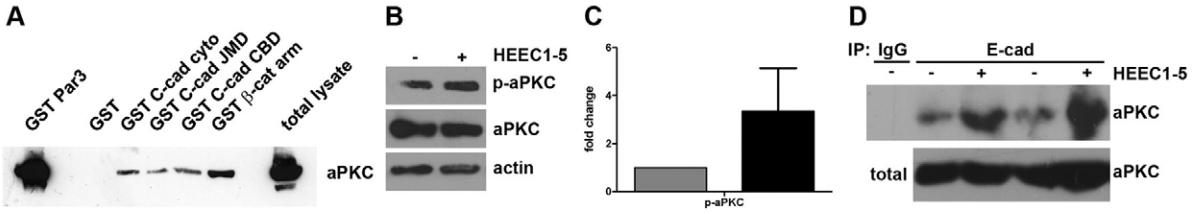

Fig. 9. The cadherin extracellular domain alters full-length cadherin-aPKC interactions. (A) GST pull down experiments. GST precipitation experiments were performed with GST fused to Par3 as positive control, GST as a negative control, C-cadherin cytoplasmic domain (cyto), C-cadherin juxtamembrane domain (JMD), C-cadherin catenin binding domain (CBD) or β-catenin armadillo repeats (β-cat arm). In the last lane 2% of total lysate input was loaded. (B) Western blot analysis of MDCK cells grown in the absence or presence of the human E-cadherin extracellular domain (HEEC1-5). Membranes were probed with phospho-aPKC antibodies, aPKC antibodies or actin as a loading control. (C) Fold activation of phospho-aPKC (p-aPKC) in cells grown in the presence of HEEC1-5. Average fold activation of phospho-aPKC in three independent experiments based on quantification of western blots shown in B and supplementary material Fig. S3, and normalized for total aPKC levels and actin. Error bar indicates the s.e. (D) Immunoprecipitation of MDCK cells grown in the absence or presence of HEEC1-5. Total lysates were precipitated with mouse mAb RR1 against dog cadherin or a non-specific IgG control, separated by SDS-PAGE and subsequently western blotted with aPKC antibodies. For total aPKC levels, 50 μg of total protein of the same lysate as used for immunoprecipitation was separated by SDS-PAGE and western blotted with aPKC antibodies. Shown are immunoprecipitations of two independently induced cell batches that were run on the same gel. In B and D MDCK-Tet-Off cells were grown in the absence of doxycycline (induces the expression of HEEC1-5), or presence of doxycycline (– and + HEEC1-5, respectively).

indicated that type I cadherins display a much wider binding specificity than previously assumed. The observation that NEC1-5 and EEC1-5 were secreted and associated with the cell surface of C-cadherin-expressing cells (supplementary material Fig. S1), although no direct evidence for heterophilic interactions, supports the data that show heterotypic interactions among type I cadherins (Duguay et al., 2003; Niessen and Gumbiner, 2002; Prakasam et al., 2006) and suggests that NEC1-5 and EEC1-5 exert their effect through endogenous C-cadherin.

The polarity protein aPKC and a constitutive active form of the small GTPase Rac restored CEC1-5-induced defects, suggesting that cadherin extracellular domains inhibit CE by altering an aPKC/Rac-dependent cadherin signalling pathway. Rac is also a component in the PCP pathway driving CE (Habas et al., 2003), most probably by mediating the cytoskeletal rearrangements necessary for the bipolar organization of cells (Tahinci and Symes, 2003). These results thus provide a direct molecular link of cadherins to polarity pathways regulating CE.

The cadherin cytoplasmic domain can interact with aPKC and this is at least partially mediated by β-catenin. The interaction is either of low stoichiometry or low affinity, because the amount of aPKC was considerably less than that precipitated by a known interaction partner of aPKC, Par3 (Fig. 9A). The cadherin extracellular domain might inhibit CE movements by altering the activity status of aPKC and the association of aPKC with the full-length cadherin. This is based on two different findings. HEEC1-5 increased the amount of phosphorylated aPKC, indicating the presence of more active aPKC in cells. This was accompanied by an increased association of full-length E-cadherin with aPKC. In *Drosophila*, planar polarity requires an inhibitory function for aPKC in non-canonical Wnt signalling (Djiane et al., 2005). Overexpression of CEC1-5 may thus induce too much of an inhibitory aPKC signal and thereby perturb CE. Unfortunately, we were unable to directly test whether CEC1-5 altered aPKC phosphorylation and cadherin association during *Xenopus* gastrulation because of problems with control antibodies in immunoprecipitations. In addition, the antibodies recognizing phospho-aPKC were made against human aPKC and showed no specific bands corresponding to the size of *Xenopus* aPKC.

Using GFP-aPKC, it was shown that aPKC displayed a polarized distribution in cells undergoing CE in *Xenopus* (Hyodo-Miura et al., 2006). However, we were unable to obtain consistent results with the aPKC antibody from Santa Cruz Biotechnology in immunofluorescence. In cultured cells the aPKC staining was predominantly in the cytoplasm. This result is in agreement with concanavalin-A precipitation experiments showing that most aPKC was not associated with membrane proteins in MDCK cells (not shown). Together, our results suggest a model whereby the cadherin type I extracellular domain regulates the local positioning of active aPKC via the full-length cadherin to drive migratory behaviour such as convergent extension.

Overall, our results reveal that cadherins play a dual role in CE movements. One prerequisite is their adhesive function, regulation of which is necessary to execute polarized cell movements (Zhong et al., 1999), and a second, aPKC-Rac-dependent contribution that may regulate the set-up of polarity. Importantly, regulation of the adhesive function of C-cadherin can occur independently of its aPKC-dependent function. Thus, the endogenous shed extracellular cadherin domain observed during gastrulation may locally alter cell polarity function to direct correct morphogenetic movements. Previously, other studies have shown a role for the cadherin cytoplasmic domain, but not its extracellular domain, in the regulation of migration in various models (Fedor-Chaiken et al., 2003; Horikawa and Takeichi, 2001; Wong and Gumbiner, 2003). Together with our results these suggest a more general role for adhesion-independent signalling in migratory movements. Lastly, our data indicate that regulation of polarity proteins may represent an important novel mechanism by which shedding of cadherins, often increased in tumour progression, regulate migratory behaviour in a three-dimensional set up.

Materials and Methods

Cell lines and antibodies

CHO cells were cultured in HAM's F12 medium supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine and 100 IU/ml each of penicillin and streptomycin. CHO cells stably expressing full-length *Xenopus* C-cadherin (C-CHO) or the C-cadherin extracellular domain fused to the Fc part of human IgG (CEC1-5FC) (Niessen and Gumbiner, 2002) were cultured in complete Glasgow glutamine-free minimal essential medium (MEM) containing 10% dialyzed FCS and 100 IU/ml each of penicillin and streptomycin. The generation of stable cell lines expressing HEEC1-5 under a tetracycline-regulated promoter (MDCK-Tet-On or MDCK-Tet-Off) will be described elsewhere (H.I., K.S. and C.M.N., unpublished). For both MDCK and MCF-7cells, three independent clones were picked and functionally tested to exclude clonal variations. The following primary antibodies were used: a rabbit polyclonal to C-cadherin extracellular domain (Yap et al., 1997), a mouse monoclonal anti-Myc (9E11; Cell Signalling), a rabbit polyclonal anti-aPKC (Sc216; Santa Cruz), a mouse monoclonal antibody to human E-cadherin extracellular domain (HECD1; Zymed), a mouse monoclonal antibody to canine E-cadherin (RR1; DSHB, University of Iowa, IA), and a mouse monoclonal antibody to human E-cadherin cytoplasmic domain (C20810; Transduction Labs).

DNA constructs and in vitro transcription

To generate CEC1-5, the coding sequence of the *Xenopus* C-cadherin extracellular domain was excised from a plasmid encoding full-length C-cadherin and ligated into pCS2+MT. cDNAs encoding the extracellular domains of *Xenopus* E-cadherin (EEC1-5) and N-cadherin (NEC1-5) and the Il2R extracellular domain and transmembrane domain fused to the C-cadherin cytoplasmic domain (I2RCtail) were cloned into pCS2+MT by PCR using plasmids encoding full-length E- and N-cadherin and IL2Rtail as templates, respectively. The cDNA for the extracellular domain of *Xenopus* cadherin 11 (Cad11EC1-5) was subcloned into pCS2+ by PCR using cadherin 11 pcDNA3 (kindly provided by Doris Wedlich, University of Karlsruhe, Karlsruhe, Germany) as a template. The W2A mutant of the C-cadherin ectodomain (CEC1-5W2A) was generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) with the CEC1-5 construct as template. The deletion mutants EC1 and EC1-3 were cloned into pCS2+ by PCR using a plasmid encoding full-length C-cadherin as template. Plasmids encoding RacDA and EGFP-aPKCζ were kindly provided by Ingo Haase (University of Cologne, Cologne, Germany) and Peter Parker (Cancer Research UK, London, UK), respectively, and coding sequences subcloned into pCS2+. CΔtail-pSP64t and PAPC DN-pCS2+ were kindly provided by Barry Gumbiner (University of Virginia, Charlottesville, VA) and Eddy de Robertis (Howard Hughes Medical Institute, UCLA, CA), respectively. All constructs were confirmed by sequencing. Capped mRNAs for injection were generated in vitro from linearized plasmids using SP6 DNA polymerase (Promega).

Xenopus embryos and explants

Xenopus egg manipulation were performed as described before (Lee and Gumbiner, 1995). Embryos were microinjected at the four-cell stage with the indicated amounts of RNA. Gal4 RNA was used for control injections. In addition, PAPC extracellular domain and *Xenopus* cadherin 11 extracellular domain RNAs were used as controls for the gastrulation phenotype and Dsh RNA for the rescue experiments. Gastrulation defects were divided into four categories (normal, mild, medium and severe) on the basis of, first, the extent of the open blastopore and second the overall condition of the embryos. Scoring was performed by an independent researcher, blind to the nature of the embryo, who distributed the different control embryos randomly in new six-well plates and tested RNAs, and the identity of the different wells was revealed after scoring by K.S. The same procedure was repeated and scoring was now performed by H.I. Animal cap explants were isolated from stage 8 embryos and incubated in 0.1% BSA, 1× MBS with or without 5 ng/ml activin (Sigma) for 1 hour at room temperature. Explants were cultured in 0.5× MBS overnight at 17°C. Animal cap elongation was determined by dividing the length of animal caps by their width. Statistical analysis was carried out using the Student's *t*-test.

Western blotting, immunoprecipitation and GST precipitation assays

Cell lines and *Xenopus* embryos were lysed in NP40 buffer (1% NP40, 150 mM NaCl, 4 mM EDTA, 50 mM Tris pH 7.4) containing protease inhibitors (Sigma) and

centrifuged at 15,000 × ***g*** for 10 minutes at 4°C. Blastocoel liquid was collected from embryos using glass capillaries, denatured with SDS sample buffer, and the liquid from 20 embryos (~2 µl) was loaded per lane. Equal amounts of protein samples were separated by SDS-PAGE and analysed by western blotting according to standard procedures. MDCK-Tet-Off #40.2.17 cells were plated and grown in the presence of doxycycline for 2 days after which half of the plates were switched to medium without doxycycline to induce the expression of HEEC1-5, and all plates were grown for another 4 days. Cells were lysed in 1% NP40 buffer and used either for western blotting or immunoprecipitations.

For immunoprecipitations, 500 µg of total lysates were precleared with protein-G Sepharose, incubated with mouse monoclonal antibody RR1 against dog E-cadherin or a non-specific mouse IgG, incubated for 2 hours at 4°C, and antibodies were precipitated with 50 µl of protein-G Sepharose by incubation for 1 hour at 4°C. Beads were washed twice with lysis buffer followed by one wash with PBS. Precipitated proteins were separated by SDS-PAGE followed by western blot analysis for aPKC.

For GST precipitations, C-CHO cells were lysed in 1% NP40 lysis buffer and incubated with 5 µg of the indicated GST fusion proteins coupled to glutathione-Sepharose beads (GE Healthcare) for 1 hour. Precipitates were washed once with lysis buffer and twice with 1× PBS, denatured with SDS sample buffer and analysed by western blotting.

Concanavalin A precipitation

Supernatant of cell lines were incubated with concanavalin A Sepharose 4B (Sigma) for 1 hour at 4°C to enrich for glycosylated proteins. Precipitates were washed twice with lysis buffer followed by one wash with PBS, denatured with SDS sample buffer and separated by SDS-PAGE.

Whole-mount immunofluorescence staining of animal caps

Animal caps were fixed in 4% paraformaldehyde (PFA) and 1× TBS for 1 hour at room temperature and permeabilized in 2% BSA in 0.3% Triton X-100 and 1× TBS. Animal caps were incubated with the indicated primary antibody overnight at 4°C and subsequently treated with goat anti-rabbit or anti-mouse Alexa Fluor 488 (Molecular Probes) for 1 hour at room temperature, and mounted in Mowiol (Fluka). Images were taken using a Leica TS2 confocal laser microscope.

Immunofluorescent staining of cell lines

Cells were fixed with 4% PFA, permeabilized in 0.5% Triton X-100, incubated with the indicated primary antibodies in combination with goat anti-rat or anti-mouse Alexa Fluor 488 (Molecular Probes) and mounted in Mowiol (Fluka). Analysis was performed at room temperature using an Eclipse E800 fluorescence microscope (Nikon) equipped with a Nikon DXM1200-F digital camera and Lucia imaging software (Cytogenetics).

Aggregation assays

Blastomeres were dissociated in 1× CMFM (calcium and magnesium free medium) and aggregation was initiated by addition of $CaCl_2$ to a final concentration of 2 mM, after which the dish was rotated at 80 r.p.m. (100 × ***g***). for 5 hours. Cell aggregation assays were performed as described previously (Nose et al., 1988), using 0.01% trypsin in the presence of 1 mM $CaCl_2$ to obtain single cells. Aggregation was measured as the number of single cells at the incubation time *t* (*Ct*) divided by the number of single cells at the incubation time 0 (*C0*).

Blastomere and laminar flow adhesion assays

Blastomere adhesion assays were performed essentially as described previously (Zhong et al., 1999). Briefly, 10 µl of different concentrations of CEC1-5FC were spotted on 6 cm dishes and incubated for 2 hours at 4°C. Non-specific binding sites were blocked with 0.5% BSA, 1× MBS overnight at 4°C. Blastomeres were incubated with or without 5 ng/ml activin in 1× CMFM for 1 hour at room temperature and subsequently added to the coated areas of the dish and allowed to adhere to the substrate for 10 minutes, after which the dish was rotated at 80 r.p.m. (100 × ***g***) for 1 minute. The percentage of cells still attached to the dish after rotation is reported. Laminar flow adhesion assays were performed as described previously (Chappuis-Flament et al., 2001). Capillary tubes were coated with CEC1-5FC (100 µg/ml) and cells were allowed to adhere to the substrate for 10 minutes, after which increasing flow rates were applied. The percentage of cells still attached to the substrate at each flow rate is reported.

Detection of β-galactosidase

Embryos injected with tracer amounts of a *lacZ* plasmid were fixed in 4% PFA on ice for 1 hour, rinsed, and transferred to staining solution [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 1× PBS] (Detrick et al., 1990) o/n at 37°C.

Isolation of RNA from explants and RT-PCR

Total RNA was extracted from animal caps using the RNeasy kit (Qiagen). cDNA was synthesized from 0.4 µg RNA using SuperScript (Invitrogen). The following primer sets were used: Brachyury, sense: 5′-GGATCGTTATCACCTCTG-3′ and antisense: 5′-GTGTAGTCTGTAGCAGCA-3′; EF-1α, sense: 5′-CAGATTGGTGCTGGATATGC-3′ and antisense: 5′-ACTGCCTTGATGACTCCTAG-3′ (Fagotto et al., 1997).

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