Molecular Biology of the Cell
Vol. 20, 3598–3607, August 1, 2009

E-cadherin Surface Levels in Epithelial Growth Factor-stimulated Cells Depend on Adherens Junction Protein Shrew-1

Julia Christina Gross,* Alexander Schreiner,* Knut Engels,† and Anna Starzinski-Powitz*

*Institute for Cell Biology and Neuroscience, Johann Wolfgang Goethe University of Frankfurt, 60323 Frankfurt, Germany; and †Senckenberg's Institute of Pathology, University Hospital Frankfurt, 60596 Frankfurt, Germany

Submitted December 24, 2008; Revised June 1, 2009; Accepted June 2, 2009
Monitoring Editor: Keith E. Mostov

Gain- and loss-of-function studies indicate that the adherens junction protein shrew-1 acts as a novel modulator of E-cadherin internalization induced by epithelial growth factor (EGF) or E-cadherin function-blocking antibody during epithelial cell dynamics. Knocking down shrew-1 in MCF-7 carcinoma cells preserves E-cadherin surface levels upon EGF stimulation. Overexpression of shrew-1 leads to preformation of an E-cadherin/EGF receptor (EGFR) HER2/src-kinase/shrew-1 signaling complex and accelerated E-cadherin internalization. Shrew-1 is not sufficient to stimulate E-cadherin internalization, but facilitates the actions of EGFR and thus may promote malignant progression in breast cancer cells with constitutive EGFR stimulation by reducing surface E-cadherin expression.

INTRODUCTION

The formation of epithelial adherens junctions (AJs) is mediated by the calcium-dependant cell adhesion protein E-cadherin (Gumbiner, 1996). AJs are highly dynamic structures that undergo rapid, but nevertheless regulated assembly and disassembly during morphological remodeling events, such as wound healing or migration during development, whereas aberrant cell motility eventually allows tumor invasion and metastasis (Takeichi, 1991; Jamora and Fuchs, 2002; Bryant and Stow, 2004; Gumbiner, 2005; Lecuit, 2005). Endocytic trafficking of E-cadherin regulates AJ dynamics and stability. On growth factor stimulation AJs are disrupted, E-cadherin is internalized and accumulates in both clathrin-coated vesicles and caveolae vesicles depending on the growth factor and the epithelia type (Akhtar and Hotchin, 2001; Al Moustafa *et al.*, 2002; Palacios *et al.*, 2002; Paterson *et al.*, 2003; Janda *et al.*, 2006). Endocytosed E-cadherin can either be degraded or recycled to the cell surface. In turn, cell adhesion can modulate growth factor signaling activities, because cadherins were found to interact with growth factor receptors at the cell surface (Francavilla *et al.*, 2007; Reshetnikova *et al.*, 2007). Therefore, endocytosis of E-cadherin and signal transduction are inseparably linked cellular functions.

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E08–12–1240) on June 10, 2009.

Address correspondence to: Anna Starzinski-Powitz (starzinski-powitz@bio.uni-frankfurt.de).

Abbreviations used: AJ, adherens junction; EGF, epithelial growth factor; EGFR, epithelial growth factor receptor; EMT, epithelial–mesenchymal transition; ERK1/2, extracellular signal–regulated kinase 1/2; GFP, green fluorescence protein; HER2, human epithelial growth factor receptor-2; HRP, horseradish peroxidase; RTK, receptor tyrosine kinase; shRNA, short hairpin RNA.

Members of the Src family of nonreceptor protein tyrosine kinases (SFK) have been proposed as regulators of AJ function (Thomas and Brugge, 1997). The activation of Src in cultured epithelial cells down-regulates E-cadherin and causes cell dissociation (Behrens *et al.*, 1993) and is implicated in promoting the epithelial–mesenchymal transition (EMT; Boyer *et al.*, 2000). In the case of AJ disassembly induced by epithelial growth factor (EGF), Src is found in complexes with the EGF receptor (EGFR) and E-cadherin (Shen *et al.*, 2008). By contrast, a recent study suggests that Src might also act positively on the maintenance of AJs (McLachlan *et al.*, 2007).

Shrew-1 is a recently described AJ-associated transmembrane protein that we identified in epithelia cells of skin, uterus, and pancreas (Bharti *et al.*, 2004; Jakob *et al.*, 2006). It can be found at the basolateral part of the plasma membrane where it colocalizes with, and apparently integrates into E-cadherin–mediated AJs. On activation of receptor tyrosine kinases (RTKs), shrew-1 seems to internalize together with E-cadherin (Bharti *et al.*, 2004).

In nonpolarized, highly migratory and invasive cells, shrew-1 interacts with the transmembrane glycoprotein CD147, an invasion-promoting protein (Lim *et al.*, 1998; Kanekura *et al.*, 2002). This interaction and the fact that shrew-1 down-regulation reduces cellular invasion implies that it is involved in the process of invasiveness (Schreiner *et al.*, 2007).

In this article we have used gain- and loss-of-function approaches to uncover a role for shrew-1 in facilitating growth factor–induced E-cadherin endocytosis. These findings are also highly relevant for cancer development because deregulated AJ disassembly is a key step in tumor progression.

MATERIALS AND METHODS

Antibodies

Human epithelial growth factor receptor-2 (HER2), Src, and phospho-tyrosine (PY100) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Monoclonal anti-green fluorescent protein (GFP) antibody was obtained

from Roche Diagnostics (Mannheim, Germany) and a polyclonal anti-GFP antibody from BD Biosciences Clontech living colors (Mountain View, CA). Monoclonal GAPDH antibody was purchased from Ambion/Applied Biosystems (Darmstadt, Germany). Monoclonal E-cadherin (clone 36) was obtained from BD Transduction Laboratories (Los Angeles, CA) and used for Western blot detection. Another mAb against E-cadherin (5H9) was purchased from Sigma-Aldrich Chemie (München, Germany) for immunofluorescence staining. DECMA-1, a rat mAb against E-cadherin, was a kind gift from R. Kemler (Max Planck Institute of Immunology, Freiburg, Germany). Polyclonal antibodies were generated against the cytoplasmic domain of shrew-1 in rats in collaboration with Genovac (Freiburg, Germany). Secondary antibodies (Alexa Fluor 488– and 594–labeled antibodies) were from Molecular Probes (Leiden, The Netherlands). Horseradish peroxidase (HRP)- and AP-coupled secondary antibodies and HRP-coupled streptavidin were purchased from Jackson Immunochemicals (Dianova, Hamburg, Germany). EGF was obtained from Immunotools (Friesoythe, Germany).

Cell Culture

Human mammary carcinoma (MCF-7) cells were cultured in DMEM supplemented with 10% FCS (both from PAA Laboratories, Cölbe, Germany), 1% penicillin, and 100 U/ml streptomycin (Invitrogen, München, Germany) 37°C with 5% CO_2.

Cell Transfection and Infection

MCF7 cells were grown on 6-cm plates until 40% confluent and transfected with pEGFP-shrew-1 (Bharti *et al.*, 2004) or PEGFP-N3 vector (BD Biosciences Clontech) alone applying magnet-assisted transfection (MaTra; IBA, Göttingen, Germany). Stable cell lines were selected by adding the eukaryotic selection marker G418 (Invitrogen, München, Germany) at a final concentration of 5 mg/ml 48 h after transfection until single colonies formed. Colonies were isolated, expanded, and grown in the presence of 0.5 mg of G418 per ml of medium and analyzed for their transfection efficiency and expression level.

Cell lines stably expressing short hairpin RNA (shRNA) against shrew-1 or mock controls were generated by infecting MCF7 cells with lentiviral particles containing the SEW vector encoding a sequence for a shRNA against shrew-1 or scrambled sequence (Schreiner *et al.*, 2007).

Knockdown of Shrew-1 RT-PCR

Knockdown efficiency was determined by semiquantative RT-PCR. Confluent MCF7 cells expressing shRNA against shrew-1 or control cells were harvested, and total RNA was extracted with the Cyto ALL Kit (Thermo Scientific, Hamburg, Germany) and subsequently RT-PCR with primers against shrew-1 cytoplasmic domain (SHR: 5′-AAAAATTGCTGTGCCCAAAGC; SHR: 3′-TTAGCAGGAGATTTCAAACCAT) and BIP (BIP: 5′-TACACTTGGTATTGAAACTG and 3′-GGTGGCTTTCCAGCCATTC) as a control was performed with the VersoTM RT-PCR-kit (Thermo Scientific, Hamburg, Germany).

Immunofluorescence and Confocal Microscopy Analysis

Cells were grown on glass coverslips and upon reaching the desired confluence were fixed for 10 min with 4% paraformaldehyde, in phosphate-buffered saline (PBS), washed two times for 5 min with PBS, and permeabilized for 5 min with 0.1% Triton X-100 (in PBS). For immunofluorescence labeling, the cells were blocked for 30 min with 10% FCS/PBS. Primary antibodies in 10% FCS/PBS were incubated either for 1 h at room temperature or overnight at 4°C. Antibody binding was visualized by fluorochrome-conjugated secondary antibodies. Nuclei were stained with Hoechst dye no. 33258 (Sigma-Aldrich Chemie). The samples were examined with a TCS SP5 confocal laser scanner microscope (Leica Microsystems, Heidelberg, Germany). Images were processed with Imaris (Bitplane, Zürich, Switzerland) and prepared for publication with Adobe Photoshop (Adobe Systems, Unterschleissheim, Germany). The images were further analyzed using ImageJ software (http://rsb.info.nih.gov/ij/; W. Rasband, National Institutes of Health, Bethesda, MD).

DECMA-1 Treatment and AJ Disruption Assay

DECMA-1 antibodies were used as a hybridoma supernatant. To determine the concentration, supernatant was incubated with Sepharose-G beads to precipitate antibodies. Concentration of DECMA-1 supernatant before and after precipitation was determined to calculate the antibody content. In a six-well dish 0.25×10^5 cells were serum-starved over night and treated with 40–50 μg/ml antibody for indicated periods and then immunostained for E-cadherin. To assay AJ dissolution, cell colonies of 6–15 cells were divided into three categories: coherent, intermediate, and scattered and then statistically analyzed.

Immunoblotting

Cells, 0.25×10^5, were seeded per six-well dish, starved overnight, stimulated as indicated, washed with PBS, and lysed with 100 μl of radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.25% sodium desoxycholate, 0.1% Nonidet P-40, and 0.1% SDS) plus proteinase inhibitor cocktail Complete (Roche Applied Science, Mannheim, Germany) and Phosphatase Inhibitor I and II (Sigma-Aldrich Chemie) for 10 min at 4°C. Total protein, 20 μg, was separated by SDS-PAGE and transferred onto nitrocellulose membranes in a semidry blotting chamber (PEQLAB Biotechnologie, Erlangen, Germany). Membranes were blocked with 5% nonfat milk powder in Tris-buffered saline/Tween (TBST; 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 0.1% Tween 20) for 1 h. After a single wash step with TBST, the membranes were incubated with primary antibody for 2 h at room temperature or overnight at 4°C. After intensive washing, the bound primary antibody was detected with alkaline phosphatase–conjugated secondary antibody (Dianova, Hamburg, Germany) or HRP-conjugated secondary antibody (Dianova). Enzyme substrates were NBT/BCIP (Roche Diagnostics, Mannheim, Germany) for alkaline phosphatase or a solution of 2.5 mM luminol, 0.4 mM *p*-coumaric acid, 100 mM Tris-HCl, pH 8.5, and freshly added 0.009% H_2O_2 for HRP.

Surface Biotinylation Experiments

Surface Protein Assay. After treatment of cells with EGF for indicated periods, the cell surface of cell colonies was labeled for 15 min on ice with 0.5 mg/ml membrane-impermeable EZ-Link Sulfo-NHS-Biotin (Perbio, Bonn, Germany) in PBS, pH 7.4. After quenching (50 mM ammonium chloride in PBS, 0.1 mM $CaCl_2$, and 1 mM $MgCl_2$), the cells were lysed in 0.1 ml of radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 0.25% SDS, and 0.1% Nonidet P-40) containing the protein inhibitor cocktail Complete (Roche Diagnostics) for 10 min at 4°C. Protein, 200 μg, from each lysate was used for precipitation (16 h at 4°C) with 30 μl of neutravidin beads (Perbio, Bonn, Germany). The precipitates were washed three times with radioimmunoprecipitation assay buffer and immunoblotted.

Degradation Assay. The cell surface of cell colonies was labeled for 60 min on ice and subsequently treated with EGF for indicated periods at 37°C, cells were processed as described above.

Endocytosis Assay. The cell surface of cell colonies was labeled for 30 min on ice with 0.5 mg/ml cleavable, membrane-impermeable EZ-Link Sulfo-SS-Biotin (Perbio) in PBS, pH 7.4. After quenching with DMEM, one sample of the cells was directly lysed in 0.1 ml of radioimmunoprecipitation assay buffer. The other samples of the cells were incubated in DMEM with or without EGF at 37°C for 10 min. Subsequently, surface biotin was glutathione stripped by two 20-min washes of glutathione solution (50 mM glutathione,

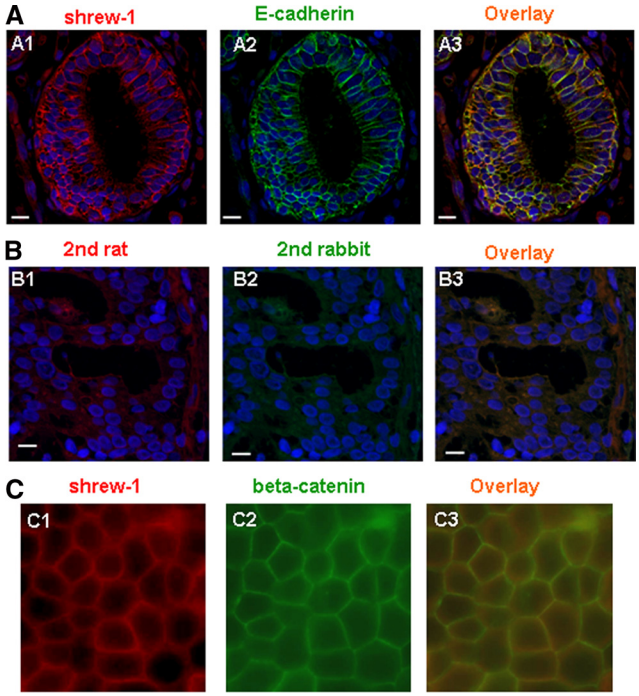

Figure 1. Endogenous shrew-1 is found in mammary gland sections and in a MCF7 carcinoma cell line. (A) Immunohistochemical staining of shrew-1 in human mammary gland section. Shrew-1 is located at cell–cell contacts where it colocalizes with E-cadherin. (B) Control for unspecific reactivity of rat or mouse secondary antibodies. (C) Low levels of endogenous shrew-1 are found in MCF7 cells at AJ and it colocalizes with ß-catenin. Scale bar, 10 μm.

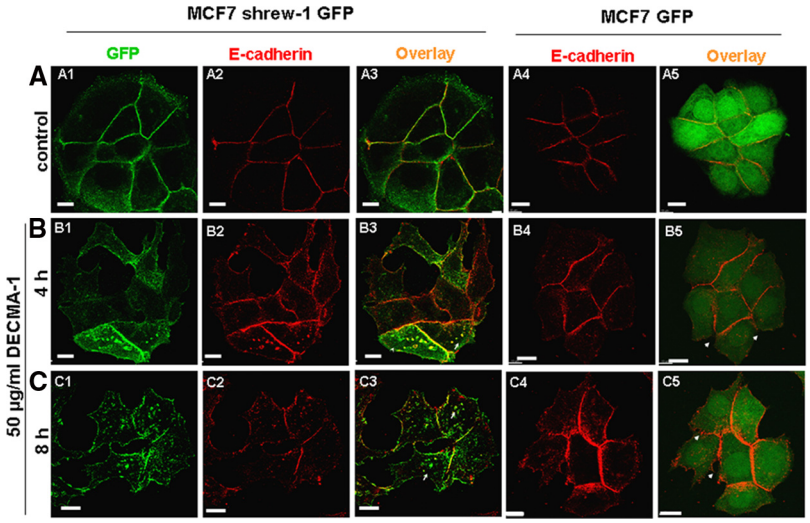

Figure 2. Direct disassembly of adherens junctions by E-cadherin function-blocking antibody DECMA-1 is enhanced by shrew-1-GFP. (A) MCF7 cells expressing shrew-1-GFP (A, 1–3) or GFP alone (A, 4 and 5) were starved overnight and stained for E-cadherin with 5H9 antibody. (B and C) Additionally cells were treated with 50 μg/ml DECMA-1 antibody for 4 h (B, 1–5) or 8 h (C, 1–5). Arrows show colocalization of internalized shrew-1-GFP and E-cadherin and arrow heads morphological signs of AJ disruption. Scale bar, 10 μm.

75 mM NaCl, 75 mM NaOH, and 1% BSA) at 0°C, as described (Le *et al.*, 1999). The effectiveness of glutathione stripping was demonstrated in cells, which were surface-biotinylated at 0°C for 30 min and then immediately subjected to glutathione stripping. Only the internalized biotinylated proteins, which were protected from glutathione stripping, could be precipitated with neutravidin beads and analyzed by immunoblotting.

Coimmunoprecipitation

Cells were washed twice with ice-cold PBS and lysed for 30 min at 4°C in a buffer containing 10 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 60 mM *n*-octyl-glucoside. Samples were precleared for 1 h at 4°C using protein G-Sepharose (20 μl, slurry 1:1; GE Healthcare, Freiburg, Germany) and subjected to immunoprecipitation overnight at 4°C using anti-HER2 IgG (rAb) followed by 2-h incubation with protein G-Sepharose (30 μl, slurry 1:1). After four washes with coimmunoprecipitation buffer, samples were separated by SDS-PAGE (8% acrylamide), transferred to nitrocellulose, and probed with appropriate antibodies.

Phospho-Immunoprecipitation

Cells were treated with freshly prepared 10 mM pervanadate (Sigma) for the time indicated before EGF stimulation for 10 min, washed in ice-cold PBS, and lysed in 0.1 ml of RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 0.25% SDS, and 0.1% Nonidet P-40) containing 100 mM pervanadate and 1/25 protein inhibitor cocktail Complete (Roche Diagnostics) for 10 min at 4°C. Two hundred micrograms protein of each lysate was used for precipitation (16 h at 4°C) with GFP antibody (Clontech) to precipitate shrew-1. The Western blots from the precipitates were probed with phosphotyrosine antibody (Cell Signaling Technology).

Wound-healing Assay

For the analysis of MCF7 cell migratory behavior, 1×10^6 cells were seeded in six-well plates and after forming a confluent monolayer, were serum-starved for 12–16 h. Then wounds were created using a 200-μl pipette tip, and medium was supplemented with 100 ng/ml EGF. Two parallel wounds were created in each well, and their location was marked on the bottom of the six-well plate; 12 wounds were created for each cell clone. Images were taken after 24 h. Covered wound closure in pixel was measured with Image J and statistically analyzed.

Statistical Analysis

The results are presented as SEMs of three experiments using MCF7 cells. Western blot intensities are presented as relative value to controls, with the control equal to 1. Data were analyzed by ImageJ (Scion) and the Student's *t* test performed at http://www.physics.csbsju.edu/stats/*t* test.html. The level of significance was $p < 0.05$.

RESULTS

Expression of Shrew-1 in Mammary Gland Sections and in Mammary Carcinoma Cells (MCF7)

Localization of endogenous shrew-1 was investigated in cultured mammary carcinoma cells and human mammary gland tissue sections by immunofluorescence microscopy. Endogenous shrew-1 colocalizes with E-cadherin at the plasma membrane and is restricted to E-cadherin junctions in lumenal mammary layer (Figure 1A). Similarly, the cell culture system for our experimental setup, MCF7 mammary carcinoma cells, showed a comparable staining pattern of endogenous shrew-1 (Figure 1B). In agreement with our

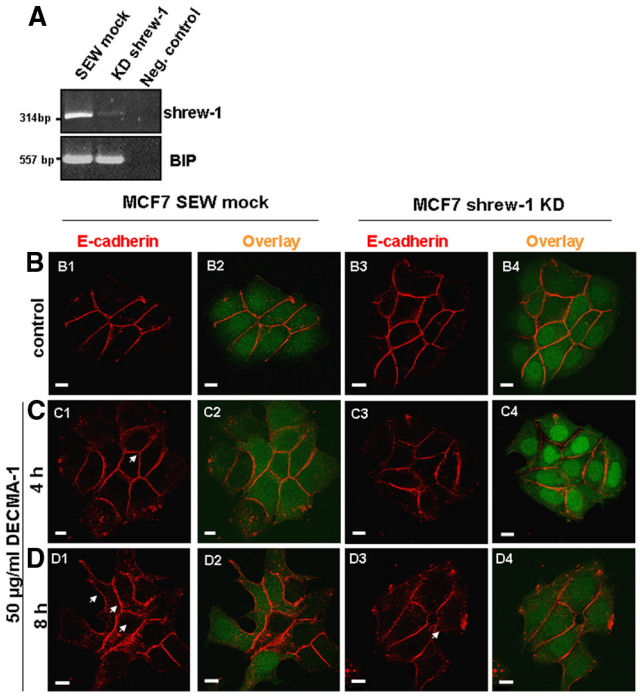

Figure 3. Direct disassembly of adherens junctions by E-cadherin function-blocking antibody DECMA-1 is decreased by knockdown of shrew-1. (A) Endogenous shrew-1 was knocked down by shRNA. Functional knockdown was observed by semiquantitative RT-PCR with BIP as a loading control. (B) MCF7 cells expressing the SEW mock control (B, 1 and 2) or shrew-1 KD (B, 3 and 4) were starved overnight and stained for E-cadherin with 5H9 antibody. (C and D) Additionally cells were treated with 50 μg/ml DECMA-1 antibody for 4 h (C, 1–4) or 8 h (D, 1–4). Arrowheads show morphological signs of AJ disruption. Scale bar, 10 μm.

previous data overexpression of shrew-1 in MCF7 cells did not result in an altered morphology and no change of E-cadherin was visible in localization or expression level, also implying that AJ in these cells were unchanged (Figure 2A, 1–3).

Shrew-1 Influences Anti-E-Cadherin Antibody-mediated Disruption of AJs

Because an increased shrew-1 expression level apparently did not influence cell morphology or the localization of E-cadherin, we raised the question of whether shrew-1 might play a role in AJ's dynamics, particularly by affecting E-cadherin. To address this, we aimed to disintegrate the AJs using E-cadherin function-blocking antibodies (mAb DECMA-1; Vestweber and Kemler 1985). When applied to the cell culture medium, DECMA-1 antibody binds to and keeps E-cadherin in a single-molecule state, so that E-cadherin–mediated contacts are disrupted within 4–8 h (Frixen and Nagamine, 1993; Nakagawa *et al.*, 2001; Kleiner *et al.*, 2007). This results in the scattering of various epithelial cells (Frixen and Nagamine, 1993; Nakagawa *et al.*, 2001; Kleiner *et al.*, 2007). We treated shrew-1–overexpressing cells as well as control cells expressing GFP alone with 50 μg/ml DECMA-1 antibody for 4 and 8 h. The cells were then analyzed by immunofluorescence staining of endogenous E-cadherin and shrew-1-GFP (Figure 2B, 1–5). After DECMA-1 treatment for 4 h the cells started to impair AJ integrity, which was reflected by detachment from each other, and the increased occurrence of E-cadherin in vesicular structures. This was much stronger in shrew-1–overexpressing cells than in GFP control cells. Furthermore, shrew-1-GFP also colocalized with E-cadherin in vesicles (Figure 2B, 1–3). These results show that shrew-1-GFP internalizes together with E-cadherin in vesicles and suggest that it enhances AJ dissolution. A prediction of this hypothesis is that shrew-1 reduction will have the opposite effect (Figure 2C, 1–3). To stably down-regulate shrew-1 in MCF7 cells, we used a nucleotide sequence against shrew-1 mRNA previously shown to be functional (Schreiner *et al.*, 2007). After lentiviral transduction of shrew-specific shRNA, RT-PCR analysis of the shrew-1 mRNA revealed prominent down-regulation in cells infected with shrew-1–specific shRNA compared with the mock control (Figure 3A). Infection efficiency, as determined by FACS analyses, was more than 80% in all cells (data not shown). Similar to shrew-1–overexpressing cells, knockdown of shrew-1 had no apparent morphological effect in unstimulated cells (Figure 3B, 1–4). However, in response to treatment with DECMA-1 knockdown of shrew-1 leads to the opposite effect on AJ disassembly compared with overexpression of shrew-1. The AJs of shrew-1 knockdown (KD) cells appeared less disturbed and the detached phenotype as well as vesicular E-cadherin staining observed in shrew-1–overexpressing cells was rarely seen in MCF7 shrew-1 KD cells (Figure 3C, 3 and 4, and D, 3 and 4). Immunofluorescence staining of mock-infected cells showed AJ detachment similar to the GFP-overexpressing control cells (Figure 3C, 1 and 2, and D, 1 and 2), which in turn resembles MCF7 wild-type cells.

To quantify shrew-1's dependence on DECMA-1 for disassembling AJ, shrew-1-GFP cells, GFP control cells, shrew-1 KD cells and mock-infected cells were treated with DECMA-1 and then stained for E-cadherin. Cell colonies of 6–15 cells from all four cell lines were scored into three categories: coherent, intermediate, or scattered (cells detach from each other; Figure 4A). As implied by E-cadherin staining in vesicular structures in these cells, the intermediate stage indicates the impairment of junctional integrity. About 50 colonies (>300 cells) per cell line were scored. After 4 h of DECMA-1 treatment, 74% of GFP and mock-infected cells and 77% of shrew-1 KD cell colonies still seem coherent, 23, 24, and 21% were in the intermediate stage and <3% were completely scattered (Figure 4B). In contrast, in shrew-1-GFP colonies only 27% of cells were coherent, whereas 59% were already in the intermediate category and 14% showed

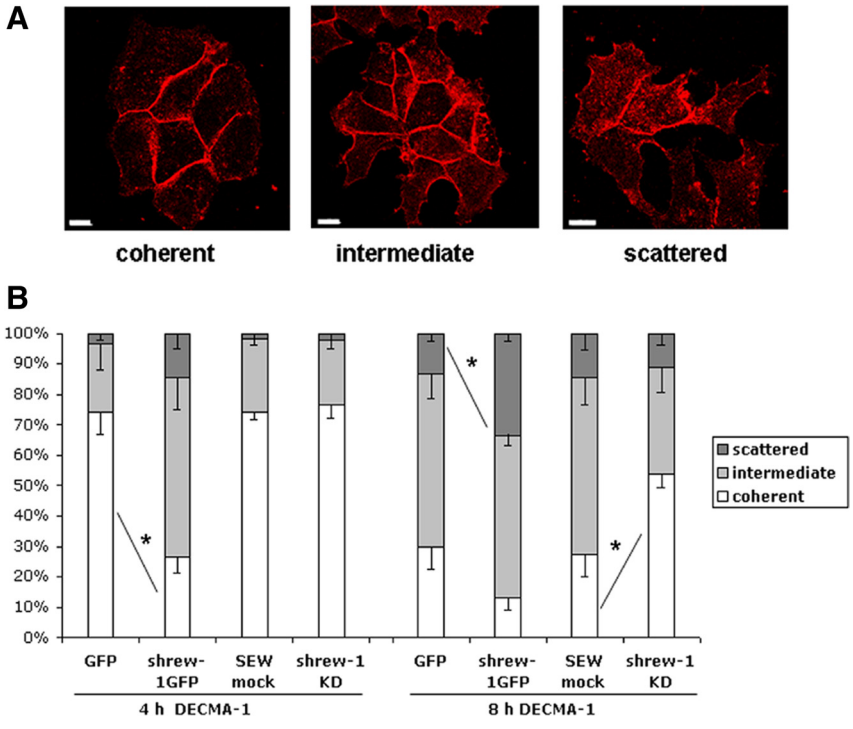

Figure 4. Quantification of shrew-1 influences on DECMA-1 induced disruption of AJs. Cells of all four cell lines treated with 50 μg/ml DECMA-1 antibody for 4 h or 8 h or left untreated were stained for E-cadherin. (A) Cell colonies (6–15 cells) were classified into three categories: colony coherent (white), intermediate (light gray), and scattered (dark gray). (B) Over 50 colonies (more than 300 cells) for each time point of three independent experiments were scored and statistically analyzed. Error bars, SEM. * $p < 0.01$. After 4 h shrew-1 overexpression facilitates AJ disassembly in contrast to control and shrew-1 KD cells, whereas knockdown slows down the disassembly process, but does not completely inhibit it.

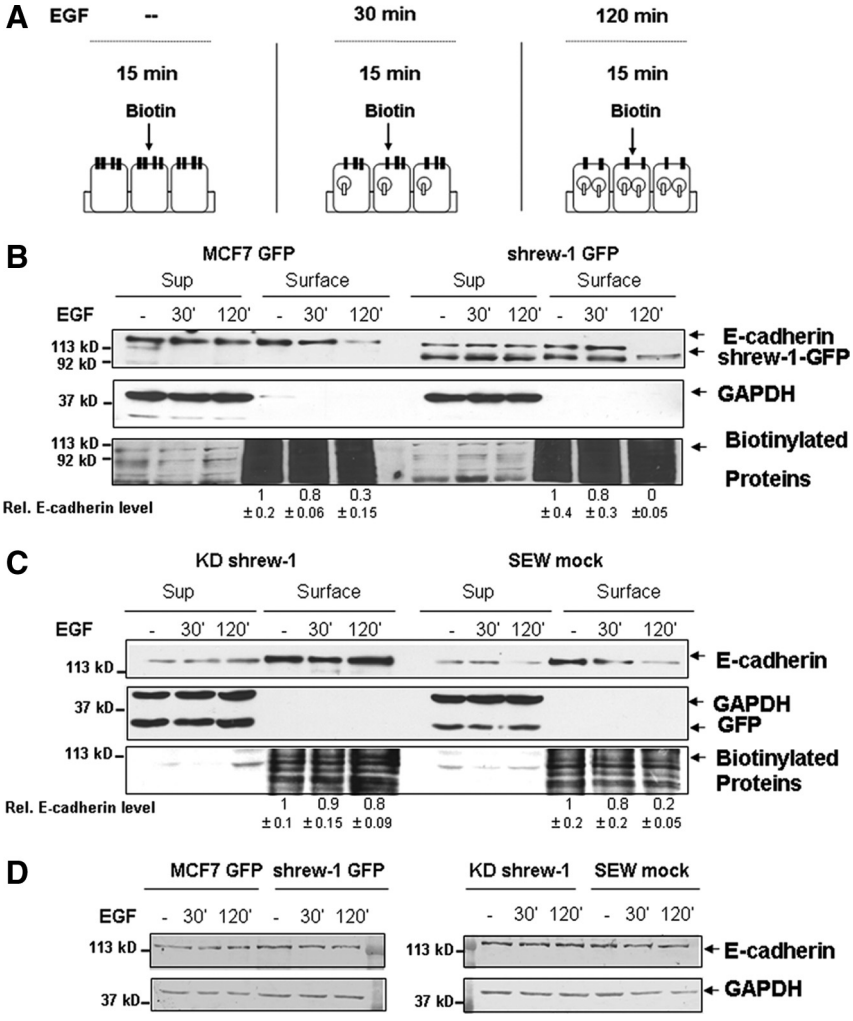

Figure 5. Shrew-1's effect on E-cadherin surface localization. (A) First cells were treated with EGF for 30 or 120 min to induce E-cadherin internalization or left unstimulated, and then cell surface proteins were biotinylated for 15 min on ice. Black bars represent biotinylated E-cadherin, white bars internalized/nonbiotinylated E-cadherin. (B) Biotinylated E-cadherin disappeared from the surface of shrew-1–overexpressing cells in a more pronounced way then control cells, seen after 120 min. (C) In contrast, shrew-1 KD cells did not show substantial loss of biotinylated E-cadherin at the cells' surface when compared with control cells. As a loading control we visualized all biotinylated proteins with HRP-streptavidin to show equal protein amounts in each sample. (D) It should be noted that the total amount E-cadherin did not change upon stimulation with EGF in either cell line. Loading of equal amounts was monitored by GAPDH antibody staining.

an almost fully scattered phenotype (Figure 4B). After 8 h, shrew-1 overexpression cells showed even more completely detached colonies (34%, shrew-GFP; compared to 15%, GFP/SEW mock; and 10%, shrew-1 KD).

After 8 h control cells exhibit a score distribution similar to shrew-1–overexpressing cells at 4 h (GFP/SEW mock: 30/27% coherent, 57/58% half-scattered and 13/15% scattered). In line with these observations, shrew-1 KD cells showed delayed impairment of junctional integrity (intermediate stage) as well as scattering compared with shrew-1–overexpressing and control cells after 8 h of treatment (54% coherent, 35% intermediate, and 11% scattered; Figure 4B).

All in all, shrew-1 overexpression facilitates impairment of AJ integrity, whereas knockdown slows down this process. Thus, it seems that disturbance and subsequent internalization of E-cadherin induced by DECMA-1 implies a modulatory role for shrew-1 in the dynamics of AJs.

Shrew-1 Modulates EGF-dependant E-Cadherin Internalization

So far our data suggest a modulatory role of shrew-1 in antibody-mediated E-cadherin internalization. This raises the important question of whether shrew-1 exhibits a similar role if E-cadherin internalization is triggered by a growth factor stimulus, as happens, for example, during tissue reorganization or tumor development. A prominent molecule in this context is EGF and its receptor family, which play a central role especially in breast carcinoma and are therapeutic antibody targets (Drebin *et al.*, 1988; Shepard *et al.*, 1991). The importance of EGF and its receptor in transient tissue reorganisation and tumor progression has been demonstrated extensively (Holbro *et al.*, 2003a,b; Lu *et al.*, 2003; Lo *et al.*, 2007). In cell culture EGF temporally disrupts AJs and leads to internalization of E-cadherin and stimulation of ERK1/2 signaling (Bryant *et al.*, 2007; Kleiner *et al.*, 2007).

To test if shrew-1 has indeed an effect on E-cadherin internalization in this context, we performed surface biotinylation experiments. For this we used the four different MCF7 cell strains: GFP, shrew-1-GFP-overexpressing, shrew-1 KD, and mock-infected cells. After stimulation with EGF for 30 or 120 min, or unstimulated cells as a control, cell surface proteins were biotinylated for 15 min on ice (Figure 5A). Using this experimental setup, we were able to show that E-cadherin disappeared from the surface of MCF7 cells. This effect was more pronounced in those cells overexpressing shrew-1 compared with GFP control cells, as best seen after 120 min (Figure 5B). Consistent with this observation, shrew-1 KD cells did not show substantial loss of E-cadherin at the cell surface compared with control cells at both time points (Figure 5, B and C). These data fit well with the effect of shrew-1 on AJ disruption by DECMA-1 antibody (Figure 4B). As a loading control, all biotinylated proteins were

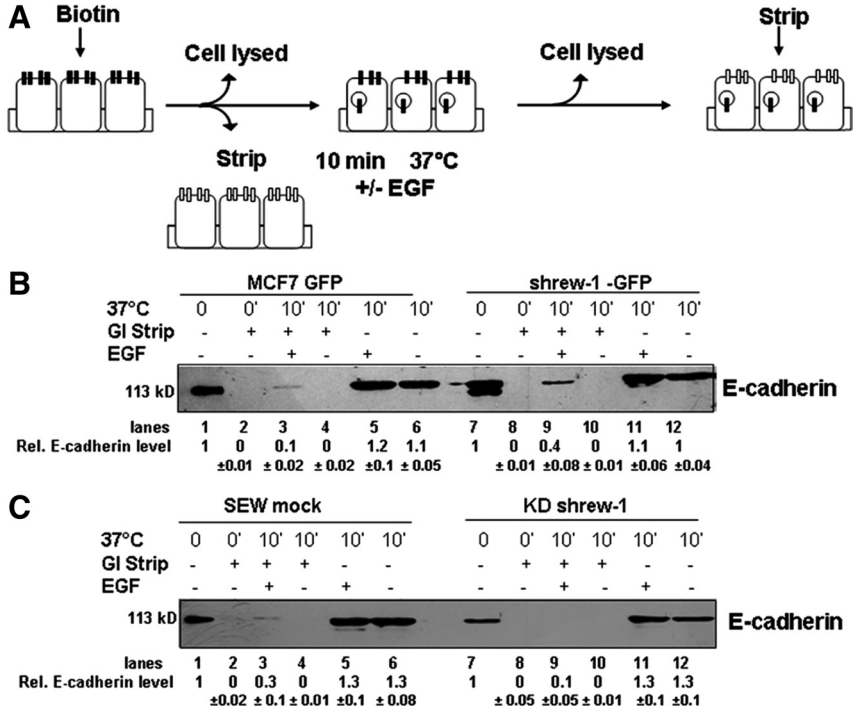

Figure 6. Shrew-1's effect on E-cadherin internalization. (A) First cells were biotinylated for 30 min on ice. Then cells were either lysed directly, or subjected to glutathione stripping or transferred to 37°C with or without EGF for 10 min to induce E-cadherin internalization, followed by glutathione stripping. Black bars represent biotinylated E-cadherin and white bars nonbiotinylated/stripped E-cadherin. (B) Lanes 1 and 7, total level of biotinylated E-cadherin; lanes 2 and 8, stripping efficiency control; lanes 3 and 9, EGF-induced internalized E-cadherin; lanes 4 and 10, constitutive internalized E-cadherin; lanes 5, 11 and 6, 12, additional controls showing total level of biotinylated E-cadherin at 37°C after 10 min with or without EGF. More biotinylated E-cadherin was precipitated from shrew-1–overexpressing cells then control cells, after 10 min of EGF treatment. (C) Endocytosis experiment with shrew-1 KD and mock control cells; lanes are as described in B. Shrew-1 KD cells do not show internalization of biotinylated E-cadherin after 10 min of EGF treatment. Western blots were probed with monoclonal E-cadherin antibodies.

visualized with HRP-streptavidin to indicate equal protein loading (Figure 5, B and C). Of note, the total amount of E-cadherin remained unchanged after EGF stimulation in all cell lines (Figure 5D).

To specifically address the question if shrew-1 influences the EGF-dependent endocytosis of E-cadherin, we performed an endocytosis assay. For this surface proteins were labeled with a cleavable EZ-Link Sulfo-SS-Biotin for 30 min. Subsequently cells were either lysed directly to visualize biotinylated surface proteins at time point 0 or immediately subjected to glutathione stripping to reverse biotinylation. Alternatively, cells were incubated in medium with or without EGF at 37°C for 10 min and subjected to stripping afterward. During this procedure internalized biotinylated proteins were protected from glutathione stripping (Figure 6A). After 10 min of EGF treatment an increased amount of biotinylated E-cadherin accumulated in the shrew-1-GFP–overexpressing cells and less in KD cells compared with control cells (Figure 6, B and C, lanes 3 and 9). The results of this experiment support the interpretation of the previous assay and indicate that shrew-1 overexpression enhances EGF-mediated internalization of E-cadherin, whereas knockdown decreases the amount of internal E-cadherin. Incubation without EGF at 37°C showed no detectable internalization of E-cadherin and excludes the idea that shrew-1 could also influence constitutive endocytosis (Figure 6, B and C, lanes 4 and 10).

To discriminate whether shrew-1 influences only E-cadherin internalization or has also some enhancing effect on its degradation, we performed the following experiment. MCF7 shrew-1-GFP, GFP, shrew-1 KD, and mock-infected cells were surface-biotinylated on ice at time point 0, transferred to 37°C, and stimulated with EGF. Cells were either harvested directly or 4 and 18 h after stimulation. Biotinylated proteins were precipitated with neutravidin-beads and analyzed by Western blots using E-cadherin antibodies. If shrew-1 overexpression affects the degradation of E-cadherin a substantial reduction of biotinylated E-cadherin could be expected after 4 h in comparison to control cells, because the reported half-life of E-cadherin in untreated cells has been shown to be ~5 h (Stewart *et al.*, 2000). As can be seen in Figure 7, in all MCF7 cell lines tested the level of E-cadherin protein was not influenced by either overexpression or knockdown of shrew-1 in comparison to control cells. As expected according to the half-life of E-cadherin substantial reduction of biotinylated E-cadherin was visible after 18 h (Figure 7, B and C). Overall, these data implicate that shrew-1 does not influence the turnover of E-cadherin but its internalization upon stimulation with EGF.

Shrew-1 Facilitates Integration of E-Cadherin into EGFR Signaling Complexes

So far, different factors have been identified that influence AJ proteins by activation of EGFRs. One of the major kinases shown to play a role in EGF-mediated AJ disruption is tyrosine kinase Src (Shen *et al.*, 2008). Thus we speculated that shrew-1 and Src may contribute to the same signaling network. Good evidence for such a speculation would be the EGF-dependent coprecipitation of HER2 (the predominant EGFR in MCF7 cells) together with E-cadherin, Src, and shrew-1. To investigate this, anti-HER2 antibodies were used for immunoprecipitation experiments (Figure 8 A). The precipitates obtained were analyzed by Western blotting, for the presence of E-cadherin, shrew-1-GFP, and Src. As expected in MCF7 GFP cells (control) E-cadherin, HER2, and Src were present in a complex 10 min after EGF stimulation. This is in agreement with a recent report showing that in unstimulated cells E-cadherin does not interact with HER2 (Najy *et al.*, 2008). In shrew-1–overexpressing cells, however, this complex was already detectable in unstimulated cells and, in addition, contained shrew-1 itself (Figure 8A). The HER2/E-cadherin/Src/shrew-1 complex remained in EGF-stimulated cells for 10 min. Similarly to control cells shrew-1 KD cells did not show preformation of the complex which, however, was visible after stimulation with EGF (data not shown). This implies that shrew-1 might modulate the cel-

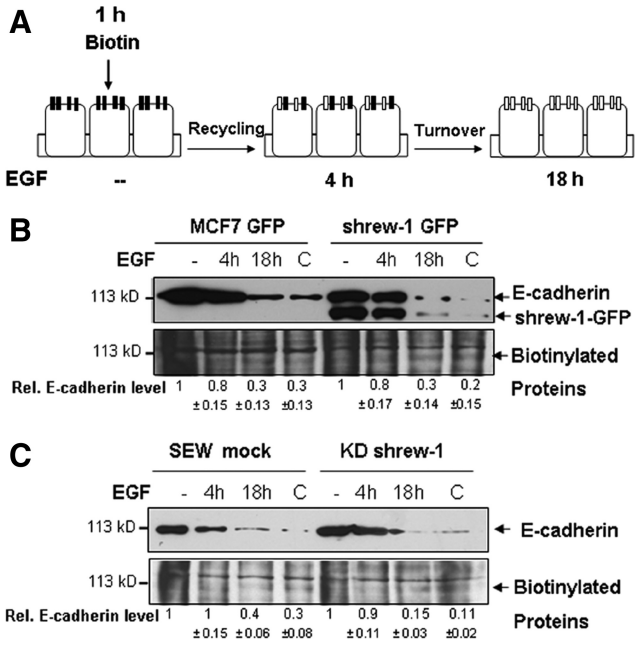

Figure 7. Shrew-1 does not influence the degradation of E-cadherin. (A) Cells were surface biotinylated for 1 h on ice and subsequently either treated with EGF for 4 h or 18 h or were left untreated, and then biotinylated proteins were isolated and analyzed for E-cadherin by Western blots. Black bars represent biotinylated E-cadherin and white bars newly emerged E-cadherin. (B and C) No significant differences between the MCF7 variants at 4 h and 18 h with regard to shrew-1 could be observed, implicating that shrew-1 does not influence degradation of E-cadherin. As a loading control all biotinylated proteins were visualized with HRP-streptavidin to show equal protein amounts in each sample.

lular response to EGF by facilitating complex preformation of E-cadherin/HER2/Src.

Therefore, if shrew-1 is able to mediate the formation of HER2/E-cadherin/Src complexes, this raises the question whether shrew-1 itself is phosphorylated in a Src-dependent manner upon EGF stimulation or DECMA-1 treatment.

To test this MCF7 cells expressing shrew-1-GFP were treated with the Src-specific inhibitor PP2 or the inactive control compound PP3 (5 μM) for 20 min and with vanadate for 5 min before stimulation with either EGF or DECMA-1 for 10 min. Inhibition of Src kinases with the inhibitor PP2 abrogated tyrosine phosphorylation of shrew-1-GFP in EGF and in DECMA-1 treated cells, whereas the inactive analog PP3 still allowed phosphorylation of shrew-1 (Figure 8C).

Another important downstream signaling component of the EGFR signaling pathway is activated ERK1/2 (Hynes and Macdonald 2009), which is also induced by DECMA-1 (Kleiner *et al.*, 2007). Therefore, we asked the question of whether overexpression of shrew-1 has an effect on phosphorylation of ERK1/2, reflecting its activation. As shown in Figure 8B enhanced ERK1/2 phosphorylation was transiently observed after 5 min of EGF stimulation compared with GFP control cells. Furthermore, shrew-1 overexpression also increases ERK1/2 phosphorylation in a DECMA-1–dependent manner but with a different time course. Noteworthy activated ERK1/2 was quantitatively indistinguishable in shrew-1 KD and control cells (data not shown).

Shrew-1 Influences Cell Migration in a Wound-healing Assay

It has been reported frequently that wound healing of epithelial cell sheets after injury requires activation of EGFR (Frey *et al.*, 2004; Wells *et al.*, 2006). Given this fact, EGFR activation should induce E-cadherin dynamics leading to internalization, which, according to our findings would be modulated by shrew-1.

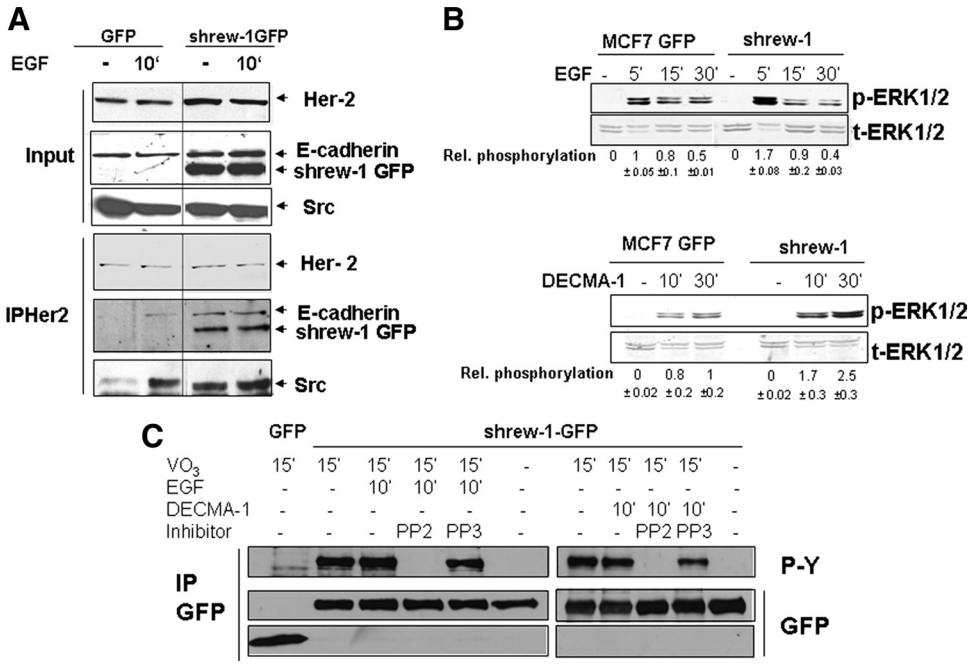

Figure 8. Shrew-1 facilitates integration of E-cadherin into RTK signaling complexes. (A) Antibodies against HER2 were used for coimmunoprecipitation experiments analyzed in Western blots using antibodies against E-cadherin, GFP (to identify shrew-1-GFP) and against Src. E-cadherin, HER2, shrew-1-GFP, and Src occurred in one complex after 10 min of EGF stimulation, but could already be detected in unstimulated cells. (B) ERK1/2 activation was analyzed by phospho-specific ERK1/2 antibodies and loading of equal amounts by total ERK1/2 antibodies in shrew-1-GFP–overexpressing cells and GFP control cells in dependence of EGF or DECMA-1. (C) EGF- and DECMA-1–dependent tyrosine phosphorylation of shrew-1 was analyzed by immunoprecipitation in the presence of vanadate and Src inhibitors. Inhibitors were applied in a final concentration of 5 μM for 20 min before stimulation. Cells were pretreated with vanadate for 5 min before DECMA-1 or EGF treatment for 10 min. Shrew-1 was tyrosine phosphorylated in a Src dependent manner, both, after treatment with EGF and DECMA-1.

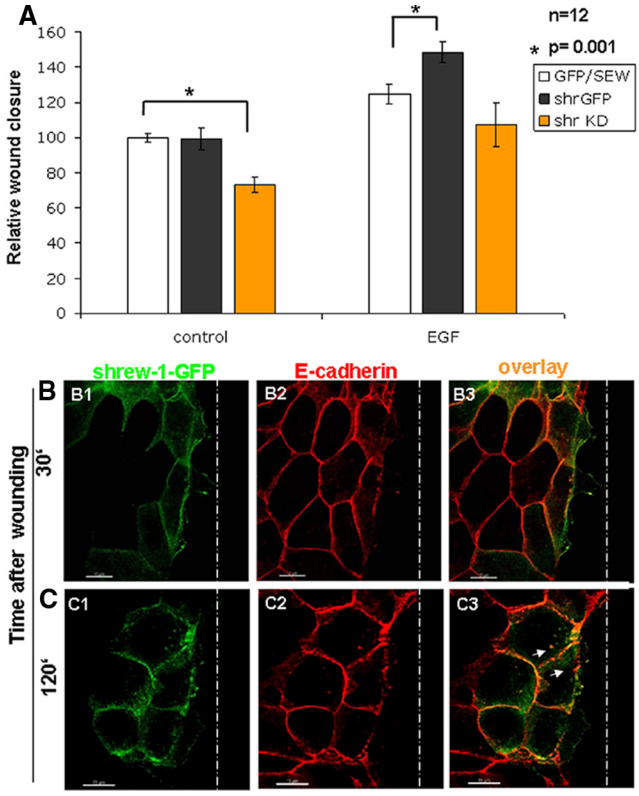

Figure 9. (A) Migration of shrew-1-GFP–overexpressing cells, shrew-1 KD, and GFP/mock control cells was observed in an in vitro wound-healing assay in the presence or absence of EGF 24 h after wounding. Twelve different wounds per cell line and condition were analyzed statistically. Error bars, SEM. (B) Layers of MCF7 cells expressing shrew-1-GFP were starved overnight, scratch-wounded, and stained for E-cadherin with 5H9 antibody after 30 or 120 min (B and C). Arrows show colocalization of internalized shrew-1-GFP and E-cadherin at wound edges. Scale bar, 15 μm.

If so, it could be anticipated that overexpression and knockdown of shrew-1 in MCF7 cells might affect wound healing if these cells are grown to sheets and scratched. Indeed, the results of our wound healing assays are in line with this hypothesis, i.e., that overexpression of shrew-1 accelerated the process. Migration of MCF7 shrew-1–overexpressing cells was significantly higher after 24 h than in GFP control cells in the presence of 100 ng/ml EGF, whereas in shrew-1 KD cells migration was moderately reduced (Figure 9A). In line with our previous results immunofluorescence staining of shrew-1-GFP cells 30 and 120 min after wounding showed vesicular staining for E-cadherin and shrew-1 at wound edges after 120 min (Figure 9C, 1–3).

DISCUSSION

In general, fine tuning of intercellular adhesion by removing E-cadherin from the cell surface is a basic feature of epithelial cells to alter parameters such as motility and invasiveness in physiological situations during development and in adult organisms (Thiery and Chopin, 1999; Gumbiner, 2005), but also occurs aberrantly in pathophysiology such as carcinoma development (Bryant and Stow, 2004). Our findings are highly relevant for these issues because they show that AJ-associated protein shrew-1 acts as a novel modulator of E-cadherin internalization during epithelial cell dynamics induced by either blocking E-cadherin directly with DECMA-1 antibody or EGF-mediated activation of HER2 RTK. In line with our results, previous reports have shown that internalization of E-cadherin and loss of epithelial morphology can indeed be induced by DECMA-1 and EGF (Kleiner *et al.*, 2007).

One of the challenges presented by our previous data were the apparent contradiction that shrew-1 influenced the invasive capacities of cells in culture (Schreiner *et al.*, 2007), yet was found in AJs in polarized epithelial cells (Bharti *et al.*, 2004). The observations of this article close the gap, because they imply that shrew-1 acts as a switch protein in epithelial cell dynamics. In the course of these processes E-cadherin–mediated AJs are disrupted, and E-cadherin is endocytosed, thus reducing its concentration in the plasma membrane. Here, shrew-1 is obviously necessary in MCF7 breast carcinoma cells, independent of whether E-cadherin internalization is induced by E-cadherin-blocking antibody DECMA-1 or by EGF.

These two stimuli both reflect situations in vivo, which finally lead to reducing intercellular adhesion and altering properties of epithelial cells. Treatment of MCF7 cells with DECMA-1 simulates blocking E-cadherin function (Vestweber and Kemler, 1985; Ozawa *et al.*, 1990; Nakagawa *et al.*, 2001) as has been described for soluble E-cadherin fragments in cell culture and that is postulated to occur in vivo during cancer development (Wheelock *et al.*, 1987; De Wever *et al.*, 2007). Furthermore, aberrant activation of HER2 by EGF has been found in a number of different carcinomas (Wheelock *et al.*, 1987; De Wever *et al.*, 2007). In nonpathological situations EGF-mediated reduction of intercellular adhesion is also critical, for example, in developmental processes (for a review see Tepass *et al.*, 2002). Based on published findings and those in this article, it is therefore reasonable to assume a critical role for shrew-1 in the dynamics of AJs' disassembly/assembly. By modulating the amount of E-cadherin on the cell surface, shrew-1 might promote processes such as motility of epithelial cells and also morphogenesis of epithelial tissues (unpublished observations).

Notably, shrew-1 appears to be internalized into vesicles together with E-cadherin. Provided that E-cadherin internalization indeed reflects a balance between signals that promote endocytosis and those that inhibit it, as proposed recently (Yap *et al.*, 2007), shrew-1 protein appears to be a regulator/modulator involved here. In line with such a hypothesis is the observation that the degree of EGF-mediated endocytosis from the plasma membrane apparently depends on the amount of shrew-1 in MCF7 cells. Similarly, endocytosis of E-cadherin induced by DECMA-1 is postponed when endogenous shrew-1 is down-regulated and accelerated in cells overexpressing it.

A critical step in the signaling triggered by activated EGFR/HER2 that leads to internalization of E-cadherin is the formation and activation of a signaling complex in which Src is a key component (Shen *et al.*, 2008). This complex is normally only detectable in stimulated MCF7 cells, but not before stimulation (Najy *et al.*, 2008). However, if shrew-1 is overexpressed, one can observe preformation of a shrew-1–positive complex containing E-cadherin and Src. This preformed complex by itself obviously does not lead to AJ disassembly and E-cadherin internalization, but requires a specific signal such as EGF. This subsequently leads to activation of Src kinase (Brunton *et al.*, 2004; Shen *et al.*, 2008) and finally enhanced motility of those cells. Quite likely, the recruitment and activation of Src into the E-cadherin/shrew-1 complex is critical for shrew-1 function, because shrew-1 phosphorylation de-

pends on Src (Figure 8C) and numerous previous reports have shown that Src activation is essential for the disruption of cell–cell adhesion (Frame, 2004).

In conclusion, we propose a model where shrew-1 chaperones E-cadherin and Src into RTK signaling platforms and further downstream compartments, leading to the internalization of E-cadherin and cell migration. Consistently, shrew-1–overexpressing cells respond faster and thus more efficiently to EGF stimulation. Further studies will be necessary to elucidate fully how engagement of shrew-1 results in the modulation of E-cadherin and signaling pathways downstream of cell adhesion–growth factor receptor complexes in normal epithelial cell–cell dynamics. Finally in cancer cells deregulated shrew-1, in concert with hyperactive RTKs, e.g., EGF receptor, might promote progression of malignancy.

ACKNOWLEDGMENTS

We thank Avril Arthur-Göttig for critical reading of the manuscript and Monika Kamprad for expert technical assistance. This work was supported through funding by Deutsche Forschungsgemeinschaft (SFB 628; STA 187/16-1) and fellowships to J.G. by Center of Membrane Proteomics (Goethe University) and Evangelisches Studienwerk Villigst e.V.

REFERENCES

Akhtar, N., and Hotchin, N. A. (2001). RAC1 regulates adherens junctions through endocytosis of E-cadherin. Mol. Biol. Cell 12, 847–862.

Al Moustafa, A. E., Yen, L., Benlimame, N., and Alaoui-Jamali, M. A. (2002). Regulation of E-cadherin/catenin complex patterns by epidermal growth factor receptor modulation in human lung cancer cells. Lung Cancer *37*, 49–56.

Behrens, J., Vakaet, L., Friis, R., Winterhager, E., Van Roy, F., Mareel, M. M., and Birchmeier, W. (1993). Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/beta-catenin complex in cells transformed with a temperature-sensitive v-SRC gene. J. Cell Biol. *120*, 757–766.

Bharti, S., Handrow-Metzmacher, H., Zickenheiner, S., Zeitvogel, A., Baumann, R., and Starzinski-Powitz, A. (2004). Novel membrane protein shrew-1 targets to cadherin-mediated junctions in polarized epithelial cells. Mol. Biol. Cell *15*, 397–406.

Boyer, B., Valles, A. M., and Edme, N. (2000). Induction and regulation of epithelial-mesenchymal transitions. Biochem. Pharmacol. *60*, 1091–1099.

Brunton, V. G., MacPherson, I. R., and Frame, M. C. (2004). Cell adhesion receptors, tyrosine kinases and actin modulators: a complex three-way circuitry. Biochim. Biophys. Acta *1692*, 121–144.

Bryant, D. M., Kerr, M. C., Hammond, L. A., Joseph, S. R., Mostov, K. E., Teasdale, R. D., and Stow, J. L. (2007). EGF induces macropinocytosis and SNX1-modulated recycling of E-cadherin. J. Cell Sci. *120*, 1818–1828.

Bryant, D. M., and Stow, J. L. (2004). The ins and outs of E-cadherin trafficking. Trends Cell Biol. *14*, 427–434.

De Wever, O., Derycke, L., Hendrix, A., De Meerleer, G., Godeau, F., Depypere, H., and Bracke, M. (2007). Soluble cadherins as cancer biomarkers. Clin. Exp. Metastasis *24*, 685–697.

Drebin, J. A., Link, V. C., and Greene, M. I. (1988). Monoclonal antibodies specific for the neu oncogene product directly mediate anti-tumor effects in vivo. Oncogene *2*, 387–394.

Frame, M. C. (2004). Newest findings on the oldest oncogene; how activated src does it. J. Cell Sci. *117*, 989–998.

Francavilla, C., Loeffler, S., Piccini, D., Kren, A., Christofori, G., and Cavallaro, U. (2007). Neural cell adhesion molecule regulates the cellular response to fibroblast growth factor. J. Cell Sci. *120*, 4388–4394.

Frey, M. R., Golovin, A., and Polk, D. B. (2004). Epidermal growth factor-stimulated intestinal epithelial cell migration requires Src family kinase-dependent p38 MAPK signaling. J. Biol. Chem. *279*, 44513–44521.

Frixen, U. H., and Nagamine, Y. (1993). Stimulation of urokinase-type plasminogen activator expression by blockage of E-cadherin-dependent cell-cell adhesion. Cancer Res. *53*, 3618–3623.

Gumbiner, B. M. (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell *84*, 345–357.

Gumbiner, B. M. (2005). Regulation of cadherin-mediated adhesion in morphogenesis. Nat. Rev. Mol. Cell Biol. *6*, 622–634.

Holbro, T., Beerli, R. R., Maurer, F., Koziczac, M., Barbas, C. F., 3rd, and Hynes, N. E. (2003a). The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. Proc. Natl. Acad. Sci. USA *100*, 8933–8938.

Holbro, T., Civenni, G., and Hynes, N. E. (2003b). The ErbB receptors and their role in cancer progression. Exp. Cell Res. *284*, 99–110.

Hynes, N. E. and Macdonald, G. (2009). ErbB receptors and signaling pathways in cancer. Curr. Opin. Cell Biol. (*in press*).

Jakob, V., Schreiner, A., Tikkanen, R., and Starzinski-Powitz, A. (2006). Targeting of transmembrane protein shrew-1 to adherens junctions is controlled by cytoplasmic sorting motifs. Mol. Biol. Cell *17*, 3397–3408.

Jamora, C., and Fuchs, E. (2002). Intercellular adhesion, signalling and the cytoskeleton. Nat. Cell Biol. *4*, E101–E108.

Janda, E., Nevolo, M., Lehmann, K., Downward, J., Beug, H., and Grieco, M. (2006). Raf plus TGFbeta-dependent EMT is initiated by endocytosis and lysosomal degradation of E-cadherin. Oncogene *25*, 7117–7130.

Kanekura, T., Chen, X., and Kanzaki, T. (2002). Basigin (CD147) is expressed on melanoma cells and induces tumor cell invasion by stimulating production of matrix metalloproteinases by fibroblasts. Int. J. Cancer *99*, 520–528.

Kleiner, S., Faisal, A., and Nagamine, Y. (2007). Induction of uPA gene expression by the blockage of E-cadherin via Src- and Shc-dependent Erk signaling. FEBS J. *274*, 227–240.

Le, T. L., Yap, A. S., and Stow, J. L. (1999). Recycling of E-cadherin: a potential mechanism for regulating cadherin dynamics. J. Cell Biol. *146*, 219–232.

Lecuit, T. (2005). Adhesion remodeling underlying tissue morphogenesis. Trends Cell Biol. *15*, 34–42.

Lim, M., Martinez, T., Jablons, D., Cameron, R., Guo, H., Toole, B., Li, J. D., and Basbaum, C. (1998). Tumor-derived EMMPRIN (extracellular matrix metalloproteinase inducer) stimulates collagenase transcription through MAPK p38. FEBS Lett. *441*, 88–92.

Lo, H. W., *et al.* (2007). Epidermal growth factor receptor cooperates with signal transducer and activator of transcription 3 to induce epithelial-mesenchymal transition in cancer cells via up-regulation of TWIST gene expression. Cancer Res. *67*, 9066–9076.

Lu, Z., Ghosh, S., Wang, Z., and Hunter, T. (2003). Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of beta-catenin, and enhanced tumor cell invasion. Cancer Cell *4*, 499–515.

McLachlan, R. W., Kraemer, A., Helwani, F. M., Kovacs, E. M., and Yap, A. S. (2007). E-cadherin adhesion activates c-Src signaling at cell-cell contacts. Mol. Biol. Cell *18*, 3214–3223.

Najy, A. J., Day, K. C., and Day, M. L. (2008). The ectodomain shedding of E-cadherin by ADAM15 supports ErbB receptor activation. J. Biol. Chem. *283*, 18393–18401.

Nakagawa, M., *et al.* (2001). Recruitment and activation of Rac1 by the formation of E-cadherin-mediated cell-cell adhesion sites. J. Cell Sci. *114*, 1829–1838.

Ozawa, M., *et al.* (1990). A possible new adhesive site in the cell-adhesion molecule uvomorulin. Mech. Dev. *33*, 49–56.

Palacios, F., Schweitzer, J. K., Boshans, R. L., and D'Souza-Schorey, C. (2002). ARF6-GTP recruits Nm23–H1 to facilitate dynamin-mediated endocytosis during adherens junctions disassembly. Nat. Cell Biol. *4*, 929–936.

Paterson, A. D., Parton, R. G., Ferguson, C., Stow, J. L., and Yap, A. S. (2003). Characterization of E-cadherin endocytosis in isolated MCF-7 and chinese hamster ovary cells: the initial fate of unbound E-cadherin. J. Biol. Chem. *278*, 21050–21057.

Reshetnikova, G., *et al.* (2007). Definition of a direct extracellular interaction between Met and E-cadherin. Cell Biol. Int. *31*, 366–373.

Schreiner, A., *et al.* (2007). Junction protein shrew-1 influences cell invasion and interacts with invasion-promoting protein CD147. Mol. Biol. Cell *18*, 1272–1281.

Shen, Y., Hirsch, D. S., Sasiela, C. A., and Wu, W. J. (2008). Cdc42 regulates E-cadherin ubiquitination and degradation through an epidermal growth factor receptor to Src-mediated pathway. J. Biol. Chem. *283*, 5127–5137.

Shepard, H. M., *et al.* (1991). Monoclonal antibody therapy of human cancer: taking the HER2 protooncogene to the clinic. J. Clin. Immunol. *11*, 117–127.

Stewart, D. B., Barth, A. I., and Nelson, W. J. (2000). Differential regulation of endogenous cadherin expression in Madin-Darby canine kidney cells by

cell-cell adhesion and activation of beta -catenin signaling. J. Biol. Chem. *275*, 20707–20716.

Takeichi, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. Science *251*, 1451–1455.

Tepass, U., Godt, D., and Winklbaur, R. (2002). Cell sorting in animal development: signalling and adhesive mechanisms in the formation of tissue boundaries. Curr. Opin. Genet. Dev. *12*, 572–582.

Thiery, J. P., and Chopin, D. (1999). Epithelial cell plasticity in development and tumor progression. Cancer Metastasis Rev. *18*, 31–42.

Thomas, S. M., and Brugge, J. S. (1997). Cellular functions regulated by Src family kinases. Annu. Rev. Cell Dev. Biol. *13*, 513–609.

Vestweber, D., and Kemler, R. (1985). Identification of a putative cell adhesion domain of uvomorulin. EMBO J. *4*, 3393–3398.

Wells, A., *et al.* (2006). Motility signaled from the EGF receptor and related systems. Methods Mol. Biol. *327*, 159–177.

Wheelock, M. J., Buck, C. A., Bechtol, K. B., and Damsky, C. H. (1987). Soluble 80-kd fragment of cell-CAM 120/80 disrupts cell-cell adhesion. J. Cell Biochem. *34*, 187–202.

Yap, A. S., Crampton, M. S., and Hardin, J. (2007). Making and breaking contacts: the cellular biology of cadherin regulation. Curr. Opin. Cell Biol. *19*, 508–514.