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Casein kinase 1 is a novel negative regulator of E-cadherin-based cell-cell contacts

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Introduction, Results, and Discussion: 4,726 words

1

2 Cadherins are the most crucial membrane proteins for the formation of tight and

3 compact cell-cell contacts. Cadherin-based cell-cell adhesions are dynamically

4 established and/or disrupted during various physiological and pathological

5 processes. However, the molecular mechanisms that regulate cell-cell contacts are

6 not fully understood. In this manuscript, we report a novel functional role of

7 casein kinase 1 (CK1) in regulation of cell-cell contacts. Firstly, we observe that

8 IC261, a specific inhibitor for CK1, stabilizes cadherin-based cell-cell contacts,

9 whereas overexpression of CK1 disrupts them. CK1 co-localizes with E-cadherin,

10 and phosphorylates the cytoplasmic domain of E-cadherin *in vitro* and in a cell

11 culture system. We show that the major CK1-phosphorylation site of E-cadherin

12 is Serine 846, a highly conserved residue between classical cadherins.

13 Constitutively phosphorylated E-cadherin (S846D) is unable to localize at cell-cell

14 contacts and has decreased adhesive activity. Furthermore, phosphorylated E-

15 cadherin (S846D) has weaker interaction with β -catenin and is internalized more

16 efficiently than wild type E-cadherin. These data indicate that CK1 is a novel

17 negative regulator of cadherin-based cell-cell contacts.

18

In multi-cellular organisms, individual cells are often connected to each other via cell-cell adhesions to form three-dimensionally structured tissues or organs. The formation of tight and compact cell-cell adhesions suppresses free cell movement and provides cells with a positional cue for proper polarization. Among many junctional proteins, cadherins, especially classical cadherins, are the most crucial membrane proteins for the establishment of intercellular adhesions (For reviews, see (15, 32)). Approximately 20 classical cadherins have been identified, and each displays a characteristic tissue distribution. For example, E-cadherin, the prototype and the best-characterized classical cadherin, is primarily expressed in epithelial cells. N-cadherin is expressed in neuronal and fibroblastic cells, while VE-cadherin is expressed in endothelial cells. The extracellular domains of cadherins form Ca^{2+} -dependent homophilic adhesions between neighboring cells. The cytoplasmic region of cadherins includes two domains: a membrane-proximal CH2 domain and a more distal CH3 domain. (CH denotes Cadherin Homology, and these domains are found in most of the classical cadherins.) (34). The CH2 and CH3 domains interact with p120 catenin and β -catenin/ γ -catenin, respectively. β -Catenin binds to α -catenin, which in turn associates with actin filaments. While the extracellular domain of cadherin induces cell-cell adhesion in the presence of Ca^{2+} , interaction between the cytoplasmic domain of cadherin and the underlying actin cytoskeleton is also required for construction of tight and compact cell-cell adhesions (For a review, see (32)).

Cadherin-based cell-cell contacts are not static, but are often dynamically modulated during various physiological and pathological processes including mitosis,

1 oncogenesis and epithelial-mesenchymal transition during embryonic development. In
 2 all these processes, cadherin has been reported to be down-regulated by endocytosis
 3 (1, 19, 29, 39). However, the molecular mechanisms for the induction of endocytosis
 4 are not clearly understood. In cell culture systems, two experimental stimuli enhance
 5 the endocytosis of E-cadherin: activation of tyrosine kinases and low Ca^{2+} treatment.
 6 Upon activation of tyrosine kinases such as src, HGF and EGF receptors, E-cadherin
 7 and its binding proteins become tyrosine phosphorylated, which induces the
 8 recruitment of the E3-ubiquitin ligase Hakai and the ubiquitination of the E-cadherin
 9 complex. The complex is then internalized and sorted into lysosomes for protein
 10 degradation (11, 31). In contrast, low Ca^{2+} treatment triggers internalization of E-
 11 cadherin that occurs independently of tyrosine phosphorylation and ubiquitination, and
 12 the internalized E-cadherin is recycled back to the plasma membrane (8, 21, 31). The
 13 molecular mechanism for this process largely remains to be clarified.

14 Casein kinase 1 (CK1) is a serine/threonine kinase that is evolutionally
 15 conserved from yeast to mammals (For a review, see (20)). In mammals, at least seven
 16 CK1 isoforms (α , β , $\gamma 1$, $\gamma 2$, $\gamma 3$, δ and ϵ) and their splice variants have been identified,
 17 which are all ubiquitously expressed. All CK1 isoforms are highly homologous within
 18 their kinase domains, but they differ in the length and primary structure of their non-
 19 catalytic domains. CK1 phosphorylates many substrates that are involved in various
 20 cellular processes including cell differentiation, proliferation, membrane transport and
 21 oncogenesis. For example, in yeast, CK1 phosphorylates and enhances endocytosis of
 22 many membrane proteins including the α -factor pheromone receptor and uracil
 23 permease (16, 24, 25). In both invertebrates and vertebrates, several CK1 isoforms
 24 have been reported to have a regulatory role in the Wnt signaling pathway (10, 12, 23,

1 40, 41). In the present study, we report that the cytoplasmic domain of cadherin is a
2 novel substrate for CK1 and that the subsequent phosphorylation of E-cadherin
3 negatively regulates cadherin-based cell-cell adhesions.

Results

Casein kinase 1 inhibitor stabilizes cadherin-based cell-cell contacts

In the process of testing the effect of several serine/threonine kinase inhibitors on HEK293 cells, we realized that an inhibitor of casein kinase 1 (CK1), IC261, induced a prominent morphological change. Although HEK293 cells express moderate levels of N-cadherin, they do not form tight cell-cell contacts and have a fibroblastic morphology (Fig. 1a; upper left panel). Upon addition of IC261, however, the cells started to adhere together and form cell-cell contacts, reminiscent of mesenchymal-epithelial transition (Fig. 1a; lower right panel). Other kinase inhibitors such as GSK-3 β and PKA inhibitor had no obvious effect (Fig. 1a). In the presence of IC261, N-cadherin was recruited to cell-cell contact sites and formed more regular and compact adhesions (Fig. 1b), though the expression levels of N-cadherin and β -catenin were not affected (Fig. 1c). IC261 is the most widely used CK1 inhibitor that blocks CK1 in a highly specific manner (26). CK1 isoforms show different sensitivity to IC261; IC₅₀ \approx 1 μ M for CK1 δ and CK1 ϵ and IC₅₀ \approx 10 μ M for CK1 α (26). The cell-cell contact stabilization effect was seen in the presence of 2.5 μ M IC261 (data not shown); however, the maximal effect was observed with 10 μ M IC261, suggesting that inhibition of not only CK1 δ and CK1 ϵ but also other isoforms including CK1 α is attributed to the effect of IC261.

Since HEK293 cells do not express E-cadherin, we examined whether IC261 also affects E-cadherin-based cell-cell contacts in MCF-7 cells. MCF-7 cells are epithelial cells derived from human breast cancer that express E-cadherin but not N-cadherin. Under low confluence conditions, MCF-7 cells adhere to each other weakly and form relatively immature E-cadherin-based cell-cell contacts (Fig. 2a; left panels).

However, upon addition of IC261, they formed more compact cell-cell contacts, at which there was a higher accumulation of E-cadherin (Fig. 2a; right panels). The localization of ZO-1, a marker for tight junctions, was not significantly affected by IC261 (data not shown). When cultured at higher density, MCF-7 cells formed tighter cell-cell contacts and under these conditions, IC261 had no significant effect (data not shown). The expression of E-cadherin and β -catenin was not affected by IC261 (Figs. 5a and b).

When MCF-7 cells are incubated in low Ca^{2+} medium ($\text{Ca}^{2+} \approx 100 \mu\text{M}$), the extracellular domains of E-cadherin form only weak and transient homophilic interactions, and cells dissociate from one another (Fig. 2b; upper panels). Interestingly, when IC261 was added to the low Ca^{2+} medium, the dissociation process was prevented. The cells remained attached to each other (Fig. 2b; lower panels), and β -catenin was still localized at cell-cell contacts (Fig. 2c), suggesting that E-cadherin-based intercellular adhesions are maintained. To quantify the effect of IC261, we incubated cells in suspension in low Ca^{2+} medium with or without $10 \mu\text{M}$ IC261 for 2 h, and cell aggregates were counted after trypsin treatment in the presence of Ca^{2+} (TC treatment) or EGTA (TE treatment) (30). The cell-dissociation index ($N_{\text{TC}}/N_{\text{TE}}$, where N_{TC} and N_{TE} are the total particle numbers after the TC and TE treatment) was 0.34 and 0.14 in the cells cultured in the absence and presence of IC261, respectively, and the difference was statistically significant ($p < 0.005$). To further examine this effect, IC261 was added to cells following dissociation in low Ca^{2+} medium. After 2-3 h of IC261 incubation, the cells reformed cell-cell contacts even in low Ca^{2+} medium (Supplementary Fig. 1a). Thus, IC261 not only blocks cell separation but also reverts cell-cell contact formation under low Ca^{2+} conditions.

1 To investigate whether the effect of IC261 depends on cadherin, we used L
2 fibroblast cells that do not express endogenous classical cadherins and thus do not
3 form stable cell-cell contacts. Addition of IC261 to L cells did not induce the
4 formation of cell-cell contacts, and some cells rounded up, probably due to effects on
5 the cytoskeleton (Fig. 2d; upper panels). In contrast, L cells stably expressing E-
6 cadherin formed weak and immature cell-cell adhesions when plated at low density
7 (Fig. 2d; lower left panels). Upon addition of IC261, they tightly adhered to each other
8 and formed compact cell-cell contacts (Fig. 2d; lower right panel), indicating that
9 IC261 requires E-cadherin to promote cell-cell contact formation.

10 To examine whether IC261 affects the disruption of E-cadherin-based cell-cell
11 contacts induced by src, we used MDCK epithelial cells transformed with a
12 temperature-sensitive src mutant (ts-src MDCK cells). At 40.5°C (the non-permissive
13 temperature for ts-src activity), cells form tight intercellular adhesions. In contrast,
14 when shifted to 35 °C (the permissive temperature for ts-src activity), cells dissociate
15 from one another and acquire a spindle-like morphology (Supplementary Fig. 1b;
16 upper panels) (2, 11). This shape change is accompanied by a redistribution of E-
17 cadherin from the plasma membrane to intracellular compartments (Supplementary
18 Fig. 1b; lower panels). Addition of IC261 did not significantly affect either this
19 morphological change or the E-cadherin re-localization at 35 °C (Supplementary Fig.
20 1b; upper and lower panels). Thus, IC261 does not affect tyrosine kinase- and
21 ubiquitin-dependent disruption of cell-cell contacts.

22 We also investigated the effect of overexpression of CK1 on E-cadherin-based
23 cell-cell contacts, by microinjecting the cDNA encoding CK1 α into the nucleus of
24 MCF-7 epithelial cells. Between microinjected cells, the level of E-cadherin and β -

catenin at cell-cell contacts was significantly reduced, compared with that between non-microinjected cells (Fig. 3). Microinjection of the cDNA encoding CK1 ϵ also induced a similar effect but to a lesser extent (data not shown), which may be due to its auto-inhibitory domain. Microinjection of the empty vector had no effect (data not shown). These data suggest that CK1 has an inhibitory role on the formation of E-cadherin-based cell-cell contacts, consistent with the observation that IC261 stabilizes them.

CK1 co-localizes and interacts with E-cadherin

We analyzed the subcellular localization of CK1 α in MDCK cells by immunostaining with anti-CK1 α antibody. At a steady state, CK1 α accumulated at cell-cell contact sites where it co-localized with E-cadherin (Fig. 4a). We further examined whether CK1 localization is dynamically regulated under Ca²⁺ switch conditions. In low Ca²⁺ medium, MDCK cells lost their tight E-cadherin-based cell-cell contacts, and CK1 α was diffusely distributed into the cytosol and was no longer concentrated at the plasma membrane (Fig. 4b; upper panels). Upon reversion to normal Ca²⁺ medium, E-cadherin accumulated at cell-cell adhesion sites. CK1 α was recruited to these newly forming cell-cell contact sites, although the time course of this recruitment was slightly slower than that of E-cadherin (Fig. 4b; middle and lower panels). By using MDCK cells stably expressing GFP-tagged CK1 α , we observed a similar subcellular localization of CK1 α (Supplementary Fig. 2). Thus, CK1 α co-localizes with E-cadherin at cell-cell contact sites and this localization is dynamically regulated during the formation of cell-cell adhesions.

We next examined the interaction between CK1 and E-cadherin using a GST pulldown assay. Endogenous CK1 α from MCF-7 cells bound to GST-E-cadherin-coupled beads, but not to GST-coupled beads (Fig. 4c; upper panel). Endogenous CK1 ϵ also showed a strong preference to GST-E-cadherin beads (Fig. 4c; middle panel). The interaction was further examined by immunoprecipitation using MCF-7 or MDCK cells. However, we could not consistently detect co-immunoprecipitation between E-cadherin and CK1 (data not shown), suggesting that the interaction occurs transiently or catalytically in cells.

CK1 phosphorylates the cytoplasmic domain of E-cadherin

We used an *in vivo* phosphorylation assay to examine whether CK1 phosphorylates the components of the E-cadherin complex. MCF-7 cells were incubated with ^{32}P -labelled orthophosphate in the presence or absence of IC261, followed by immunoprecipitation with anti-E-cadherin antibody, SDS-PAGE and autoradiography. In the E-cadherin complex, phosphorylation of E-cadherin, α -catenin and β -catenin was detected (Figs. 5a and b). Under low confluence conditions, addition of IC261 reduced the phosphorylation of E-cadherin and α -catenin by approximately 50% (Fig. 5a). Both proteins remained phosphorylated even in the presence of IC261, suggesting that kinases other than CK1 also phosphorylate E-cadherin and α -catenin. When cells were cultured at higher density, phosphorylation of E-cadherin was not affected by IC261 (Fig. 5b), thus confluence of cells affects phosphorylation of E-cadherin. However, when cells were incubated in low Ca^{2+} medium, IC261 suppressed the phosphorylation of E-cadherin (Fig. 5b). IC261 reduced the phosphorylation of α -catenin under both normal and low Ca^{2+} conditions, but had no effect on the

1 phosphorylation of β -catenin. The amounts of β -catenin and α -catenin bound to E-
2 cadherin were not affected under these experimental conditions (Figs. 5a & b, and data
3 not shown). These data indicate that CK1 phosphorylates both E-cadherin and α -
4 catenin in MCF-7 cells.

5 It has been reported that CK1 phosphorylates Serine 45 of β -catenin, which
6 primes the subsequent phosphorylation by GSK-3 β and enhances the proteasomal
7 degradation of β -catenin (23, 40). We produced non-phosphorylatable (S45A) and
8 pseudo-phosphorylated (S45D) β -catenin mutants and studied whether such mutations
9 affect the adhesive properties of β -catenin in epithelial cells. However, both mutants
10 localized at cell-cell contact sites in MDCK cells and bound equally to E-cadherin in a
11 GST-E-cadherin pulldown assay (data not shown). Thus, we could not obtain any data
12 indicating a significant role of CK1-mediated phosphorylation of β -catenin in the
13 formation of cell-cell contacts. Therefore, we focused on CK1-mediated
14 phosphorylation of E-cadherin. The significance of CK1-mediated phosphorylation of
15 α -catenin is currently being investigated.

16 Next, we studied whether CK1 can directly phosphorylate E-cadherin. Using an
17 *in vitro* phosphorylation assay, we found that purified CK1 δ protein efficiently
18 phosphorylates GST-E-cadherin, but not GST (Fig. 5c). By contrast, another kinase
19 PKC ζ did not phosphorylate GST-E-cadherin, suggesting that CK1 specifically
20 phosphorylates E-cadherin. About 0.8 mol of phosphate was maximally incorporated
21 into 1 mol of E-cadherin (data not shown), indicating that E-cadherin is indeed a
22 prominent substrate of CK1.

1 There are three known substrate consensus sequences for CK1 phosphorylation:
 2 D/EXXS/T, PO₄-S/TXXS/T and SLS (X: any amino acid). In the case of β -catenin,
 3 S45 matches with the third consensus sequence SLS (Fig. 5d). To determine the CK1-
 4 phosphorylation sites of E-cadherin, we first aligned the amino acid sequences of the
 5 cytoplasmic domain of several classical cadherins from different species.
 6 Interestingly, in the β -catenin-binding site there is a highly conserved region that
 7 includes SLS, at the amino acid position of 846 in murine E-cadherin (Fig. 5d). There
 8 are other conserved serine/threonine residues, at the position of amino acids 849, 852
 9 and 855 in murine E-cadherin. They satisfy the second consensus sequence PO₄-
 10 S/TXXS/T that could be sequentially phosphorylated following the primed S846
 11 phosphorylation. To identify the CK1-phosphorylation sites on E-cadherin, we tested a
 12 series of non-phosphorylatable mutants of E-cadherin (at amino acids 846, 849, 852
 13 and/or 855) for phosphorylation *in vitro*. The single amino acid substitution S846A
 14 reduced CK1-catalyzed phosphorylation by 70-80%, while mutations at 849, 852 and
 15 855 did not affect the level of phosphorylation (Fig. 5e; left panels). This indicates that
 16 the S846 is the major phosphorylation site for CK1. We also tested mutations of other
 17 residues (T750 and S848), but phosphorylation was not affected by these mutations
 18 (data not shown). Thus other minor CK1 phosphorylation sites on E-cadherin remain
 19 to be clarified. Casein kinase 2 (CK2) is another serine/threonine kinase that has been
 20 reported to phosphorylate E-cadherin and enhance E-cadherin- β -catenin interaction
 21 (22). For CK2, the mutation at 846 did not affect the phosphorylation; instead the
 22 mutations at 849, 852 and 855 reduced the phosphorylation (Fig. 5e; right panels).
 23 These data indicate that CK1 and CK2 phosphorylate distinct serine residues.

It has been shown that CK1-catalyzed phosphorylation primes the subsequent phosphorylation of β -catenin by GSK-3 β (23). E-cadherin also contains a serine residue at S842, though not conserved in fly, which matches with a substrate consensus sequence for GSK-3 β phosphorylation (S/TXXXS-PO₄) following the CK1-catalyzed phosphorylation at S846 (see Fig. 5d). However, CK1-catalyzed phosphorylation of E-cadherin did not prime the phosphorylation by GSK-3 β in *in vitro* phosphorylation assays (Supplementary Fig. 3), and GSK-3 β inhibitor did not affect the effect of IC261 on cell dissociation (data not shown).

CK1-induced phosphorylation of E-cadherin attenuates its adhesive function at cell-cell contact sites

To explore the functional significance of the CK1-induced phosphorylation of E-cadherin, we mutated the major phosphorylation residue S846 into alanine (S846A) and aspartic acid (S846D) to produce non-phosphorylatable and pseudo-phosphorylated mutant forms of E-cadherin, respectively. The myc-tagged wild type and mutant forms of E-cadherin were stably expressed in L fibroblast cells, and more than five independent clones were analyzed for each of the different forms of E-cadherin. Upon expression of the wild type or non-phosphorylatable E-cadherin, L cells formed stable cell-cell contacts under high confluence conditions and became epithelial-like (Fig. 6b; left and middle panels). In contrast, L cells expressing the pseudo-phosphorylated E-cadherin mutant did not form tight cell-cell contacts and remained fibroblastic (Fig. 6b; right panel), despite a similar level of E-cadherin expression (Fig. 6a). Consistently, when cultured in suspension, L cells expressing the wild type or non-phosphorylatable E-cadherin formed large cell aggregates, while

those expressing the pseudo-phosphorylated E-cadherin did not (Fig. 6c). Differences in strength of cell-cell adhesions were quantified using trypsin treatment in the presence of Ca^{2+} (TC treatment) or EGTA (TE treatment) (30). Cell aggregates were counted, and the cell-dissociation index ($N_{\text{TC}}/N_{\text{TE}}$, where N_{TC} and N_{TE} are the total particle numbers after the TC and TE treatment) was calculated (Table 1). This index was 0.64, 0.46 and 0.88 in the cells expressing wild type, S846A and S846D E-cadherin, respectively. The differences between wild type and S846A and between wild type and S846D are statistically significant ($p < 0.01$ and $p < 0.005$, respectively). Furthermore, we examined the effect of the mutations on the localization of E-cadherin by immunofluorescence. Both wild type and non-phosphorylatable E-cadherin accumulated at cell-cell contact sites, while pseudo-phosphorylated E-cadherin was diffusely distributed in the cytosol and on the plasma membrane (Fig. 6d). Taken together, these data indicate that the single amino acid substitution in the major CK1 phosphorylation site of E-cadherin influences the adhesive activity of E-cadherin and that, upon phosphorylation on S846, E-cadherin is unable to localize at cell-cell contact sites nor to mediate stable intercellular adhesions.

We also tested the effect of IC261 on these transfected cells. At low cell density, L cells expressing wild type E-cadherin formed more stable cell-cell contacts in the presence of IC261 (Fig. 2d and Fig. 6e; upper panels). Interestingly, IC261 also affected cells expressing non-phosphorylatable E-cadherin in a similar manner (Fig. 6e; middle panels). In contrast, IC261 did not clearly promote cell-cell adhesions between cells expressing pseudo-phosphorylated E-cadherin (Fig. 6e; lower panels), though the cell-dissociation index showed a minor effect (Table 1). The effect of IC261 on these cells was also confirmed by quantifying the cell-dissociation index

(Table 1). Thus, the stabilizing effect of IC261 on cell-cell contacts is partly attributed to modulation of S846 of E-cadherin. However, phosphorylation of other proteins and/or other minor phosphorylation sites on E-cadherin are also required for the IC261 effect.

To further investigate the significance of CK1-mediated phosphorylation of E-cadherin, we established MDCK epithelial cells stably expressing the E-cadherin mutants (Fig. 7A). Expression of wild type or S846A E-cadherin did not induce obvious morphological changes, and both exogenous and endogenous E-cadherin accumulated at cell-cell contact sites (Fig. 7b; left and middle panels). In contrast, cells expressing S846D E-cadherin were more flattened with some fibroblastic characteristics also observed, and both exogenous S846D and endogenous E-cadherin failed to accumulate at cell-cell adhesions (Fig. 7b; right panel). As the cell density increased, however, both endogenous and exogenous S846D E-cadherin accumulated at cell-cell contact sites (data not shown). Thus, in epithelial cells, expression of pseudo-phosphorylated E-cadherin induced a dominant-negative effect, inhibiting the formation of cell-cell contacts under conditions of low confluence.

CK1-phosphorylation of E-cadherin decreases the interaction between E-cadherin and β -catenin and enhances endocytosis of the E-cadherin complex

Finally, we explored the molecular mechanism by which CK1 regulates cell-cell contacts. First, we examined the effect of CK1 on the interaction of E-cadherin with the binding proteins. GST-E-cadherin wild type or S846A was first incubated with ATP and CK1 δ at 30°C, and then mixed with HEK293 cell lysates, followed by GST-pull down assays. The amounts of E-cadherin-bound β -catenin or p120 catenin from

the lysates were examined by Western blotting. CK1 δ bound both E-cadherin wild type and S846A, and enhanced the interaction of E-cadherin with β -catenin but not with p120 (Fig. 8a, upper panels). This effect of CK1 δ was observed even when CK1 δ and E-cadherin were incubated with ATP γ S (Supplementary Fig. 4a). Taken together with the data that CK1 δ can directly interact with β -catenin (Fig. 8a, lower panels), the effect of CK1 δ on the increased binding between E-cadherin and β -catenin is not through phosphorylation, but through formation of a ternary complex between three proteins. Interestingly, in the presence of CK1 δ , the higher amount of β -catenin bound to E-cadherin S846A than to E-cadherin wild type (Fig. 8a, upper panel). Next, we tested whether CK1-phosphorylation of E-cadherin itself affects the affinity of E-cadherin for its binding proteins. Beads coupled to GST-E-cadherin S846A or S846D were incubated with HEK293 cell lysates. When titration was performed with decreasing amounts of cell lysates, it was revealed that the interaction of β -catenin with pseudo-phosphorylated E-cadherin was weaker than that with non-phosphorylatable E-cadherin (Fig. 8b; upper panel). Interaction with p120 was not suppressed by the mutations (Fig. 8b; middle panel). These results indicate that E-cadherin, β -catenin and CK1 δ can form a ternary protein complex, but that, once E-cadherin is phosphorylated, direct interaction between E-cadherin and β -catenin is reduced.

We also studied whether the interaction between E-cadherin and β -catenin blocks CK1-induced phosphorylation of E-cadherin. E-cadherin was immunoprecipitated from MCF-7 cell lysates under mild or harsh detergent conditions to precipitate the E-cadherin- β -catenin complex or E-cadherin free from β -catenin,

1 respectively (11), followed by an *in vitro* phosphorylation assay. E-cadherin that did
2 not bind to β -catenin was more efficiently phosphorylated by CK1 than E-cadherin
3 that was bound to β -catenin (Fig. 8c). When recombinant β -catenin was added to E-
4 cadherin, phosphorylation of E-cadherin was reduced (Supplementary Fig. 4b). Both
5 α -catenin and β -catenin that bound to E-cadherin were phosphorylated by CK1 (Fig.
6 8c). Taken together, these data suggest that dissociation from β -catenin promotes
7 phosphorylation of E-cadherin by CK1.

8 We then examined whether CK1 is involved in the endocytosis of E-cadherin
9 by using surface biotinylation assays. Under low Ca^{2+} conditions, endocytosis of E-
10 cadherin is enhanced, leading to disruption of E-cadherin-based cell-cell contacts (21).
11 In the presence of IC261, this low Ca^{2+} -induced separation of cell-cell adhesions is
12 strongly suppressed in MCF-7 cells (Figs. 2b & c). Indeed, the biotinylation assay
13 revealed that the internalization of E-cadherin induced by low Ca^{2+} treatment was
14 suppressed by IC261 (Fig. 9a). We also examined whether CK1-phosphorylation of E-
15 cadherin affects endocytosis of E-cadherin in MDCK cells. Upon low Ca^{2+} -treatment,
16 non-phosphorylatable E-cadherin (S846A) was not efficiently endocytosed, compared
17 with wild type E-cadherin (Fig. 9b). In contrast, pseudo-phosphorylated E-cadherin
18 (S846D) was more efficiently internalized from the plasma membrane than wild type
19 E-cadherin (Fig. 9c). Endocytosis of E-cadherin S846D was not significantly
20 suppressed by IC261 (Supplementary Fig. 4c). Internalization of E-cadherin wild type
21 and S846D was further compared by using immunofluorescent analysis. As shown in
22 Fig. 9d, higher amounts of E-cadherin S846D were localized in early endosomes than
23 those of E-cadherin wild type. The quantification of the immunostaining intensity
24 showed that percentage of endosomal E-cadherin relative to overall E-cadherin was

1 5.7% and 13.3% for E-cadherin wild type and S846D, respectively (statistically
2 different, $p < 0.01$ ($n=10$)). These data indicate that CK1 plays a positive role in
3 endocytosis of E-cadherin and that E-cadherin is an important substrate of CK1 in this
4 process.

5 Since association of E-cadherin with β -catenin has been reported to affect
6 cadherin targeting to the plasma membrane (7), we also examined the involvement of
7 CK1-catalyzed phosphorylation in this process by combining pulse chase and surface-
8 biotinylation assays. Newly synthesized E-cadherin was metabolically labeled with
9 [^{35}S] and its transport to the plasma membrane was monitored by biotinylation with
10 NHS-SS-Biotin in the presence or absence of IC261 for 45 min (data not shown) or 2
11 h (Supplementary Fig. 4d). We found that addition of IC261 did not significantly
12 influence the transport of newly synthesized E-cadherin to the plasma membrane,
13 suggesting that CK1 is not involved in cadherin membrane targeting.

Discussion

In this study, we provide evidence for a novel mechanism that regulates E-cadherin-based cell-cell adhesions: CK1 phosphorylation of the cytoplasmic domain of E-cadherin. Upon phosphorylation, endocytosis of E-cadherin is enhanced, and E-cadherin loses its stable localization at cell-cell contact sites. In yeast, CK1 has been reported to be involved in endocytosis of many membrane proteins, including the α -factor pheromone receptor and uracil permease (16, 24, 25). CK1-induced phosphorylation of these membrane proteins primes the subsequent ubiquitination, leading to their internalization and/or sorting to the vacuole for protein degradation. In mammals, however, the mode of regulation of endocytosis by CK1 may be different. Our findings did not suggest the involvement of ubiquitination in the CK1-induced endocytosis of E-cadherin. Firstly, IC261 did not affect the E-cadherin endocytosis or disruption of E-cadherin-based cell-cell contacts induced by src, a process that is dependent on ubiquitination of the E-cadherin complex (11). In addition, we did not detect ubiquitination of the pseudo-phosphorylated (S846D) E-cadherin (data not shown).

The function of CK1 is regulated by both its subcellular localization and catalytic activity (for a review, see a ref (20)). In epithelial cells, we found that CK1 α is localized at cell-cell contact sites, and its localization is dynamically regulated during the modulation of cell-cell contacts. When cells separate from each other, CK1 α does not accumulate on the plasma membrane. However, as cells reform intercellular adhesions, CK1 α is recruited at cell-cell contact sites. The molecular mechanism for this recruitment remains to be resolved. In addition, several molecular mechanisms regulate the catalytic activity of CK1. Firstly, the C-terminal domain of

CK1 contains inhibitory autophosphorylation sites. Truncation of the C-terminus or de-phosphorylation of autophosphorylation sites by phosphatases increases CK1 activity (5, 6, 13). Secondly, in some cell types, an increase of the plasma membrane concentration of phosphatidylinositol 4, 5-bisphosphate (PIP₂) reduces CK1 α activity (4, 14). It is not clearly understood what physiological stimuli regulate CK1 activity in epithelial cells. One possible stimulus is the wingless (Wnt) signaling pathway. Activation by Wnt-3a has been reported to activate CK1 ϵ in HEK293 cells (37). Moreover, CK1 phosphorylates various components of the Wnt signaling pathway, regulating this pathway either positively or negatively (10, 12, 23, 40, 41). Taken together with our finding that CK1-mediated phosphorylation of E-cadherin attenuates its interaction with β -catenin, CK1 may regulate both Wnt signaling and cell-cell contacts simultaneously in epithelial cells.

There are 7 isoforms (α , β , γ 1, γ 2, γ 3, δ and ϵ) of CK1 that have distinct substrate specificities and subcellular localizations (For a review, see a ref (20)). Whether these isoforms have distinct functions or compensate for each other seems signaling pathways- and cell-context dependent. For example, in a canonical Wnt signaling pathway, CK1 α is a negative regulator (23), whereas CK1 γ 3, CK1 δ and CK1 ϵ positively regulate the pathway (10, 12, 27, 33, 35). In contrast, in a non-canonical Wnt pathway, CK1 α cooperates with CK1 ϵ positively in the PCP (planar cell polarity) signaling (36). We show here that overexpression of either CK1 α or CK1 ϵ destabilizes E-cadherin-based cell-cell contacts. RNAi of CK1 α and/or CK1 ϵ in MCF-7 cells, inducing maximally 50% reduction of endogenous CK1 proteins, did not induce any significant effects on cell morphology (data not shown). Thus, it is possible that multiple CK1 isoforms may be involved in the regulation of cell-cell

1 contacts, depending on the cell type, which needs to be studied in the future study. To
2 further characterize the functional role of CK1 *in vivo*, it may be advantageous to
3 study lower organisms that contain fewer CK1 isoforms.

4 Within the CH3 domain of cadherins, there are serine/threonine clusters that
5 largely overlap with the β -catenin-binding site. The finding that IC261 did not
6 completely abolish E-cadherin phosphorylation suggests that other kinase(s) also
7 phosphorylate E-cadherin. Indeed, CK2 has been shown to phosphorylate
8 serine/threonine residues in the CH3 domain, which enhances the interaction of E-
9 cadherin with β -catenin (18, 22). However, the suggested CK2 phosphorylation sites
10 (S840, S853 and S855) are not well conserved between classical cadherins of different
11 species. In contrast, we found that S846 shows striking conservation within classical
12 cadherins and that it is the major CK1-phosphorylation site of E-cadherin. In addition,
13 the phosphorylation at S846 decreases the interaction with β -catenin. Thus, the
14 balance of CK1- and CK2-catalyzed phosphorylation may determine the binding
15 affinity between E-cadherin and β -catenin. Furthermore, the effect of IC261 on the
16 stabilization of cell-cell contacts cannot be solely attributed to the S846
17 phosphorylation of E-cadherin (Fig. 6e), suggesting the existence of other substrate
18 proteins for CK1. Indeed, CK1 has been reported to phosphorylate other cell-cell
19 adhesion proteins such as occludin and connexin, although the functional significance
20 of these phosphorylations remains to be clarified (9, 28). It is plausible that CK1
21 phosphorylates multiple junctional proteins in order to dynamically regulate the
22 different types of cell-cell junctions in a coordinated manner.

23 Cadherin-based cell-cell adhesions are dynamically regulated during cancer
24 metastasis, mitosis and epithelial-mesenchymal transition in embryonic development.

1 It now becomes important to determine whether CK1-mediated phosphorylation of
2 cadherin is involved in these processes. To resolve this, efficient experimental assays
3 to monitor the activity of CK1 and specific antibodies against phosphorylated cadherin
4 need to be established. Other important questions are what stimuli activate CK1 *in*
5 *vivo* and how CK1-mediated phosphorylation of E-cadherin enhances endocytosis of
6 E-cadherin. Future investigation into these questions will lead us to further understand
7 this vital molecular mechanism that dynamically regulates cell-cell adhesions.
8

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Materials and Methods

Antibodies, plasmids and materials

The antibody to the cytoplasmic portion of E-cadherin from Transduction Laboratories (San Diego, California) was used for immunoprecipitation and Western blotting. Antibodies to the extracellular portion of E-cadherin (ECCD2 and HECD1) from Zymed (South San Francisco, California) were used for immunofluorescence of MDCK cells and MCF-7 cells, respectively. Anti-N-cadherin, - α -catenin, - β -catenin, -CK1 ϵ and -EEA1 antibodies were from Transduction Laboratories. Chicken anti-CK1 α antibody from EnCor Biotechnology (Alachua, Florida) was used for Western-blotting, and goat anti-CK1 α antibody from Santa Cruz (Santa Cruz, Calif.) was used for immunofluorescence. Mouse anti-myc (4A6) antibody was from Upstate (Charlottesville, Virginia) and rabbit anti-myc (A14) antibody was from Santa Cruz. All antibodies were used at dilutions of 1:1,000 for Western blotting and 1:100 for immunofluorescence, except for anti-CK1 α antibody, which was used at a dilution of 1:5,000 for Western blotting.

To construct pCAN-myc-CK1 α (full-length) and -CK1 ϵ (full-length), the cDNAs of rat CK1 α and *Xenopus* CK1 ϵ were amplified from pCS2-CK1 α and pCS2-CK1 ϵ by PCR using the primers 5'-CGAATTCGGATCCGCATGGCGAGCAGCAGCGGCTCC-3' & 5'-GGAATTCGCGGCCGCTTAGAAACCTGTGGGGGTTTGGGCC-3', and 5'-CATCGATGTCTGACACATGGAGCTGAGAGTGGGGAAC-3' & 5'-CATCGATATCGATACATGGAGCTGAGAGTGGGGAAC-3', respectively. pCS2-

1 CK1 α and pCS2-CK1 ϵ were kindly provided by X. He (Harvard Medical School,
 2 Boston). The amplified cDNAs of CK1 α and CK1 ϵ were cloned into BamHI/NotI and
 3 ClaI/NotI site of the pCAN-myc vector, respectively. To construct pcDNA4/TO/GFP-
 4 CK1 α , the cDNA of rat CK1 α was excised from pCAN-myc-CK1 α (BamHI/NotI)
 5 and, after blunting the ends, inserted into an EcoRI site of pcDNA/TO/GFP vector. To
 6 obtain pcDNA4/TO/GFP, the cDNA of GFP was amplified from pEGFP-C2 by PCR,
 7 and was inserted into BamHI/ApaI site of the pcDNA4/TO vector. pcDNA6/TR and
 8 pcDNA4/TO vectors were obtained from Invitrogen (Paisley, United Kingdom).
 9 pcDNA-E-cadherin-myc and pGEX-E-cadherin were described before (11, 17).
 10 pGEX-TEV- β -catenin was kindly provided by B. Weise (Stanford University School
 11 of Medicine, Stanford, CA). Recombinant β -catenin was produced by cleavage of
 12 GST-tag using AcTEVTM protease (Invitrogen) at 4°C for overnight, followed by
 13 affinity chromatography, according to manufacture's instruction.

14 IC261 (CK1 inhibitor), H-89 (PKA inhibitor), GSK-3 β inhibitor II and
 15 cycloheximide were purchased from Calbiochem (Darmstadt, Germany). His₆-tagged
 16 constitutively active form of CK1 δ protein was purchased from Sigma, and PKC ζ and
 17 CK2 proteins were from Calbiochem. Active form of GSK-3 β protein was obtained
 18 from Upstate. Lipofectamine PlusTM reagent and essential amino acids were obtained
 19 from Invitrogen. [γ -³²P] ATP and ³²P-orthophosphate were purchased from GE
 20 Healthcare (Piscataway, NJ). Site directed mutagenesis was performed using
 21 QuikChange[®] Site-Directed Mutagenesis kit from Stratagene (La Jolla, California).

22

Immunoprecipitation, GST-E-cadherin pulldown assay and Western blotting

Immunoprecipitation was performed as described before by using 1% Triton X-100 lysis buffer (20 mM Tris/HCl pH 7.5, 150 mM NaCl and 1% Triton X-100) containing 5 $\mu\text{g ml}^{-1}$ leupeptin, 50 mM PMSF, and 7.2 trypsin inhibitor units of aprotinin (17). To exclude β -catenin from the E-cadherin immunoprecipitate, MCF-7 cells were lysed in 1% Triton X-100 lysis buffer containing 1% SDS. The lysate was then diluted 10-fold with 1% Triton X-100 lysis buffer before immunoprecipitation.

For GST-E-cadherin pulldown assays, 10 μl of glutathione-Sepharose beads (Pharmacia) attached to 6 μg GST or GST-E-cadherin protein were incubated with cell lysates in 1% Triton X-100 lysis buffer, followed by the same procedures as described above. For GST-E-cadherin mutants pulldown assay, RIPA buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate and 0.1% SDS containing the same protease inhibitors as above) was used. Western blotting was performed as described (17).

Cell culture, immunofluorescence, microinjection, RNA interference, endocytosis assay and membrane targeting experiment

HEK293, MCF-7, MDCK and L fibroblast cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin at 37°C and ambient air supplemented with 5% CO₂. ts-Src MDCK cells were cultured as described (2). To obtain cells expressing E-cadherin mutants, MDCK and L fibroblast cells were transfected with pcDNA-myc-E-cadherin wild type, S846A or S846D using Lipofectamine-PlusTM reagent, and stably-transfected cells were selected in a medium containing 800 $\mu\text{g ml}^{-1}$ of G418

1 (Calbiochem). More than five stable clones were obtained from two independent
 2 transfections for each construct. It should be noted that expression of E-cadherin
 3 S846D decreases in both MDCK and L fibroblast cells as the passage proceeds. Thus,
 4 cells in earlier passages were used for the experiments. Since we could not obtain
 5 MDCK cells stably expressing CK1 by a conventional method, Tet-ON inducible
 6 system was used. First, MDCK cells were transfected with pcDNA6/TR, followed by
 7 selection in a medium containing $5 \mu\text{g ml}^{-1}$ of blasticidin (Invitrogen). Then,
 8 pcDNA4/TO/GFP-CK1 α was used for the second transfection and the doubly
 9 transfected cells were selected in a medium containing $5 \mu\text{g ml}^{-1}$ of blasticidin and 400
 10 $\mu\text{g ml}^{-1}$ of Zeocin (Invitrogen). 16 h after addition of tetracycline, induced expression
 11 of GFP-CK1 α was examined. For the experiments indicated as “under low confluence
 12 conditions” or “at low density”, 2×10^5 and 5×10^5 cells were plated in 6-well plates
 13 and 6-cm dishes, respectively, and after 16 h experiments were carried out. Otherwise,
 14 6×10^5 , 1.5×10^6 and 5×10^6 cells were plated in 6-well plates, 6- and 9-cm dishes,
 15 respectively, and experiments were started after 24-48 h. IC261 ($10 \mu\text{M}$), H-89 (200
 16 nM) and GSK-3 β inhibitor II ($1 \mu\text{M}$) were added for 4 h if not indicated.

17 Calcium was depleted from fetal calf serum and the low calcium medium was
 18 reconstituted as described (3) Immunofluorescence was performed as previously
 19 described (17). Microinjection was performed as described before (3). pCAN-myc-
 20 CK1 α or -CK1 ϵ ($0.1 \mu\text{g } \mu\text{l}^{-1}$ PBS) was microinjected into the nucleus of MCF-7 cells.
 21 After microinjection, cells were incubated in normal calcium medium for 6 h,
 22 followed by immunostaining with the indicated antibodies. Cell aggregation and cell
 23 dissociation assays were performed as described (30, 38). In a dissociation assay for
 24 cells with IC261, cell clumps were incubated for 2 h after medium was replaced for

1 that containing 10 μ M IC261. Validated siRNA oligos for CK1 α or CK1 ϵ were
2 obtained from Qiagen. Oligos were transfected into MCF-7 cells using Hi-Perfect
3 reagent (Qiagen) according to manufacture's instructions. Maximally 50% reduction
4 of endogenous CK1 protein was obtained by either siRNA. The endocytosis assay was
5 performed as previously described (11), except that after biotinylation cells were
6 incubated at 18°C to block the recycling of internalized E-cadherin back to the plasma
7 membrane (21), and bafilomycin was not used. To examine targeting of newly
8 synthesized E-cadherin into the plasma membrane, we combined pulse-chase and
9 surface-biotinylation assays. First, cells were metabolically labeled as described
10 before (11), except that [35 S] methionine and cysteine were used for 30 min. Then,
11 cells were incubated with 0.5 mg ml $^{-1}$ Sulfo-NHS-SS-Biotin in the presence or
12 absence of 10 μ M IC261 in Krebs-Ringer buffer at 37°C for 30 min or 2 h. Surface-
13 biotinylated E-cadherin was pulled down with 20 μ l of monomeric-avidin beads,
14 followed by elution with 2 mM D-biotin in PBS and immunoprecipitation with anti-E-
15 cadherin antibody. Immobilized Monomeric Avidin Kit (PIERCE, Rockford, IL) was
16 used for purification of biotinylated proteins.

17 Immunofluorescent images were analyzed by confocal microscopy, if not
18 otherwise indicated. To quantify the images in Fig. 9d, images were captured at every
19 0.5 μ m interval and the image where co-localization of E-cadherin and EEA-1 was
20 maximally observed was chosen for analysis. To obtain epifluorescence and confocal
21 images, we used a Zeiss Axioskop 1 with a Roper Scientific Coolsnap camera and a
22 Bio-Rad mounted on a Nikon Optiphot 2 microscope, respectively. To obtain phase
23 contrast images, we used a Leica DMIRB microscope with a Hamamatsu C4742-95
24 Orca camera. Images were captured and analyzed using Openlab (Improvision) and

ImageJ 1.36b (National Institutes of Health).

In vitro phosphorylation assay

Prior to phosphorylation assays, 2 µg of GST-E-cadherin wild type or mutant proteins were coupled to glutathione-Sepharose beads, otherwise endogenous E-cadherin protein was immunoprecipitated with anti-E-cadherin antibody using MCF-7 cells cultured in a 15-cm dish, followed by intensive washing with phosphorylation buffer (20 mM Tris/HCl pH 7.5, 5 mM MgCl₂, 1 mM EGTA and 40 µM cold ATP). The E-cadherin beads were then incubated in 30 µl of phosphorylation buffer with 0.36 µCi [γ-³²P] ATP and the indicated kinase at 30°C for 8 min while shaking at 1,400 rpm, followed by washing with ice-cold phosphorylation buffer 3 times, boiling in SDS-PAGE sample buffer and SDS-PAGE. For CK1, to optimize phosphorylation conditions, the time-dependent and kinase dose-dependent reactions were first studied, and we used an 8 min reaction time and 0.2 µg CK1δ protein for all the experiments except for maximal phosphorylation. To examine maximal phosphorylation of E-cadherin by CK1δ, the phosphorylation reaction was performed with 1.2 µg CK1δ for 30 min. 0.2 µg PKCξ, 0.2 µg GSK-3β or 250 Units CK2 was also used for a phosphorylation assay.

In vivo phosphorylation assay

Krebs-Ringer buffer (20 mM Hepes/NaOH pH 7.4, 118 mM NaCl, 4.75 mM KCl, 1.2 mM MgCl₂, 0.26 mM CaCl₂, 25 mM NaHCO₃, 0.45% glucose and 1X essential amino acids) was used for the phosphorylation assay. For low Ca²⁺ treatment, CaCl₂ was excluded from the buffer. MCF-7 cells in a 6-cm dish were first pre-incubated in

Krebs-Ringer buffer for 1 h, and further incubated in 1 ml of Krebs-Ringer buffer containing 0.5 mCi ^{32}P -orthophosphate in the presence or absence of IC261 for 2 h, when ^{32}P -orthophosphate was converted into ^{32}P -ATP inside cells followed by phosphorylation of proteins. IC261 did not grossly affect the radioactivity of total cell lysates, suggesting that it did not block the conversion from orthophosphate to ATP. Cells were then washed twice by PBS and pre-cleared by mouse control IgG beads prior to immunoprecipitation with anti-E-cadherin antibody.

Statistical analysis

Descriptive statistics on the Dissociation Index (DI) were calculated with NCSS software. Student's t tests were then generated with a threshold equal to 5% ($\alpha=0.05$). The DI's data were transformed to arcsin (square DI) and Student's t tests were achieved with NCSS software, using these values because this test requires variables with no fixed limits.

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Figure 1. Casein kinase 1 inhibitor IC261 stabilizes N-cadherin-based cell-cell contacts in HEK293 cells. **(a)** Effect of various kinase inhibitors on HEK293 cells. HEK293 cells were incubated with 1 μ M GSK-3 β inhibitor II, 200 nM H-89 (PKA inhibitor) or 10 μ M IC261 (CK1 inhibitor) for 4 h. The effect was examined by phase contrast microscopy. Scale bar: 20 μ m. **(b)** Effect of IC261 on the localization of N-cadherin in HEK293 cells. HEK293 cells were incubated in the presence or absence of 10 μ M IC261 for 4 h and the localization of N-cadherin was examined by immunofluorescence with anti-N-cadherin antibody using epifluorescence microscopy. Scale bar: 20 μ m. **(c)** Effect of IC261 on the expression of the N-cadherin complex. HEK293 cells were incubated in the presence or absence of 10 μ M IC261 for 4 h, followed by immunoprecipitation with anti-N-cadherin antibody and Western blotting with anti-N-cadherin and anti- β -catenin antibodies.

Figure 2. CK1 inhibitor IC261 stabilizes E-cadherin-based cell-cell contacts in MCF-7 and L fibroblast cells. **(a)** Effect of IC261 on MCF-7 cells under low confluence conditions. MCF-7 cells cultured at low density were incubated in the presence or absence of 10 μ M IC261 for 4 h. The effect of IC261 was examined by phase contrast and immunofluorescence microscopy with anti-E-cadherin antibody. Scale bars: 20 μ m. **(b & c)** Effect of IC261 on low Ca²⁺-induced cell separation in MCF-7 cells. MCF-7 cells were incubated in low Ca²⁺ medium in the presence or absence of 10 μ M IC261 for the indicated times. The effect of the treatment was examined by phase contrast (b) and immunofluorescence microscopy with anti- β -catenin antibody (c). Scale bars: 20 μ m. **(d)** Requirement of E-cadherin for the effect of IC261 on cell-cell

contacts. L cells or L cells expressing E-cadherin were incubated in the presence or absence of 10 μ M IC261 for 4 h. The effect of IC261 was examined by phase contrast microscopy. Scale bar: 20 μ m.

Figure 3. Overexpression of CK1 disrupts E-cadherin-based cell-cell contacts. The cDNA of myc-tagged CK1 α was microinjected into the nucleus of MCF-7 cells. The effect of expression of CK1 α on E-cadherin-based cell-cell contacts was analyzed by immunofluorescence microscopy with the indicated antibodies. Scale bar: 40 μ m.

Figure 4. CK1 co-localizes and interacts with E-cadherin. **(a & b)** Co-localization between CK1 α and E-cadherin in MDCK cells at a steady state (a) or during Ca²⁺ switch (b). The subcellular localization of E-cadherin and CK1 α was examined in MDCK cells using anti-E-cadherin and anti-CK1 α antibodies. Scale bars: 10 μ m. **(c)** Interaction between CK1 and E-cadherin by GST pulldown assays. Beads coupled to GST- or GST-tagged E-cadherin cytoplasmic domain were incubated with MCF-7 cell lysates. The proteins bound to the beads were analyzed by Coomassie protein staining and Western blotting with anti-CK1 α and anti-CK1 ϵ antibodies. Arrow and arrowhead indicate the positions of GST-E-cadherin and GST, respectively.

Figure 5. CK1 phosphorylates the cytoplasmic domain of E-cadherin. **(a & b)** Effect of IC261 on phosphorylation of the E-cadherin complex in MCF-7 cells in an *in vivo* phosphorylation assay. MCF-7 cells were cultured at low (a) or high (b) density, and incubated with ³²P-orthophosphate in the presence or absence of 10 μ M IC261 for 2 h.

1 In (b), cells were incubated in either normal (N) or low (L) Ca^{2+} medium. Cell lysates
 2 were immunoprecipitated with anti-E-cadherin antibody, followed by SDS-PAGE,
 3 autoradiography and Western blotting with the indicated antibodies. The proteins in
 4 the autoradiography bands were identified by comparing with the accompanying
 5 Western blotting results. (c) Phosphorylation of the cytoplasmic domain of E-cadherin
 6 by an *in vitro* phosphorylation assay. GST or GST-tagged cytoplasmic domain of E-
 7 cadherin was incubated with $[\gamma\text{-}^{32}\text{P}]$ ATP in the presence of CK1 δ or PKC ζ .
 8 Phosphorylated proteins were subjected to SDS-PAGE, followed by autoradiography
 9 and Coomassie protein staining. (d) Amino acid sequence alignment of the
 10 cytoplasmic domain of classical cadherins. The sequence of E-, N-, OB-, VE-, and P-
 11 cadherin are from a mouse protein database. C- and DE-cadherin are E-cadherin
 12 counterparts from frog and fly, respectively. The numbers indicate the amino acid
 13 number for mouse E-cadherin. The potential major CK1 phosphorylation site of
 14 cadherins is highlighted in red. An analogous CK1 phosphorylation site of β -catenin is
 15 also shown. (e) Determination of the major CK1 phosphorylation site of E-cadherin by
 16 an *in vitro* phosphorylation assay. GST-tagged wild type and non-phosphorylatable E-
 17 cadherin mutants were incubated with $[\gamma\text{-}^{32}\text{P}]$ ATP in the presence of CK1 δ or CK2,
 18 followed by autoradiography and Coomassie protein staining. ASSS: S846A; SAAA:
 19 S849A, S852A, S855A; AAAA: S846A, S849A, S852A, S855A.

20

21 **Figure 6.** Mutations in the major CK1-phosphorylation residue of E-cadherin affect
 22 adhesiveness of cell-cell contacts in L fibroblast cells stably expressing E-cadherin
 23 mutants. L fibroblast clones stably expressing myc-tagged wild type, non-
 24 phosphorylatable (S846A) and pseudo-phosphorylated (S846D) mutants of E-cadherin

were obtained. More than five independent clones were analyzed for each of the different types of E-cadherin, and analogous data were obtained between clones expressing the same type of E-cadherin. The data using representative clones (WT2, A5 and D13 for wild type, S846A and S846D E-cadherin, respectively) are shown. **(a)** Expression level of E-cadherin mutants in the L fibroblast clones. Cell lysates (20 μ g proteins) from the indicated clones were analyzed by Western blotting with anti-myc antibody. **(b)** Effect of mutations of E-cadherin on cell-cell contact formation. Non-transfected cells or the indicated clones were cultured at high density and analyzed by phase contrast microscopy. Scale bar: 40 μ m. **(c)** Effect of mutations of E-cadherin on formation of cell aggregates. Non-transfected cells or the indicated clones were cultured in suspension and examined by phase contrast microscopy. Scale bar: 40 μ m. **(d)** Effect of mutations on subcellular localization of E-cadherin. The indicated clones were cultured at low density, and analyzed by immunostaining with anti-myc antibody. Scale bar: 20 μ m. **(e)** Effect of mutations of E-cadherin on IC261-induced stabilization of cell-cell contacts. The indicated clones were cultured at low density in the presence or absence of 10 μ M IC261 for 4 h, and analyzed by phase contrast microscopy. Scale bar: 20 μ m.

Figure 7. Mutations in the major CK1-phosphorylation site of E-cadherin affect localization of E-cadherin in MDCK cells stably expressing E-cadherin mutants. MDCK cell clones stably expressing myc-tagged wild type, non-phosphorylatable (S846A) and pseudo-phosphorylated (S846D) mutants of E-cadherin were obtained. More than five independent clones were analyzed for each of the different types of E-cadherin, and analogous data were obtained between clones expressing the same type

of E-cadherin. Data using representative clones (WT25, WT28, A28 and D23 for wild type, S846A and S846D E-cadherin, respectively) are shown. **(a)** Expression level of E-cadherin mutants in MDCK cell clones. Cell lysates (20 μ g proteins) from the indicated clones were analyzed by Western blotting with anti-myc and anti-E-cadherin antibodies. The arrowhead and arrow indicate the positions of exogenous myc-E-cadherin and endogenous E-cadherin, respectively. **(b)** Effect of mutations in the major CK1-phosphorylation site of E-cadherin on its localization in MDCK cells. The indicated clones were examined by phase contrast microscopy and immunofluorescence microscopy with anti-myc and anti-E-cadherin antibodies. It should be noted that anti-E-cadherin antibody detects both exogenous and endogenous E-cadherin. Scale bars: 20 μ m.

Figure 8. CK1-catalyzed phosphorylation of E-cadherin affects the interaction between E-cadherin and β -catenin, and vice versa. **(a)** Effect of CK1 on the interaction between E-cadherin and the binding proteins. (Upper panel) GST or GST-tagged cytoplasmic domain of wild type or non-phosphorylatable (S846A) E-cadherin was coupled to glutathione-Sepharose beads, and incubated with ATP in the presence or absence of His₆-tagged CK1 δ at 30°C for 30 min. The beads were then incubated with HEK293 cell lysate at 4°C, followed by GST-pull down assays. The proteins bound to the beads were analyzed by Coomassie protein staining and Western blotting with anti-His₆ antibody. (Lower panel) GST or GST- β -catenin was coupled to glutathione-Sepharose beads, and incubated with His₆-CK1 δ , followed by GST-pull down assays. The proteins bound to the beads were analyzed by Coomassie protein staining and Western blotting with the indicated antibodies. **(b)** Effect of mutations of E-cadherin

on its interaction with the binding proteins. GST or GST-tagged cytoplasmic domain of non-phosphorylatable (S846A) or pseudo-phosphorylated (S846D) E-cadherin was coupled to glutathione-Sepharose beads. The beads were then incubated with the titrated amount of HEK293 cell lysate (1/2, 1/4, 1/8 of cell lysate from 80% confluent culture in 9-cm plate). The proteins bound to the beads were analyzed by Coomassie protein staining and Western blotting with the indicated antibodies. (c) Interaction between E-cadherin and β -catenin prevents phosphorylation of E-cadherin by CK1. To uncouple β -catenin from E-cadherin, MCF-7 cells were lysed in 1% SDS lysis buffer. The lysate was then diluted 10-fold prior to immunoprecipitation. Otherwise, immunoprecipitation was performed in 1% Triton X-100 lysis buffer. After immunoprecipitation, beads were intensively washed, and *in vitro* phosphorylation assays were performed with [γ - 32 P] ATP and CK1 δ , followed by autoradiography and Western blotting with anti-E-cadherin, - α -catenin and - β -catenin antibodies. Arrows indicate the positions of E-cadherin, α -catenin and β -catenin.

Figure 9. Mutations in the major CK1-phosphorylation residue of E-cadherin affect endocytosis of E-cadherin. (a) Effect of IC261 on endocytosis of endogenous E-cadherin in MCF-7 cells. E-cadherin in MCF-7 cells was surface-biotinylated and incubated in normal (N) or low (L) Ca^{2+} medium in the presence or absence of 10 μM IC261 for 5 h. Biotinylated proteins on the plasma membrane were then stripped off by glutathione treatment. Biotinylated E-cadherin inside cells were recovered on streptavidin beads and analyzed by Western blotting with anti-E-cadherin antibody. Input indicates total biotinylated E-cadherin. (b and c) Effect of E-cadherin mutation on its endocytosis in MDCK cells. (b) Wild type and non-phosphorylatable (S846A)

E-cadherin on the respective stable clones (WT28 and A28) were surface-biotinylated and incubated in a normal (N) or low (L) Ca^{2+} medium for 8 h. After glutathione treatment, biotinylated E-cadherin inside cells was recovered on streptavidin beads and analyzed by Western blotting with anti-myc antibody. Input indicates total biotinylated myc-E-cadherin. (c) Wild type and pseudo-phosphorylated (S846D) E-cadherin on the respective stable clones (WT25 and D23) were analyzed as described above, except that cells were incubated in a normal (N) or low (L) Ca^{2+} medium for 3 h. Input indicates total biotinylated myc-E-cadherin. The lower panel shows the quantification of endocytosed E-cadherin compared with total biotinylated E-cadherin from the results of three independent experiments. It should be noted that different incubation times were used in (b) (8 h) and (c) (3 h) in order to maximize the difference in endocytosis between respective mutants. (d) Effect of E-cadherin mutation on its endocytosis in MDCK cells by immunofluorescence staining. Myc-tagged wild type or pseudo-phosphorylated (S846D) E-cadherin was transiently expressed in MDCK cells and incubated with $2.5 \mu\text{g ml}^{-1}$ cycloheximide for 8 h. Cycloheximide was used to block the entry of newly synthesized E-cadherin into endosomes. The subcellular localization of exogenously expressed E-cadherin and early endosomes was analyzed using anti-myc and anti-EEA1 antibodies. Scale bars: 20 μm . Expression of E-cadherin S846D induced flattening of cells as seen in Fig. 7b, and more early endosomes were observed in the same plane as E-cadherin in confocal microscopic analyses. Neither E-cadherin wild type nor S846D affected the morphology of early endosomes.

Supp Figure 1. (a) IC261 reverts the cell separation induced by low Ca^{2+} treatment of MCF-7 cells. After an overnight culture in low Ca^{2+} medium, MCF-7 cells were further incubated in low Ca^{2+} medium containing 10 μM IC261 for the indicated times. The effect of IC261 was examined by phase contrast microscopy. Scale bar: 20 μm . **(b)** IC261 does not affect cell separation induced by activation of src in MDCK cells. ts-Src MDCK cells were cultured at 40.5°C (the non-permissive temperature) and further incubated at 35°C (the permissive temperature) for 8 h in the presence or absence of 10 μM IC261. (Upper panels) Phase contrast images. (Lower panels) Immunofluorescence staining of E-cadherin. Scale bars: 30 μm .

Supp Figure 2. CK1 co-localizes with E-cadherin. **(a & b)** Co-localization between CK1 α and E-cadherin in MDCK cells at a steady state (a) or during Ca^{2+} switch (b). The subcellular localization of CK1 and E-cadherin was examined in MDCK cells stably expressing GFP-tagged CK1 α . Scale bars: 10 μm .

Supp Figure 3. CK1-catalyzed phosphorylation of E-cadherin does not prime subsequent phosphorylation by GSK-3 β . GST or GST-E-cadherin (wild type) was coupled to glutathione-Sepharose beads, and first incubated with cold ATP in the presence or absence of CK1 δ at 30°C for 30 min, followed by intensive washing. The beads were further incubated with [γ - ^{32}P] ATP in the presence of GSK-3 β or CK1 δ at 30°C for 8 min, followed by autoradiography and Coomassie protein staining. IC261 was added in the second phosphorylation reaction except for lane 7, since significant amounts of CK1 δ bound to E-cadherin during the first phosphorylation. GSK-3 β -

catalyzed phosphorylation of E-cadherin was not clearly observed following phosphorylation by CK1 even without addition of IC261 (data not shown).

Supp Figure 4. (a) The CK1-catalyzed phosphorylation of E-cadherin is not involved in the increased interaction between E-cadherin and β -catenin. GST-E-cadherin (wild type) was coupled to glutathione-Sepharose beads and incubated with ATP or ATP γ S in the absence or presence of CK1 δ at 30°C for 30 min. The beads were then incubated with HEK293 cell lysate, and the proteins bound to the beads were analyzed by Coomassie protein staining and Western blotting with anti- β -catenin antibody. The arrow indicates the position of GST-E-cadherin. **(b)** Interaction with β -catenin suppresses the CK1-catalyzed phosphorylation of E-cadherin. Immunoprecipitation was performed using MCF-7 cell lysates in 1% SDS lysis buffer as described in Fig. 8c. The immunoprecipitates were further incubated with or without 2 μ g of recombinant β -catenin protein, and an *in vitro* phosphorylation assay was performed with [γ - 32 P] ATP and CK1 δ , followed by autoradiography and Western blotting with anti-E-cadherin and anti- β -catenin antibodies. Arrows indicate the positions of E-cadherin and recombinant β -catenin. **(c)** IC261 does not affect endocytosis of pseudo-phosphorylated (S846D) E-cadherin. Endocytosis of pseudo-phosphorylated (S846D) E-cadherin on the MDCK stable clones (D23) were analyzed in the presence or absence of IC261 as described in Figure 9c. **(d)** IC261 does not affect transport of newly synthesized E-cadherin to plasma membranes. MCF-7 cells were cultured in the presence of [35 S] methionine and cysteine for 30 min, followed by incubation with NHS-SS-Biotin in the presence or absence of 10 μ M IC261 in Krebs-Ringer buffer at

1 37°C for 2 h. Surface-biotinylated E-cadherin was then pulled down with monomeric-
2 avidin beads and eluted by 2 mM D-biotin, followed by immunoprecipitation with
3 anti-E-cadherin antibody. Immunoprecipitated proteins were examined by
4 autoradiography and Western blotting with anti-E-cadherin antibody. Arrows indicate
5 the positions of E-cadherin, α -catenin and β -catenin.
6

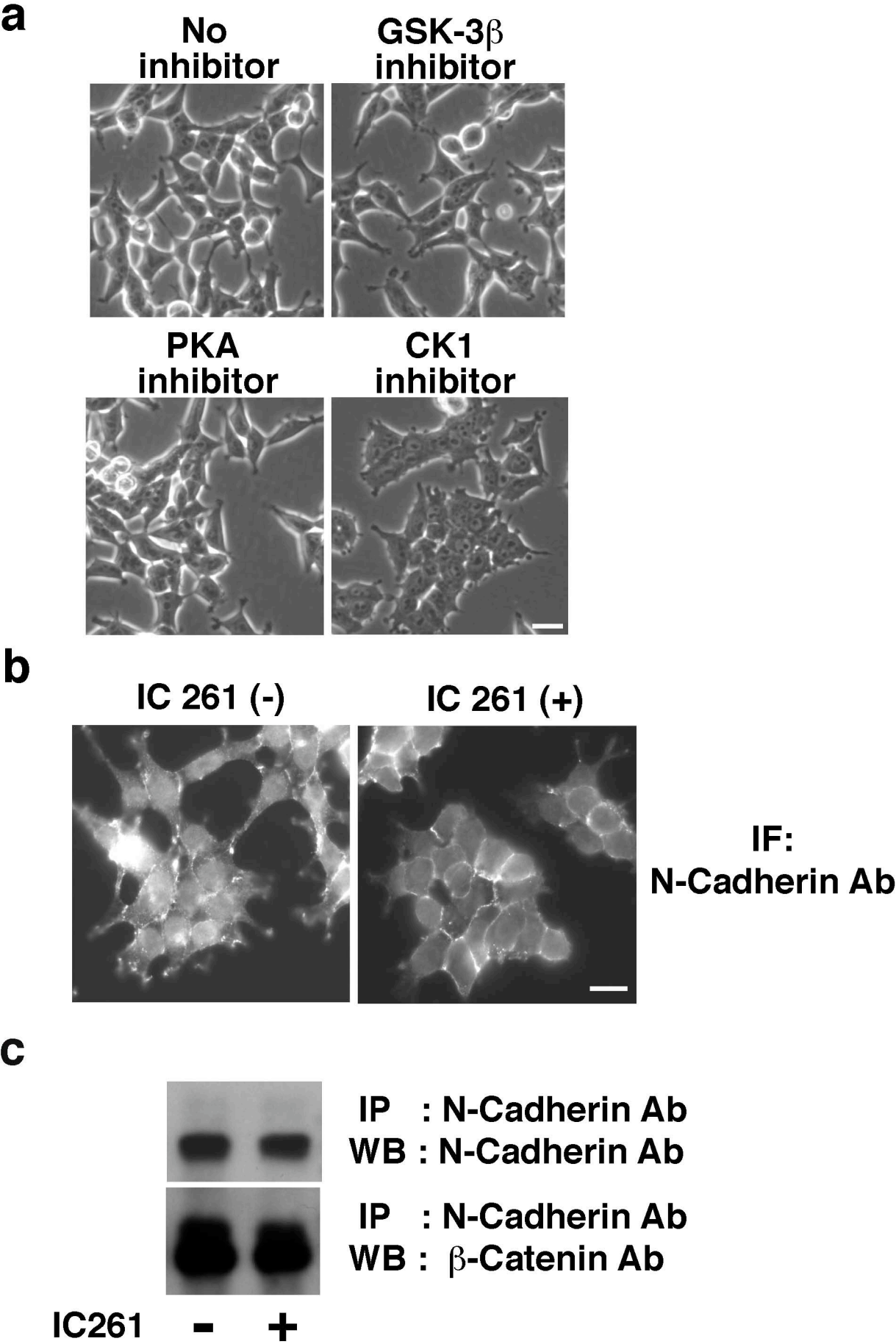


Fig. 1
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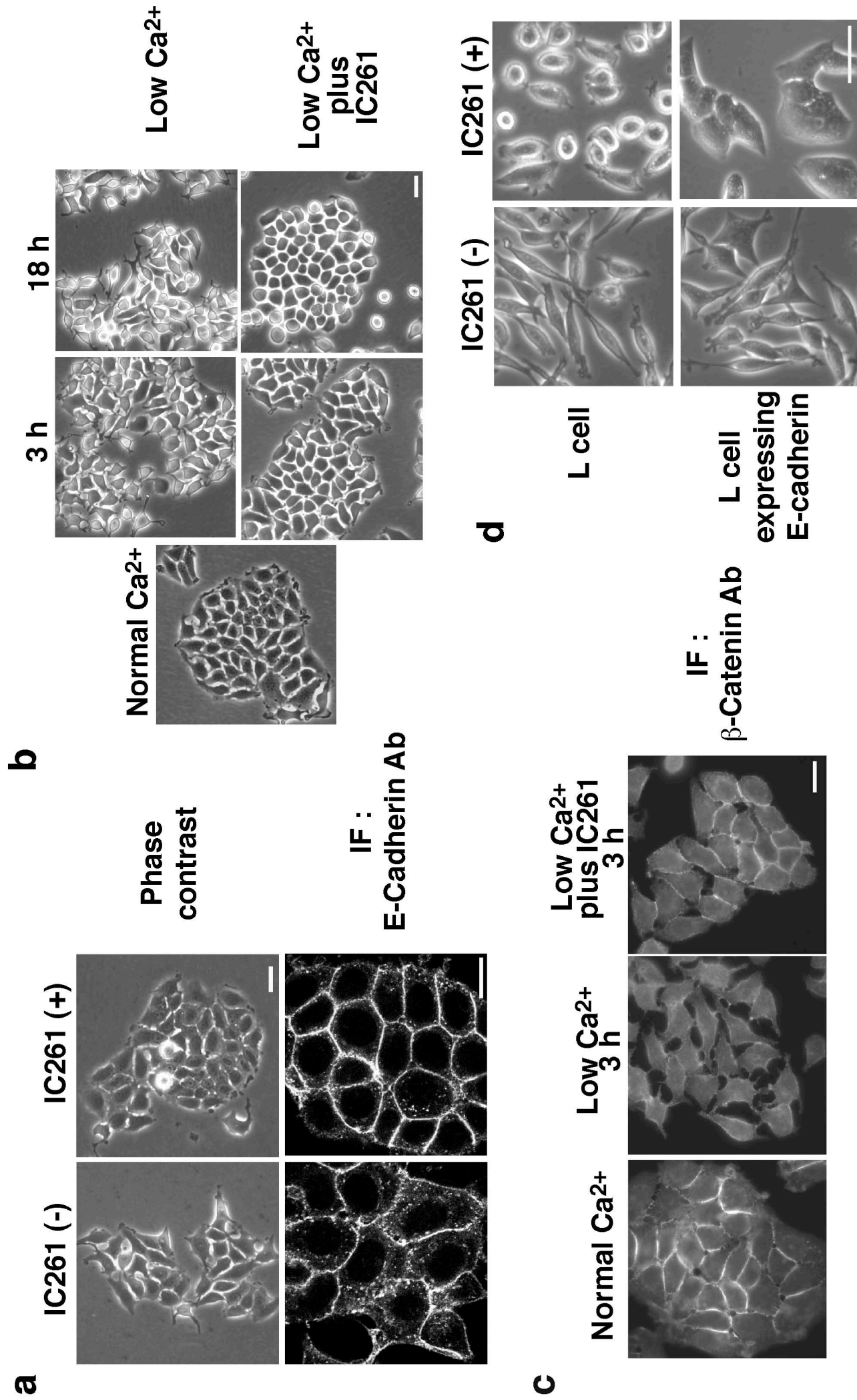


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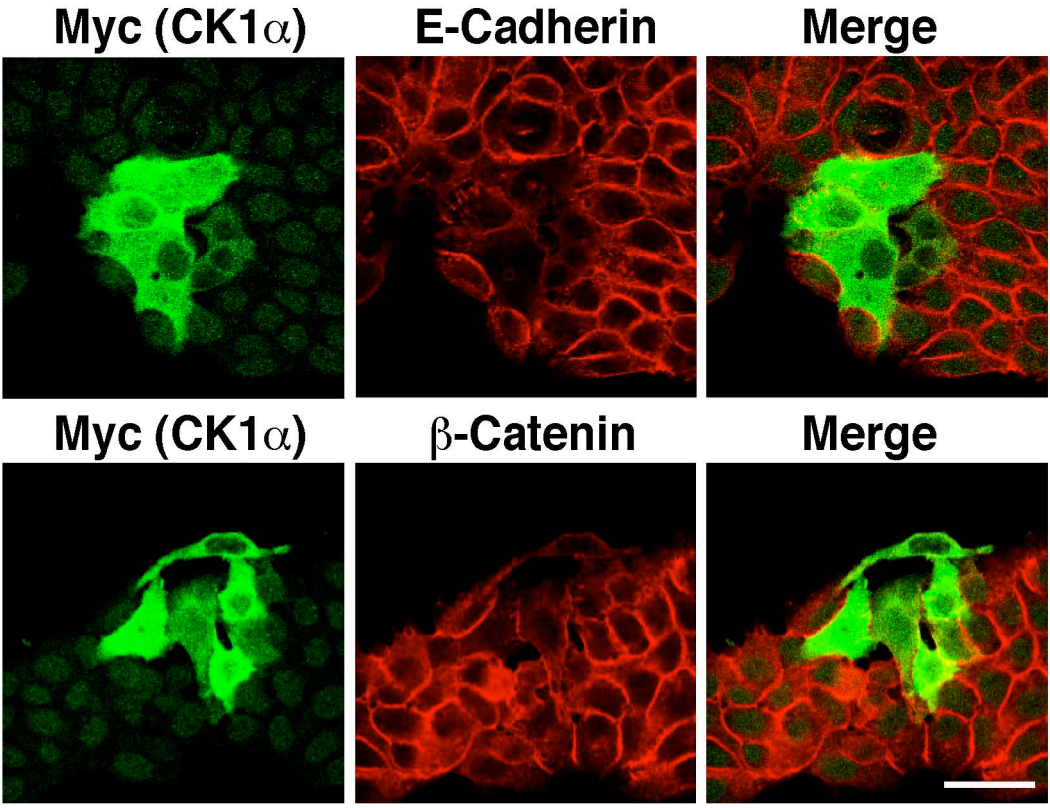


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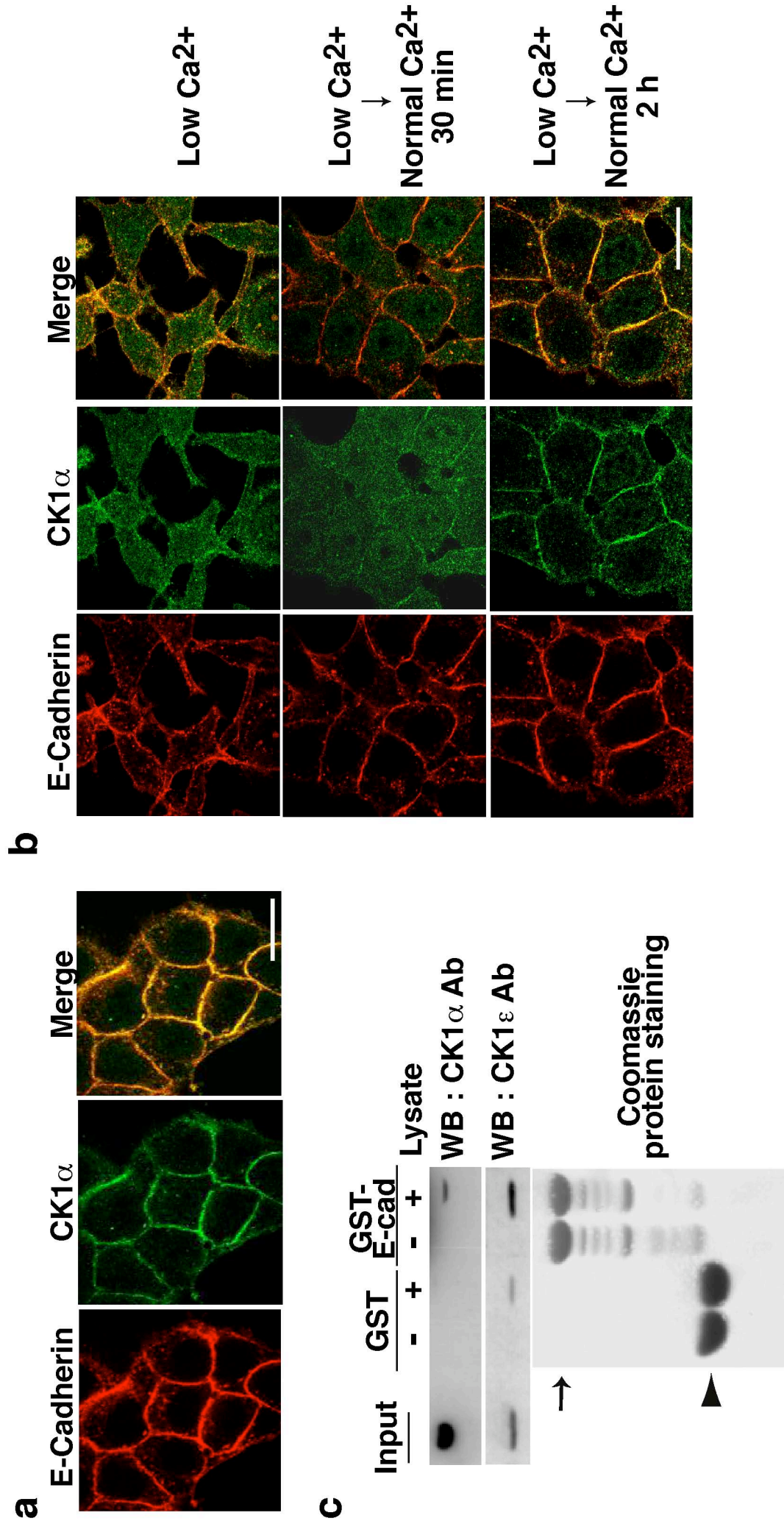


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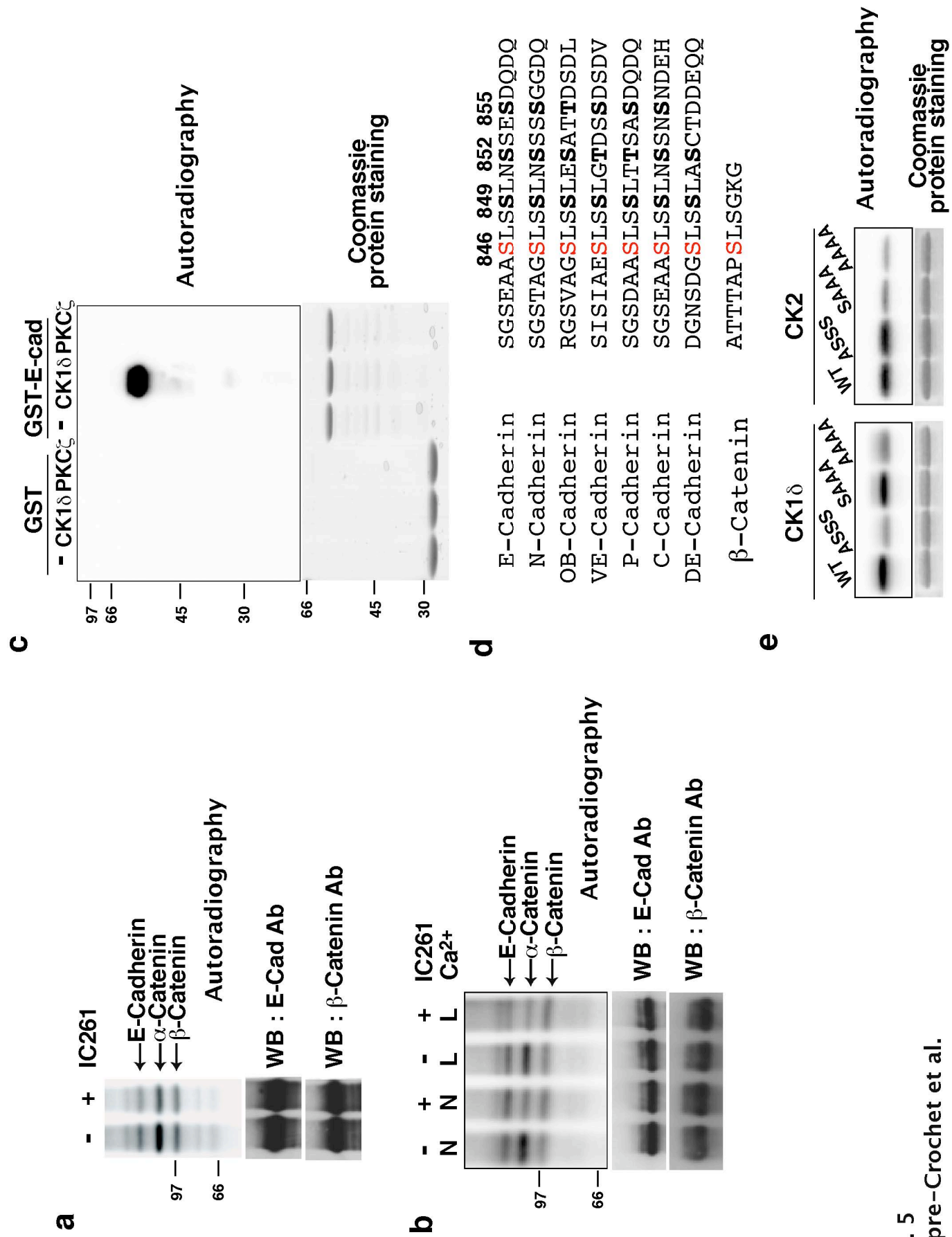


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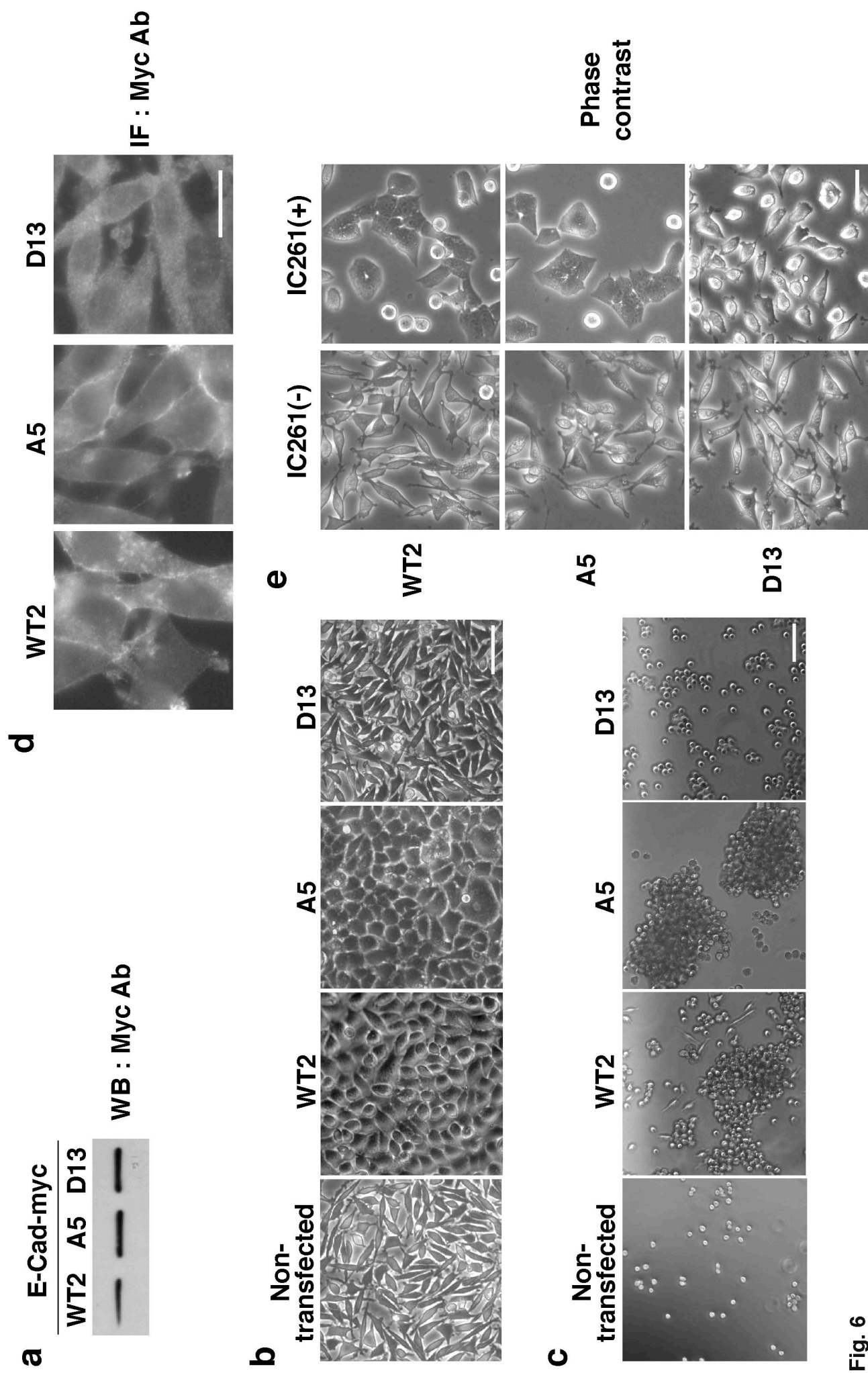


Fig. 6
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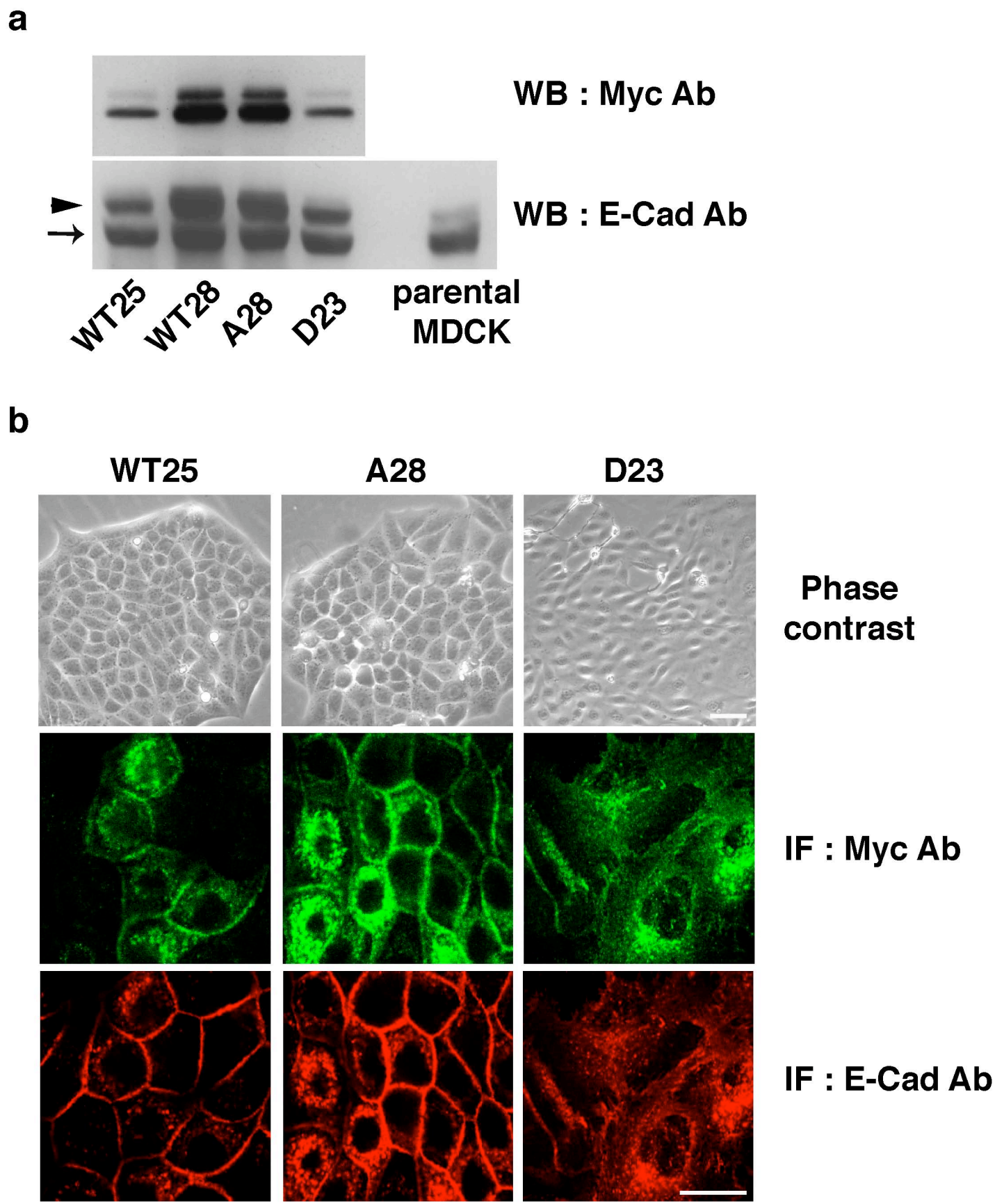
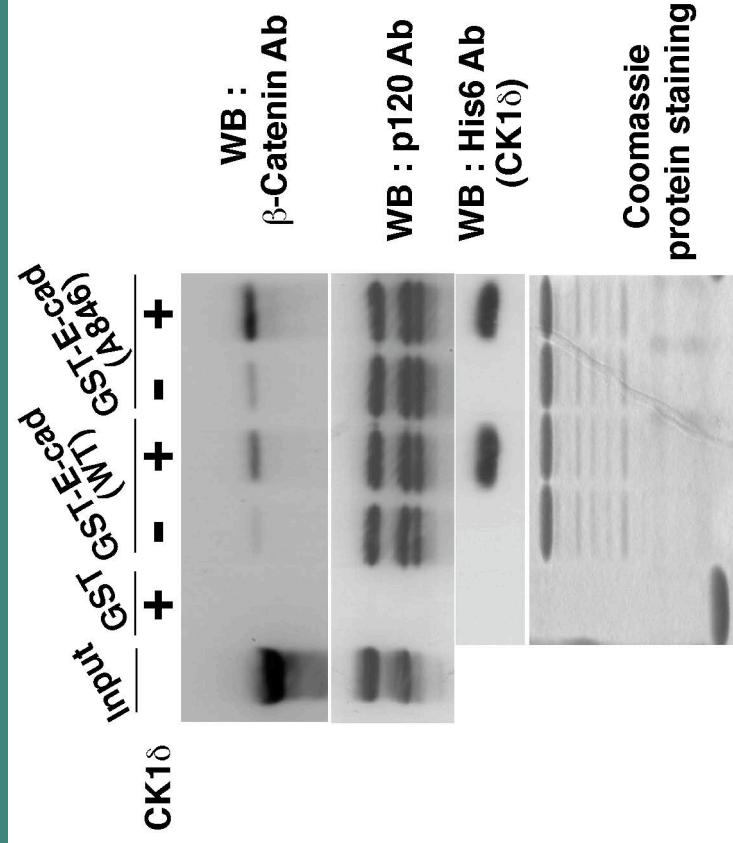
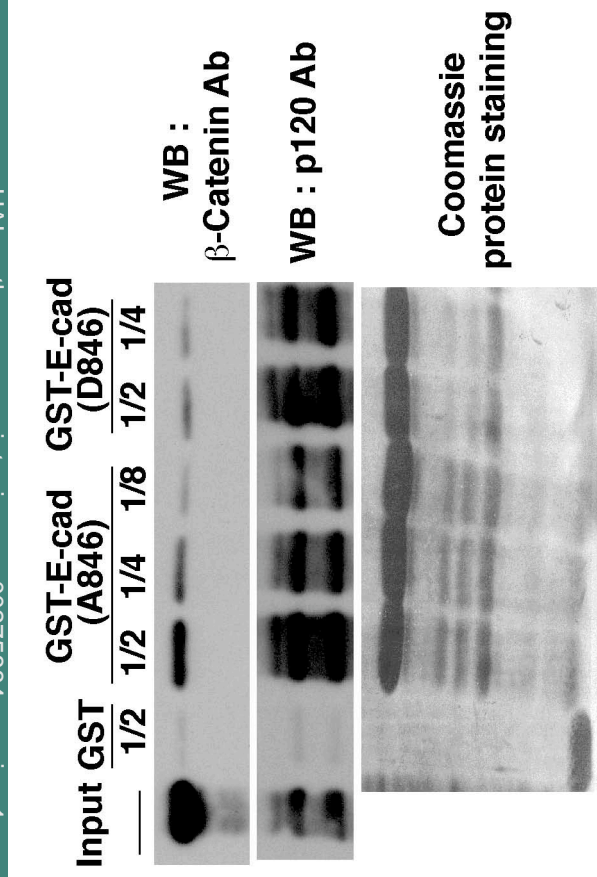


Fig. 7
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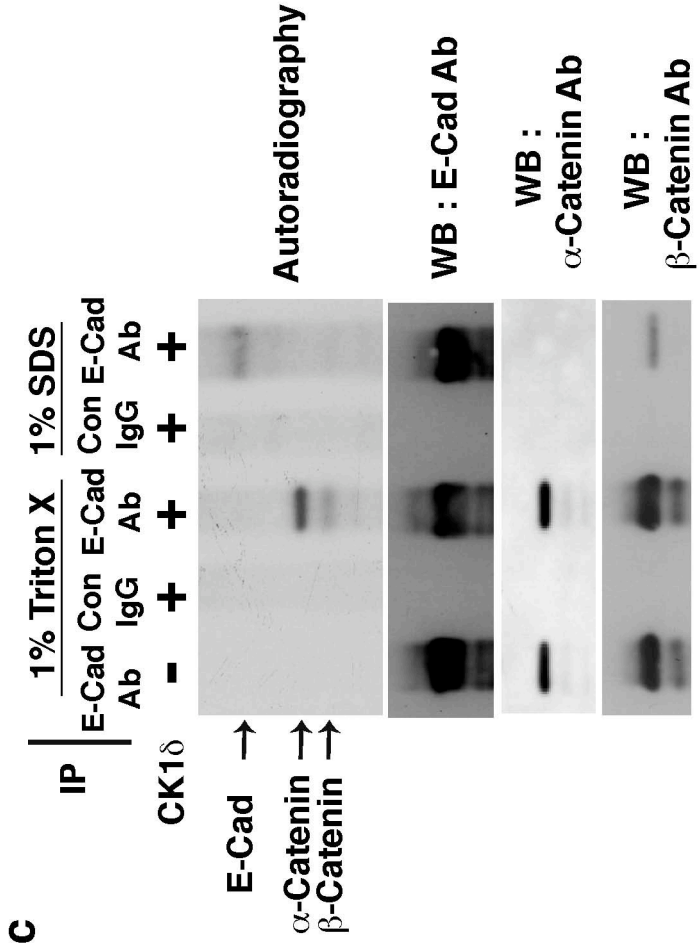
a



b



c



Figs. 8 a-d
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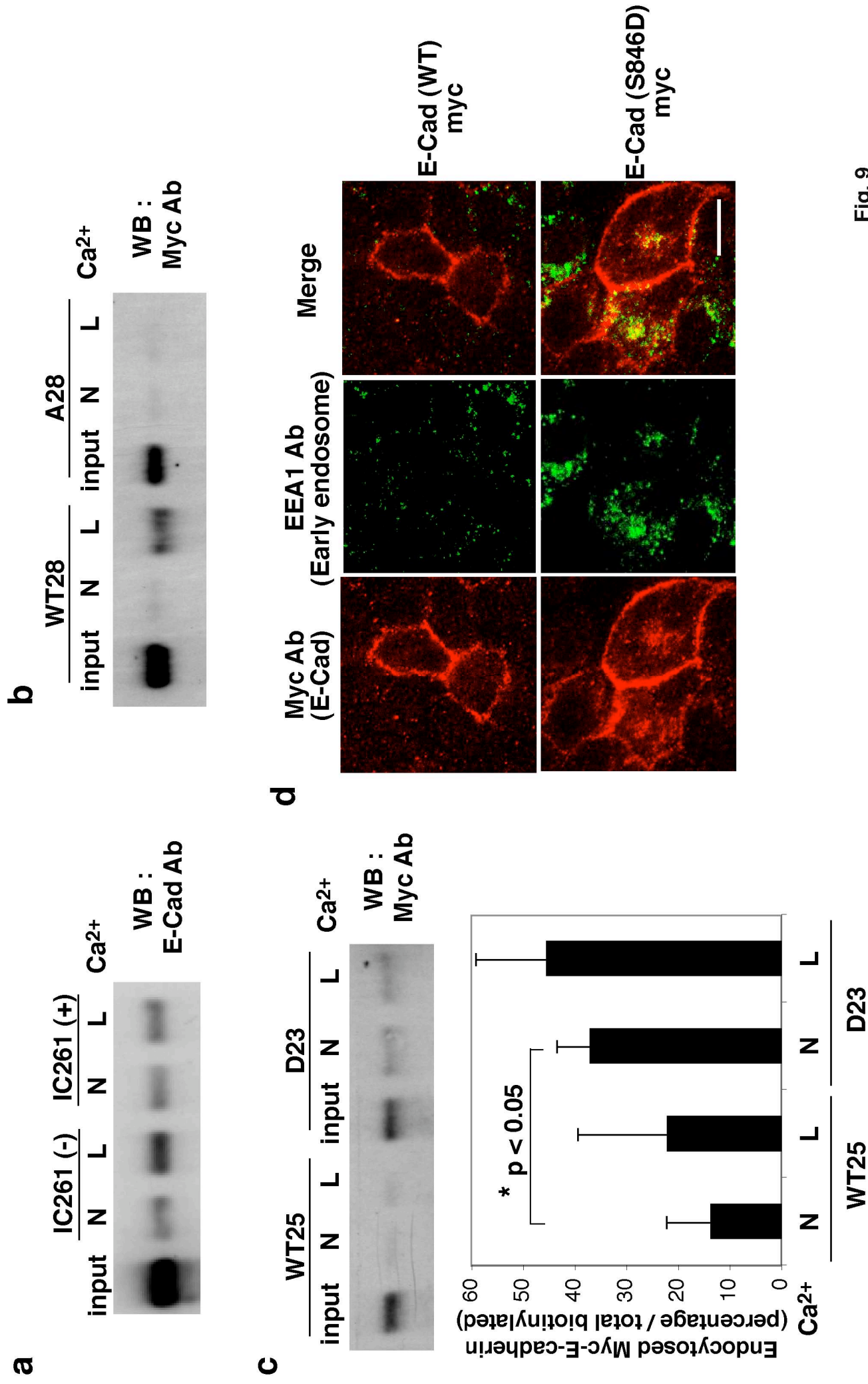


Fig. 9
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TABLE 1. Cell dissociation-index (N_{TC}/N_{TE}) of L cells expressing E-cadherin mutants in the presence or absence of IC261

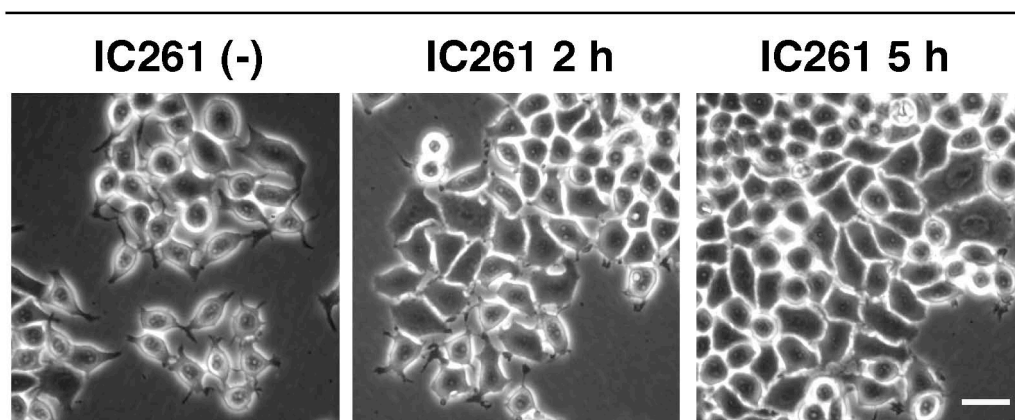
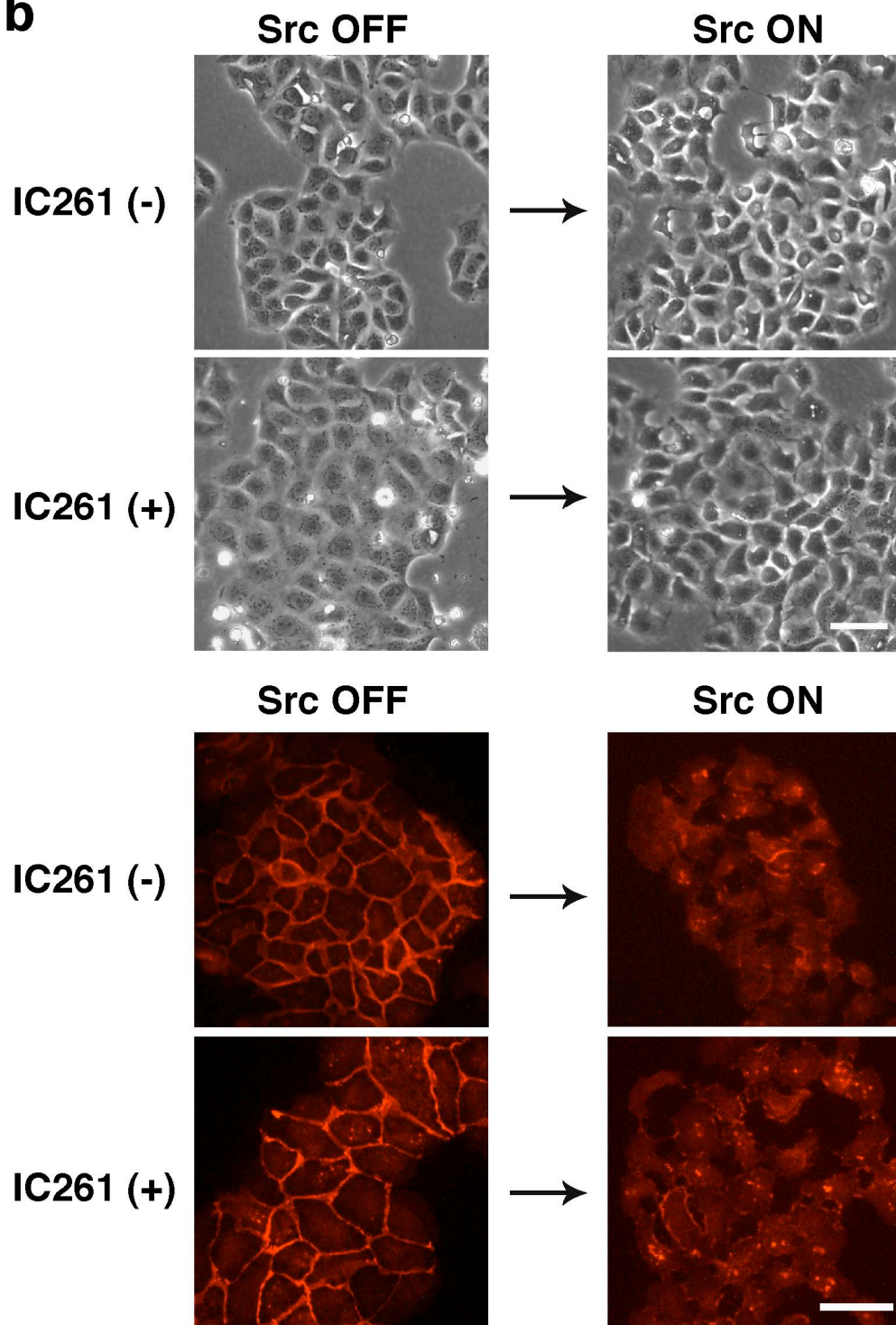
	Parental L cell	L cell expressing E-cad (WT) WT2	L cell expressing E-cad (S846A) A5	L cell expressing E-cad (S846D) D13
IC261				
-	0.83 ± 0.09	^(a) 0.64 ± 0.09	^(b) $0.46 \pm 0.09^{**}$	^(c) $0.88 \pm 0.04^{***}$
+	0.91 ± 0.05	^(d) $0.52 \pm 0.05^{*}$	^(e) $0.41 \pm 0.09^{*}$	0.72 ± 0.06

* $p < 0.05$; (a) & (d), (b) & (e)

** $p < 0.01$; (a) & (b)

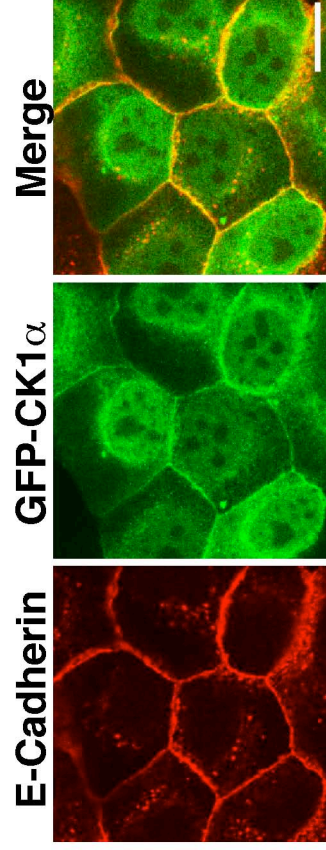
*** $p < 0.005$; (a) & (c)

The data are obtained from more than 4 independent experiments.

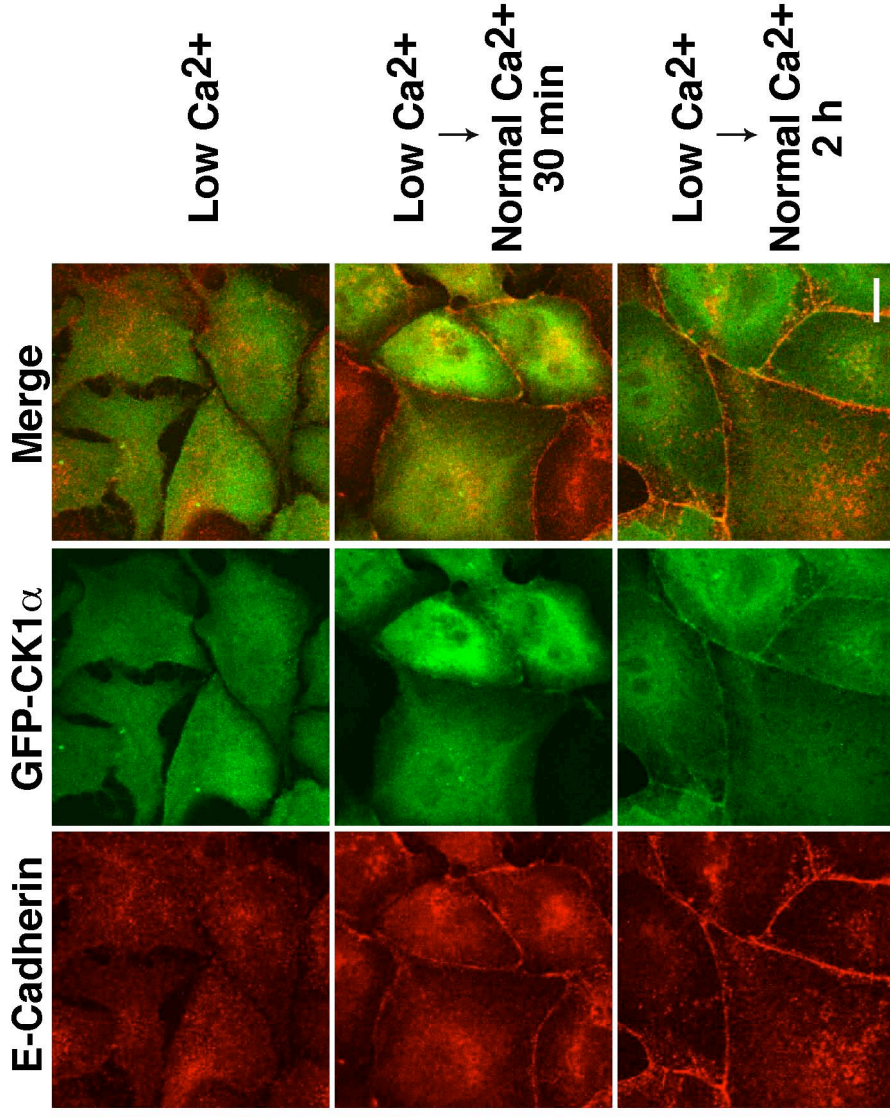
a**Low Ca²⁺****b**

Supp Fig. 1
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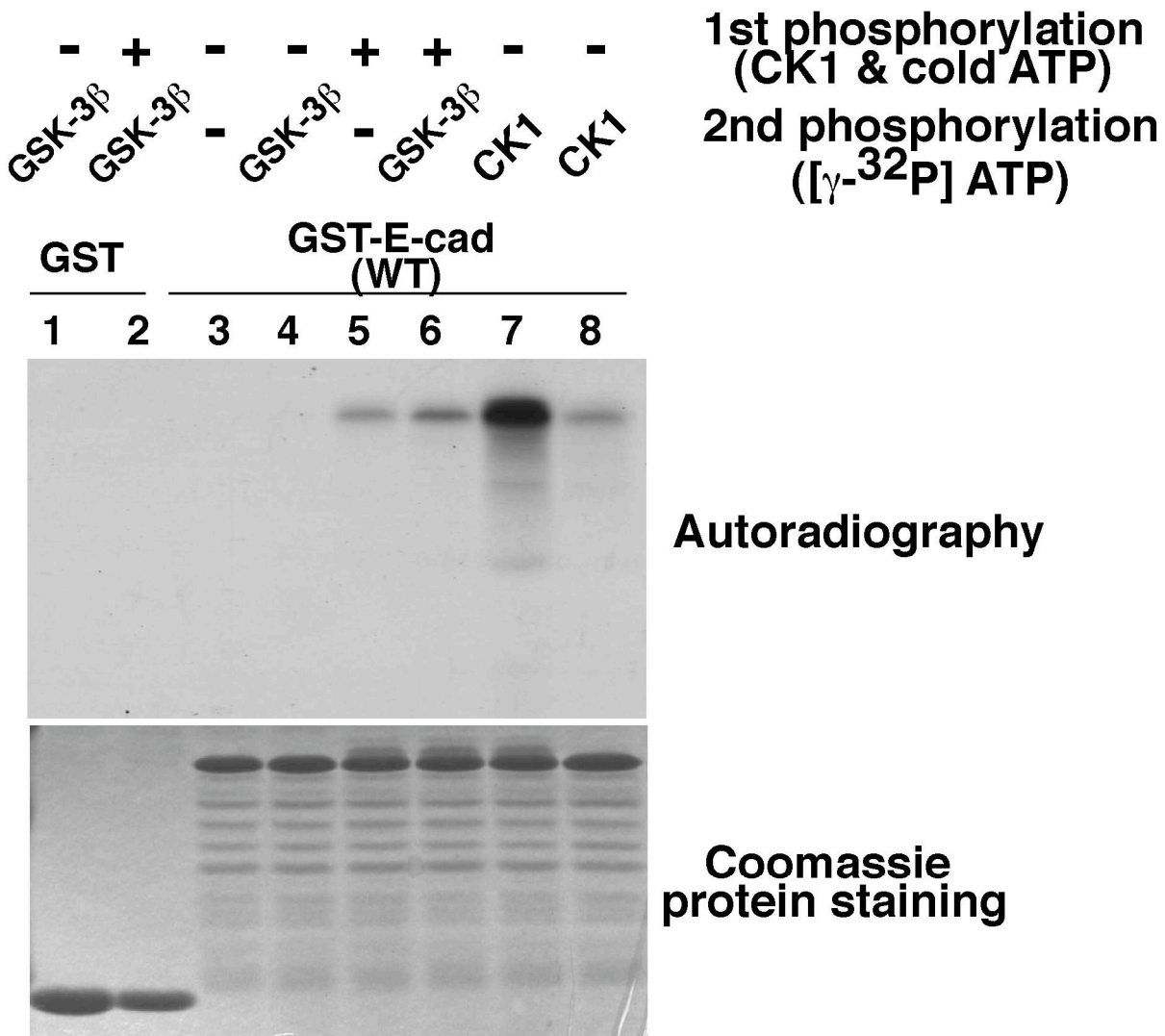
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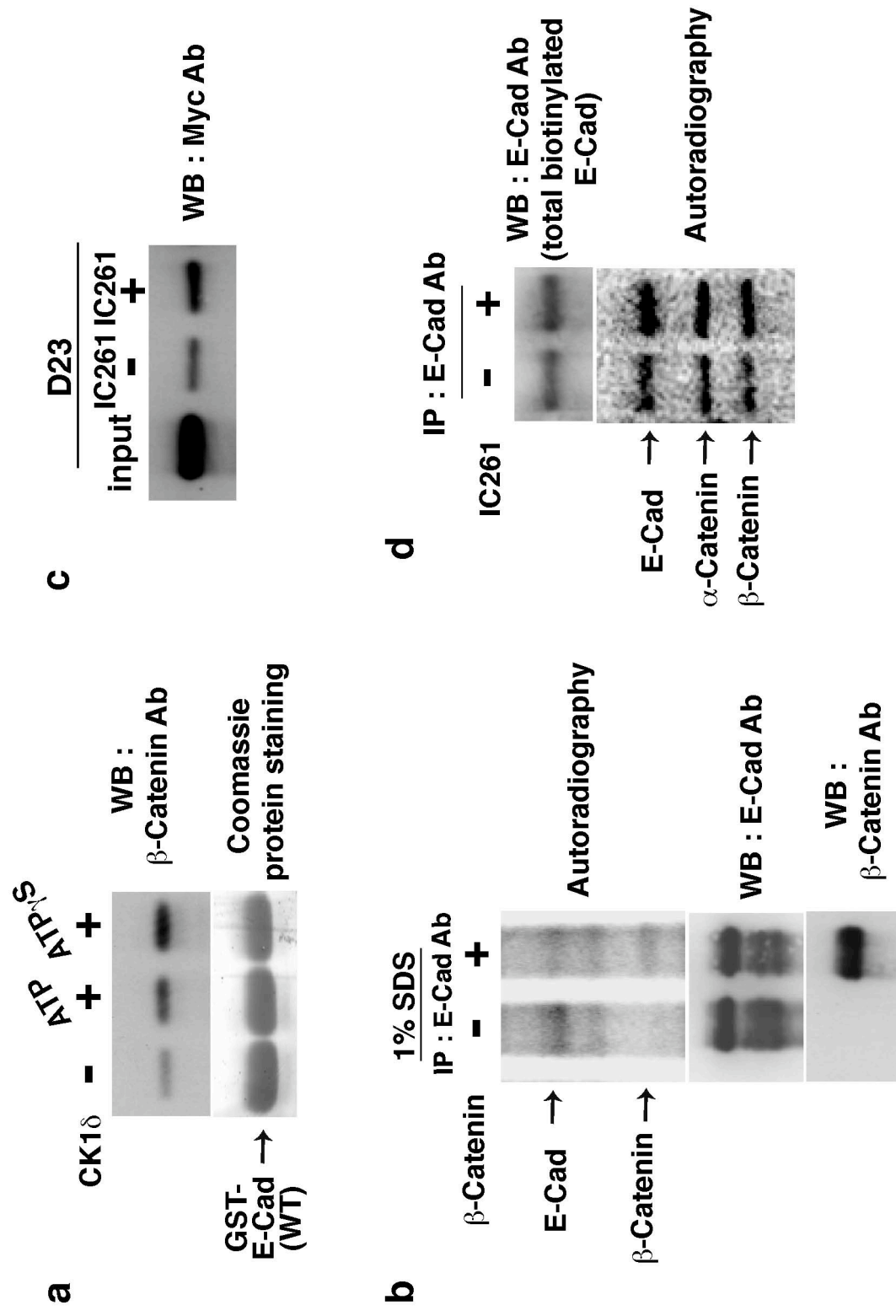
b



Supp Fig. 2
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Supp Fig. 3
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Supp Fig. 4
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