# EGF induces macropinocytosis and SNX1-modulated recycling of E-cadherin

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*Accepted 5 April 2007*
*Journal of Cell Science 120, 1818-1828 Published by The Company of Biologists 2007*
*doi:10.1242/jcs.000653*

## Summary

**In epithelia, junction proteins are endocytosed for modulation of cell-cell adhesion and cell polarity. In response to growth factors, the cell-cell adhesion protein E-cadherin is internalized from the cell surface with degradation or recycling as potential fates. However, the cellular machinery involved in cadherin internalization and recycling remains controversial. Here we investigated EGF-induced E-cadherin internalization. EGF stimulation of MCF-7 cells resulted in Rac1-modulated macropinocytosis of the E-cadherin-catenin complex into endosomal compartments that colocalized with EEA1 and the sorting nexin, SNX1. Depletion of cellular SNX1 levels by siRNA resulted in increased intracellular accumulation and turnover of E-cadherin internalized from the cell surface in response to EGF. Moreover, SNX1 was also required for efficient recycling of internalized E-cadherin and re-establishment of epithelial adhesion. Together, these findings demonstrate a role for SNX1 in retrieval of E-cadherin from a degradative endosomal pathway and in membrane trafficking pathways that regulate E-cadherin recycling.**

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/10/1818/DC1

Key words: Sorting Nexin, EGF, Cadherin, Macropinocytosis, Recycling, Cell-cell adhesion

## Introduction

For epithelial cells in sheets or tubes, alteration of stable cell-cell adhesion facilitates morphological remodeling events, such as migration during development or in metastasis during cancer (Gumbiner, 2005; Thiery, 2002). Internalization of junction proteins is one mechanism for temporary or permanent modulation of cell-cell adhesion (reviewed in Bryant and Stow, 2004; D'Souza-Schorey, 2005; Ivanov et al., 2005). A number of cell-cell junction proteins have been demonstrated to internalize in response to various physiological stimuli. For instance, autoantibodies against desmoglein-3 generated in pemphigus disorders induce desmosome disassembly and internalization in epidermal cells (Calkins et al., 2006), whereas IFNγ stimulation of intestinal epithelial sheets increases barrier permeability by temporarily internalizing and recycling tight-junction proteins, such as JAM-A and occludin (Bruewer et al., 2005; Utech et al., 2005). In canine kidney epithelial cells, the adhesion protein E-cadherin undergoes constitutive endocytosis and recycling, and it is also internalized in response to hepatocyte growth factor (HGF) for modulation of cell-cell adhesion (Fujita et al., 2002; Le et al., 1999).

E-cadherin is the prototypical epithelial cadherin and, at steady-state, it is localized to the lateral surface of polarized epithelia, where homodimers interact in trans with E-cadherin dimers on apposing cell membranes (reviewed in Yap et al., 1997). The cytoplasmic domain of E-cadherin supports interactions with multiple binding partners, most notably the catenin proteins, β-catenin or γ-catenin (plakoglobin), and p120ctn (Yap, 1998). Through the interaction of β-catenin with α-catenin, E-cadherin dynamically associates with cortical actin, although the molecular details of this interaction have recently become controversial (Drees et al., 2005; Yamada et al., 2005). In mammalian cells, binding of p120ctn to the juxtamembrane cytoplasmic region of E-cadherin delimits cadherin endocytosis and turnover, thereby stabilizing cadherin-based adhesion (Davis et al., 2003; Xiao et al., 2003; Yap et al., 1998).

We have previously shown that one mechanism to regulate E-cadherin function at cell-cell contacts is to internalize this adhesion receptor, with the potential for its recycling to the cell surface (Le et al., 2002; Le et al., 1999). FGF stimulation of mammary epithelial cells (MCF-7 cells) causes E-cadherin endocytosis as a precursor to subsequent disruption of cell-cell adhesion (Bryant et al., 2005). In recent years, a number of molecular regulators of cadherin endocytosis have been identified, including some in clathrin-dependent and clathrin-independent pathways (Bryant and Stow, 2004; Le et al., 1999; Palacios et al., 2002; Paterson et al., 2003), and a caveolin-1-associated pathway (Lu et al., 2003). Endocytosed E-cadherin can either be degraded or recycled to the cell surface – either mechanism leading to long-term or short-term modulation of cell-cell adhesion. What regulates the fate of cadherin once it is internalized from the cell surface is not known.

One potential set of candidates for regulation of E-cadherin intracellular traffic is the sorting nexins (SNXs), a family

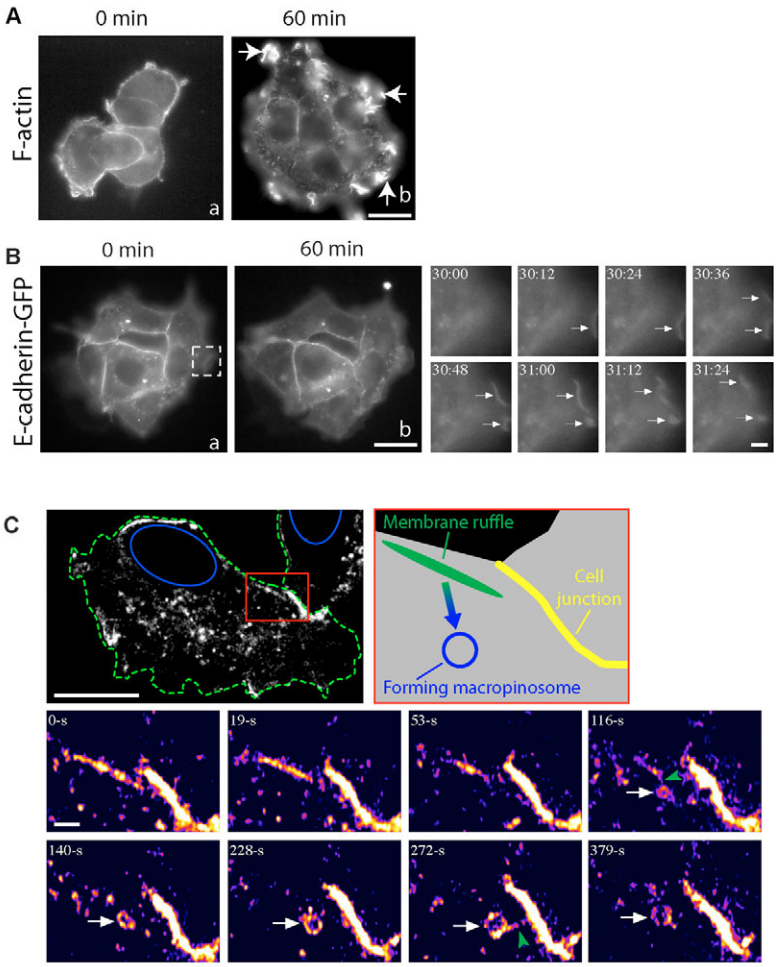


**Fig. 1.** EGF induces membrane ruffling and internalization of E-cadherin-GFP. (A) MCF-7 cells were fixed and labeled for F-actin using Alexa Fluor-488-conjugated Phalloidin either (a) without or (b) with prior EGF stimulation for 1 hour. Arrows indicate stimulation of actin-rich membrane ruffles in response to EGF. (B) MCF-7 cells stably overexpressing GFP-tagged E-cadherin (hE-GFP-MCF-7 cells) were imaged live every 12 seconds over a 1-hour treatment period with EGF using a TILLvision live imaging microscope. An example of E-cad-GFP-positive membrane ruffling is depicted in cropped movie frames 150-157 (30:00-31:24 minutes) of the boxed region of interest in a. Arrows depict ruffling membranes. See accompanying Movie 1 in supplementary material. (C) hE-GFP-MCF-7 cells were stimulated with EGF and imaged live. See accompanying Movie 2 in supplementary material. The diagram pinpoints the macropinocytosis event and cell features depicted in the movie and the subsequent intensity-coloured still frames show the main steps in the internalization of surface hE-cad-GFP (green arrow) into a macropinosome (white arrow) and its recycling (green arrowhead) back to the surface in a region of cell-cell contact. Bars, 20 μm for a and b; 2 μm for cropped movie frames.

of phosphatidylinositol 3-phosphate [PtdIns(3)*P*]-binding proteins defined by a common PX domain, with varied roles in intracellular trafficking (Carlton et al., 2005; Worby and Dixon, 2002). SNX1 was originally identified as a binding partner and regulator of epidermal growth factor receptor (EGFR) trafficking and degradation (Kurten et al., 1996). More recent studies have assigned a function for SNX1, in conjunction with the mammalian retromer complex, in retrieval of internalized proteins, such as the cation-independent mannose 6-phosphate receptor (CI-M6PR), away from the degradative pathway (reviewed in Carlton et al., 2005). Different SNX proteins have now emerged as participating in alternate endosomal membrane sorting events, particularly in the traffic of internalized proteins into and out of degradative endosomal pathway compartments (see above) (Carlton et al., 2005; Carlton and Cullen, 2005; Xu et al., 2001).

We here set out to examine EGF-induced internalization of E-cadherin and the potential role of SNX1 in the intracellular trafficking of E-cadherin in MCF-7 cells. Unlike the FGF-induced endocytosis of E-cadherin into early endosomes we previously described in these cells (Bryant et al., 2005), we here report EGF-induced, Rac1-modulated macropinocytosis of E-cadherin, β-catenin and p120ctn. Interestingly, this occurs without downregulation of total E-cadherin levels, suggesting recycling of internalized E-cadherin. SNX1 localized to E-cadherin-positive endocytic compartments and is shown here to function in the recycling of internalized E-cadherin back to the cell surface and in the re-establishment of epithelial polarity.

## Results

### EGF induces membrane ruffling and macropinocytosis of the E-cadherin-catenin complex

EGF stimulation can induce pleiotropic effects on epithelial cells, including proliferation, migration, invasion and transformation (for a review, see Thiery and Chopin, 1999). We first examined the morphological consequence of EGFR activation in our system by stimulating poorly motile human breast adenocarcinoma (MCF-7) cells with EGF. Cell shape was assessed by F-actin labeling. Cells grown in serum-supplemented medium displayed F-actin labeling mainly at cell-cell junctions, but also at surface membrane ruffles (Fig. 1A). EGF induced prominent ruffling of the cell surface membranes not in contact with other cells but did not result in overt disruption of junctional labeling or cell-cell adhesion (Fig. 1A). MCF-7 cells stably expressing E-cadherin-GFP (hE-GFP-MCF-7 cells) (Bryant et al., 2005) were examined by live cell imaging. E-cadherin-GFP was concentrated at cell-cell contacts and was also visible in intracellular vesicles (Fig. 1B), probably representing exocytic and recycling E-cadherin (Bryant and Stow, 2004). Similar to parental MCF-7 cells (Fig. 1A), EGF stimulation caused ruffling of free cell surface

membranes whereas at other points cell-cell contact was maintained (Fig. 1B, insets; see Movie 1 in supplementary material). To determine whether we could also visualize the potential internalization of E-cadherin we next imaged hE-GFP-MCF-7 cells using 4-D confocal microscopy (Lock et al., 2005). Similar to Fig. 1B, E-cadherin-GFP was localized to junctional regions and to intracellular vesicles (Fig. 1C). After EGF stimulation, E-cadherin-GFP on surface membrane ruffles was incorporated into large endosomal structures, strongly resembling macropinosomes (Fig. 1C, white arrows; see Movie 2 in supplementary material). Tubular intermediates containing E-cadherin-GFP were seen to emanate to and from these structures and surrounding regions, both early during macropinosome formation and once the mature structure was formed (Fig. 1C, green arrowheads). These are reminiscent of tubules observed from macropinosomes in other systems (Kerr et al., 2006). These findings reveal that EGF induces uptake of E-cadherin, potentially into macropinosomes, from ruffling cell-surface membranes in MCF-7 cells.

To characterize macropinocytosis as a mechanism for EGF-induced uptake of E-cadherin we next examined localization of endogenous E-cadherin in fixed cells. Although E-cadherin is typically localized to a variety of intracellular trafficking organelles, we have concentrated here on macropinosomes. Similar to E-cadherin-GFP (Fig. 1C), endogenous E-cadherin was also observed in macropinosomal structures near free edges of semi-polarized cell patches after short periods of EGF (10 minutes), and these could be co-labeled for EEA1 (Fig. 2A). Ectopic expression of EGFR-GFP revealed that it is co-internalized into these EEA1-positive structures together with E-cadherin (Fig. 2A). Notably, macropinosomal structures containing endogenous E-cadherin or E-cadherin-GFP, were ~2-2.5 μm in diameter and seen near regions of membrane ruffling, predominantly in semi-polarized cells (Fig. 2Aa,b, yellow arrowheads), in accord with previous studies on EGF-induced macropinocytosis (Hamasaki et al., 2004; Schnatwinkel et al., 2004).

To further verify their identity as macropinosomes, the structures were co-labeled with rabankyrin-5, a recently characterized regulator and marker of macropinosomes (Schnatwinkel et al., 2004). Expression of YFP-rabankyrin-5 in MCF-7 cells revealed a cytosolic pool of YFP-rabankyrin-5, plasma-membrane labeling and also its localization to numerous small vesicular puncta, reminiscent of classical early endosomes (Fig. 2B). YFP-rabankyrin-5 colocalized with E-cadherin in macropinosomes and expression of YFP-rabankyrin-5 moderately increased vesicular labeling of E-cadherin (Fig. 2B). Similar to other reports, expression of YFP-rabankyrin-5 also appeared to enhance macropinocytosis; upon stimulation with EGF (10 minutes) numerous clusters of macropinosomes labeled for YFP-rabankyrin-5 were observed in the periphery of cells, all of which also co-labeled for E-cadherin (Fig. 2B). This further supports the identity of these E-cadherin-containing structures as macropinosomes.

E-cadherin is normally complexed with β-catenin and p120ctn at stable cell-cell contacts (Yap et al., 1997). In EGF-treated cells p120ctn was localized to cell-cell junctions, and was noticed with E-cadherin on macropinosomes, which occasionally formed in monolayers of cells (Fig. 3a; white arrow). β-catenin was localized to cell-cell contacts, membrane ruffles and E-cadherin-positive macropinosomes in less confluent patches of cells (Fig. 3). Colocalization of p120ctn or β-catenin with E-cadherin was not observed on classical endosomal structures, represented by smaller vesicular

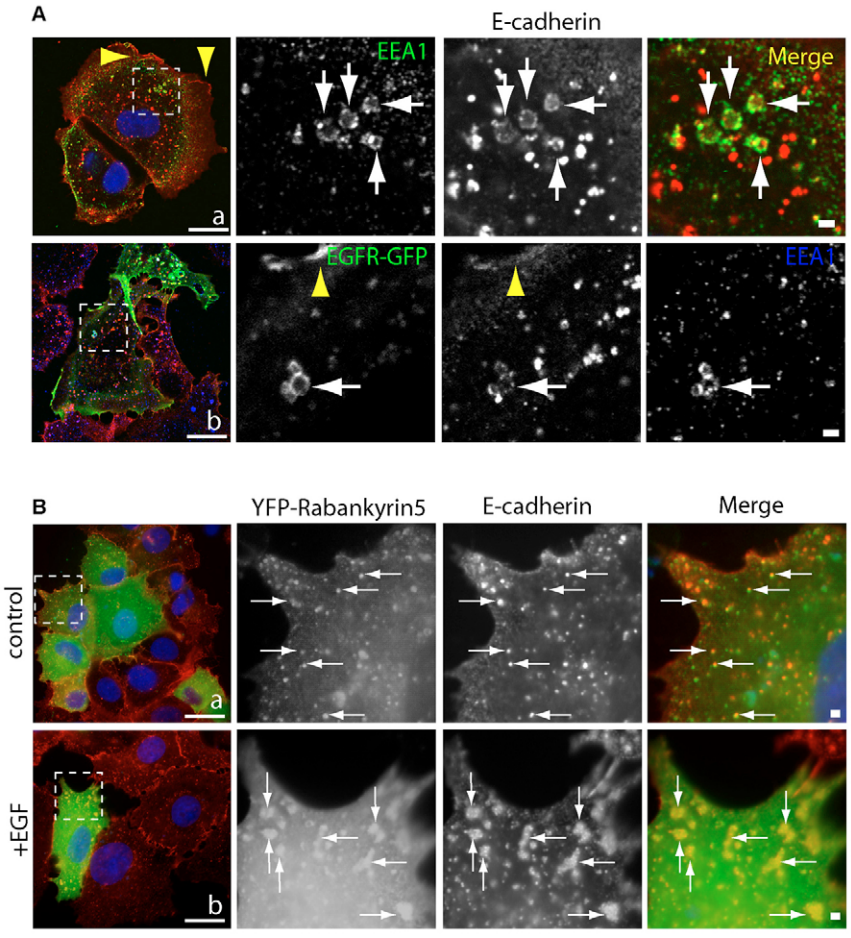


**Fig. 2.** EGF induces macropinocytosis of E-cadherin. (A) MCF-7 cells were treated with EGF for 10 minutes, fixed and immunolabeled for endogenous E-cadherin with EEA1 (a) or EGFR-GFP (b). Nuclei were stained with DAPI. Arrows denote localization of E-cadherin (a) and EGFR-GFP (b) to macropinosomes, arrowheads denote membrane ruffles. (B) MCF-7 cells were transiently transfected with YFP-rabankyrin-5, fixed and immunolabeled for endogenous E-cadherin (red) either (a) without or (b) with prior EGF stimulation for 10 minutes, and examined by epifluorescence. Arrows denote overlap of staining between intracellular E-cadherin and YFP-rabankyrin-5. Note formation of numerous macropinosomes upon EGF stimulation of YFP-rabankyrin-5-expressing cells. Second, third and fourth panels are magnifications of boxed areas shown in respective first panels. Bars, 20 μm for all first panels; 2 μm for all other panels.

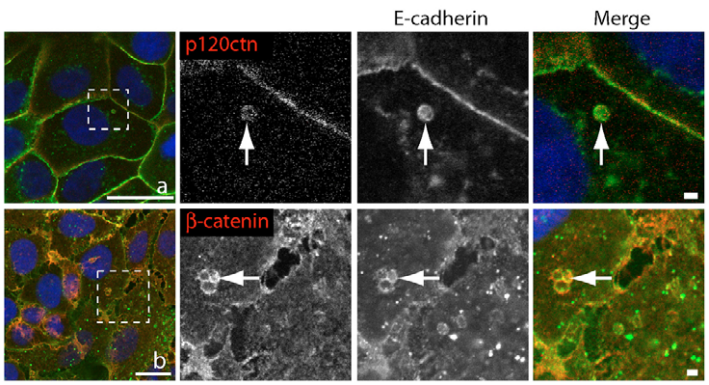


**Fig. 3.** Macropinocytosis of catenins in conjunction with E-cadherin. MCF-7 cells were treated with EGF for 10 minutes, fixed and immunolabeled for E-cadherin (green) and nuclei (DAPI; blue), and for either (a) p120ctn (red) or (b) β-catenin (red). Note localization of a pool of p120ctn and β-catenin to E-cadherin-positive macropinosomes (arrows). Second, third and fourth panels are magnifications of boxed areas shown in respective first panels. Bars, 20 μm for all first panels; 2 μm for all other panels.

early endosomes. p120ctn and a subset of β-catenin are thought to be removed from E-cadherin during classical endocytosis (Davis et al., 2003; Le et al., 1999; Xiao et al., 2003). The fact that p120ctn and β-catenin are found with E-cadherin in macropinosomes now suggests a different scenario in response to EGF.

## Rac1 promotes macropinocytosis of E-cadherin

To characterize some of the molecular machinery that facilitates internalization of E-cadherin, we next turned to the small GTPase Rac1. Rac1 has previously been implicated in the regulation of macropinocytosis, however, there are conflicting reports as to its role in E-cadherin internalization in other cell types (Akhtar and Hotchin, 2001; Izumi et al., 2004). Stimulation of MCF-7 cells with EGF resulted in an ~40% increase in endogenous GTP-Rac1 levels compared with unstimulated cells, whereas total cellular levels of Rac1 were unchanged (Fig. 4A). To determine whether activation of Rac1 alone was sufficient to induce macropinocytosis of E-cadherin, we expressed GFP-tagged versions of Rac1 and the related GTPase, Cdc42, in semi-polarized patches of cells. Overexpression of wild-type Rac1 (wt-Rac1-GFP), which localized predominantly to the plasma membrane, had no apparent effect on localization of E-cadherin, which was also localized to the plasma membrane and to cell-cell contacts (Fig. 4B). By contrast, expression of a constitutively GTP-bound mutant of Rac1 (CA-Rac1-GFP) resulted in the striking appearance of numerous enlarged endosomal structures reminiscent of enlarged macropinosomes, and labeled for both CA-Rac1-GFP and E-cadherin (Fig. 4Bb) (see also Akhtar and Hotchin, 2001). Expression of a constitutively GTP-bound mutant of Cdc42 (CA-Cdc42-GFP) was not sufficient to phenocopy this effect, whereby E-cadherin and Cdc42 were localized to plasma membrane and intracellular puncta, similar to control cells (Fig. 4B). Interestingly, neither CA-Rac1 nor CA-Cdc42 induced macropinocytosis of E-cadherin in polarized monolayers of cells (data not shown), consistent with previous reports (Izumi et al., 2004). These data suggest that Rac1 is activated downstream of EGFR, and that GTP-Rac1 induces

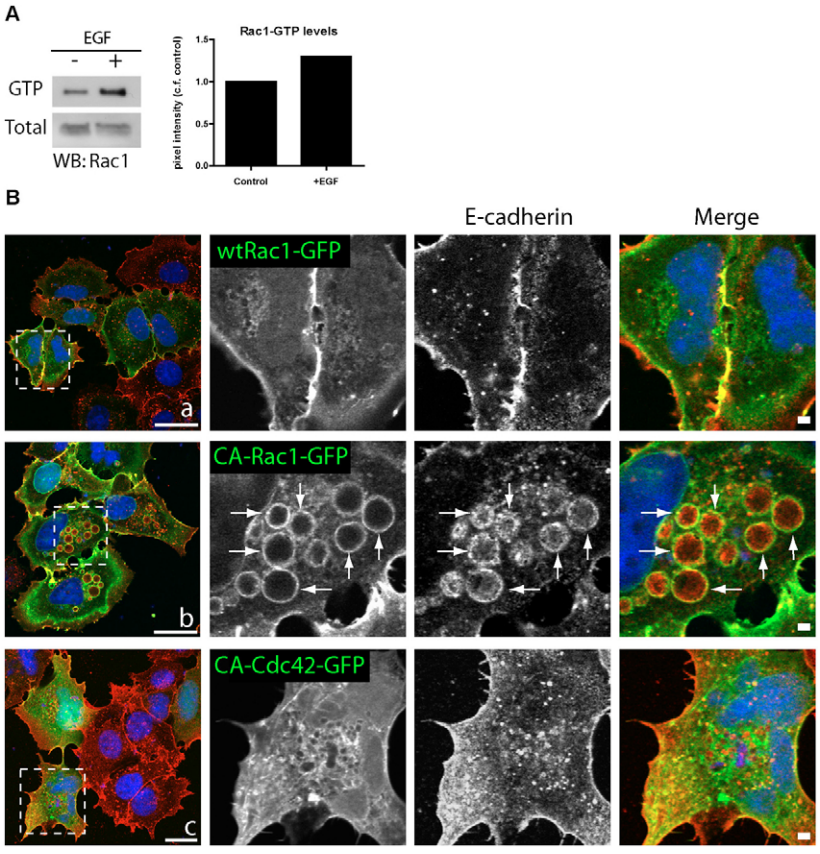


**Fig. 4.** Rac1 modulates macropinocytosis of E-cadherin. (A) Cells were serum-starved overnight, stimulated with EGF for 10 minutes and lysed. Then, levels of Rac1-GTP in equivalent concentrations of cell lysate were determined by PAK assay and compared with total Rac1 levels. Change in Rac1-GTP levels was quantified by densitometry. (B) MCF-7 cells were transiently transfected with either (a) wt-Rac1-GFP, (b) CA-Rac1-GFP or (c) CA-Cdc42-GFP (all in green), fixed and stained for endogenous E-cadherin (red) and nuclei (DAPI, blue) before confocal microscopy analysis. Note localization of E-cadherin to enlarged endosomal compartments labeled for CA-Rac1-GFP in b (arrows). Second, third and fourth panels are magnifications of boxed areas shown in respective first panels. Bars, 20 μm for all first panels; 2 μm for all other panels.

macropinocytosis of E-cadherin in semi-polarized epithelial cells.

### Internalized E-cadherin is delivered to the late endosome

To determine the fate of internalized E-cadherin, cells were stained after longer periods of EGF stimulation. In all instances, labeling of E-cadherin was seen to accumulate in the juxtanuclear region of cells by 30 minutes in EGF. Co-labeling with markers of either the Golgi complex (GM130, cis-Golgi; Fig. 5) or the recycling endosome (Rab11; Fig. 5) revealed little or no overlap with the punctate E-cadherin labeling. Co-labeling with alternate Golgi markers (e.g. p230) or SNARE proteins localized to endocytic or exocytic compartments (e.g. VAMP8, syntaxin6), similarly displayed no overlap with E-cadherin puncta (data not shown). Co-labeling of cells with the late endosomal/lysosomal marker cathepsin D (Fig. 5), which displayed as punctate intracellular labeling, revealed overlap with a proportion of intracellular E-cadherin. However, consistent with the inability of EGF to induce gross disruption to adhesion in these cells (see Fig. 1), we did not observe significant downregulation of total E-cadherin levels upon EGF stimulation (data not shown), suggesting that the majority of internalized E-cadherin is recycled back to the cell surface. Together, these data reveal that EGF induces traffic of a small pool of internalized E-cadherin to a cathepsin-D-positive-, probably late-endosomal structure.

### SNX1 regulates traffic and turnover of internalized E-cadherin

The role of selected endosomal sorting machinery in the intracellular trafficking of E-cadherin was next examined. Members of the sorting nexin protein family have been implicated in the protein trafficking of the internalized EGFR and other membrane receptors (Carlton and Cullen, 2005). Recent studies have also associated sorting nexins with EGF-induced macropinosomes and, therefore, SNX1 represents a potential modulator of E-cadherin trafficking in response to EGF (Kerr et al., 2006). Co-labeling of MCF-7 cells revealed strong overlap between SNX1 and intracellular E-cadherin at steady-state on endosomal structures (Fig. 6), reminiscent of early endosomes in cells grown in serum-supplemented medium. At short time points (10 minutes) after EGF stimulation, a common pool of E-cadherin and SNX1 was localized to macropinosomes (Fig. 6b, yellow arrowheads). Some tubular extensions emanated from macropinosomes, similar to live cell imaging of E-cadherin-GFP (Fig. 1C), and these were also labeled for SNX1 (Fig. 6). At later time points (30 minutes), intracellular E-cadherin had coalesced in the juxtanuclear region of cells, where it still overlapped with some SNX1 labeling (Fig. 6). These data reveal that E-cadherin and SNX1 colocalize to endosomal structures, including endosomes and macropinosomes, presenting SNX1 as being optimally positioned to regulate E-cadherin trafficking.

For functional analysis, SNX1 levels in MCF-7 cells were depleted by transfection of small interfering RNAs (siRNAs). SNX1-specific siRNAs effectively reduced total cellular levels of SNX1, whereas a corresponding SNX1-scrambled siRNA did not affect cellular levels of the protein (Fig. 7A). Reduction of SNX1 levels resulted in a slight increase in total levels of E-cadherin at steady-state, but did not affect cellular levels of the related SNX2 protein (Fig. 7A). In response to EGF stimulation, E-cadherin was still able to be macropinocytosed in SNX1-depleted cells (Fig. 7B). These data reveal that SNX1 can be efficiently depleted from cells without affecting internalization of E-cadherin.

To determine whether SNX1 functions in E-cadherin trafficking at a post-internalization step, we analyzed intracellular pools of E-cadherin and other membrane receptors by trypsin protection assay (Gavard and Gutkind, 2006; Xiao et al., 2003). Total cellular levels of E-cadherin, EGFR or TfnR were each slightly increased by depletion of SNX1 (Fig. 7C). Intracellular pools of these proteins sequestered from surface trypsinization were tested after various times of EGF treatment. SNX1-depleted cells displayed no significant change in the intracellular accumulation of either EGFR or TfnR in response to EGF (Fig. 7C), in accord with previous reports (Carlton et al., 2004). By contrast, SNX1-depleted cells displayed a 45% increase in the internal pool of E-cadherin at 30 minutes of EGF treatment, compared with cells treated with scramble siRNA (Fig. 7C). To confirm that this increase in intracellular E-cadherin corresponded to an accumulation of E-cadherin internalized from the cell surface, we performed antibody-uptake experiments (Gavard and Gutkind, 2006; Paterson et al., 2003; Xiao et al., 2003). Incubation of SNX1-depleted and scramble-siRNA-treated cells with an E-cadherin antibody at 4°C revealed labeling of

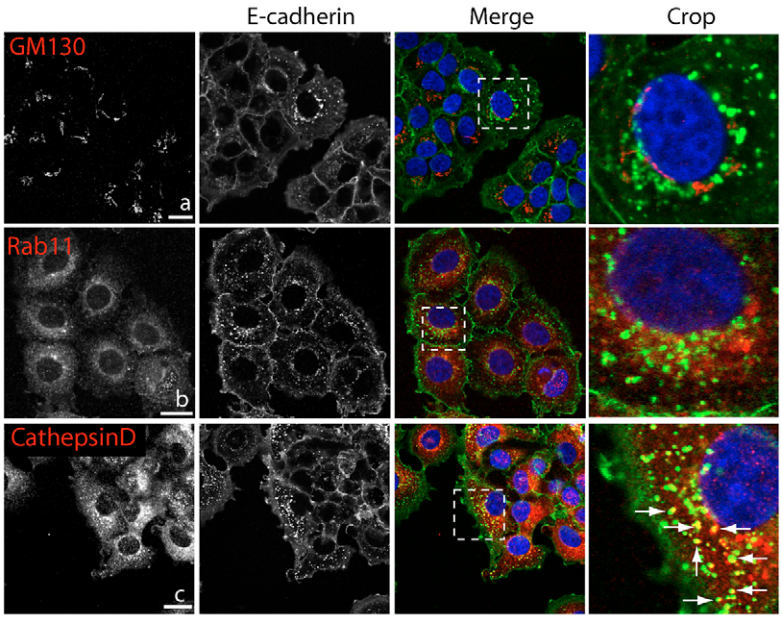


**Fig. 5.** EGF induces trafficking of a pool of E-cadherin to the late endosome. MCF-7 cells were stimulated with EGF (30 minutes), fixed and immunolabeled for E-cadherin (green) and nuclei (DAPI; blue). Antibodies against (a) GM130, (b) Rab11, or (c) cathepsin D (all in red) were used. White arrows denote co-labeling of E-cadherin and cathepsin D. Cropped panels (fourth column of panels) depict boxed region of interest at higher magnification. Bars, 20 μm.

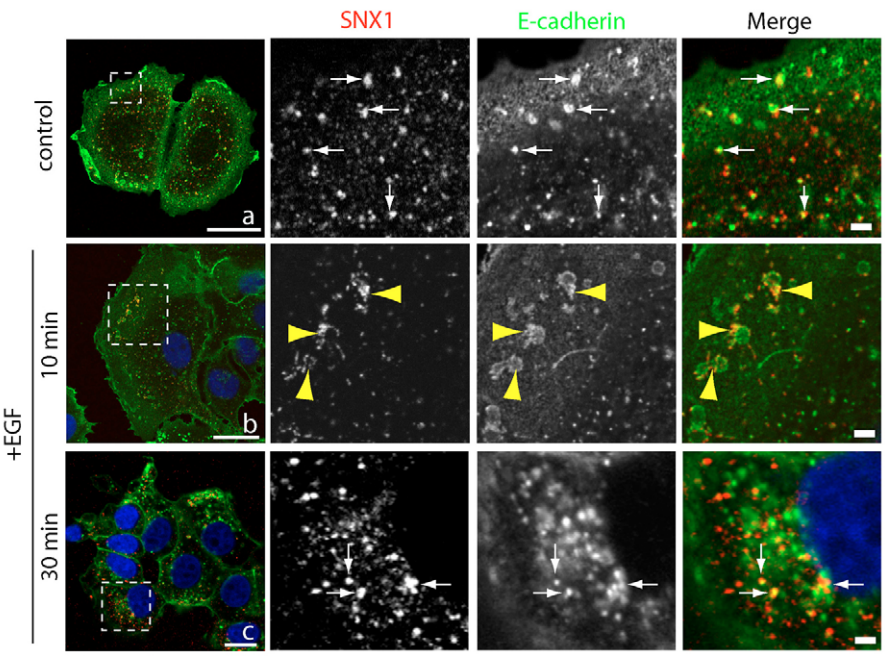


**Fig. 6.** SNX1 localizes to E-cadherin-positive endosomal membranes. MCF-7 cells were fixed and stained for E-cadherin (green), nuclei (DAPI, blue) and SNX1 (red), without (a) or with prior EGF stimulation for 10 minutes (b) or 30 minutes (c) before confocal microscopy analysis. Arrows denote co-labeling of E-cadherin and SNX proteins on small typical endosomal puncta, arrowheads indicate localization of SNX1 to E-cadherin-positive macropinosomes. Note tubular extensions from macropinosomes in (b). Second, third and fourth panels are magnifications of boxed areas shown in respective first panels. Bars, 20 μm for all first panels; 2 μm for all other panels.

surface E-cadherin (Fig. 7D), particularly at cell-cell contacts. After removal of the surface antibody under mildly acidic conditions, the intracellular staining of internalized antibody was analyzed in EGF-treated cells. Internalized E-cadherin antibody appeared in puncta throughout the cytoplasm but in SNX1-depleted cells the punctate staining was significantly more abundant (~2.5-fold increase in vesicle numbers) and was concentrated in juxtanuclear clusters (Fig. 7D). These data demonstrate that depletion of SNX1 results in the abnormal accumulation of E-cadherin internalized from the cell surface in response to EGF but not of the other recycling proteins examined.

To determine whether the accumulation of intracellular E-cadherin observed in SNX1-depleted cells resulted in enhanced turnover of the protein, we biotinylated surface E-cadherin in cells treated with SNX1 siRNA and scramble siRNA, followed by stimulation with EGF for either 4 hours or 18 hours. In scramble-siRNA-treated control cells, the level of biotinylated E-cadherin was unchanged between non-EGF treated cells and those stimulated with EGF for 4 hours (Fig. 7E). By 18 hours of EGF stimulation, biotinylated E-cadherin was largely depleted from cells, representing normal turnover of E-cadherin from the cell surface. However, in cells depleted for SNX1, a significant reduction in biotinylated E-cadherin was observed much earlier than in control cells, with a slight reduction at steady-state and becoming particularly obvious at 4 hours of EGF stimulation. By contrast, no significant alteration was observed in the turnover of TfnR in response to SNX1 depletion. This is consistent with reduced recycling of E-cadherin to the surface and it indicates a functional role for SNX1 in the turnover of E-cadherin from the cell surface in response to EGF.

### SNX1 regulates recycling of internalized E-cadherin

Our data thus far indicated that, in response to EGF, SNX1 functions to re-route E-cadherin internalized from the cell surface away from the degradative pathway. Such a function is likely to impact on whether internalized E-cadherin is available to be recycled. To assess recycling of internalized E-cadherin the '$Ca^{2+}$-switch' assay was used. We have previously shown that re-establishment of cell-cell adhesion in this assay requires recycling of internalized E-cadherin (Le et al., 1999). Both control (scramble-siRNA-treated) and SNX1-depleted cells retained patent epithelial 'cobblestone' morphology in monolayers, and displayed comparable levels of internal E-cadherin labeling (Fig. 8A; see also Fig. 7C). Depletion of extracellular $Ca^{2+}$ resulted in disruption of cell-cell adhesion and internalization of E-cadherin in both control and SNX1-depleted cells (Fig. 8A), further emphasizing that SNX1 does not regulate the internalization of E-cadherin. Re-introduction of $Ca^{2+}$ to wild-type cells (60 minutes) was sufficient to induce recycling of E-cadherin back to the cell surface and to restore cell-cell adhesion (Fig. 8A). By contrast, SNX1-depeleted cells were retarded in their ability to reform cell-cell junctions (Fig. 8A). Instead, these cells continued to display large intracellular accumulations of E-cadherin (white arrows), and were unable to efficiently reform a monolayer. As reformation of cell-cell contacts in this assay requires recycling of internalized E-cadherin back to the cell surface, these findings reveal that SNX1 is required for efficient recycling of membrane containing internalized E-cadherin.

To confirm that the level of E-cadherin recycled back to the surface was reduced in SNX1-depleted cells, we performed surface biotinylation experiments at 4°C at the different stages of the $Ca^{2+}$ switch. In scramble-siRNA-treated control cells, surface levels of E-cadherin were approximately equivalent before $Ca^{2+}$ chelation and after restoration of $Ca^{2+}$ to cell monolayers, whereas the surface levels of E-cadherin were greatly reduced upon chelation of extracellular $Ca^{2+}$ (Fig. 8B), all representing internalization and subsequent recycling of surface E-cadherin. By contrast, in SNX1-siRNA-treated cells, surface E-cadherin levels were notably reduced (an ~45% drop in recycling) upon restoration of $Ca^{2+}$ to EDTA-treated cells, representing a deficiency in the normal recycling of internalized E-cadherin (Fig. 8B). In addition, parallel

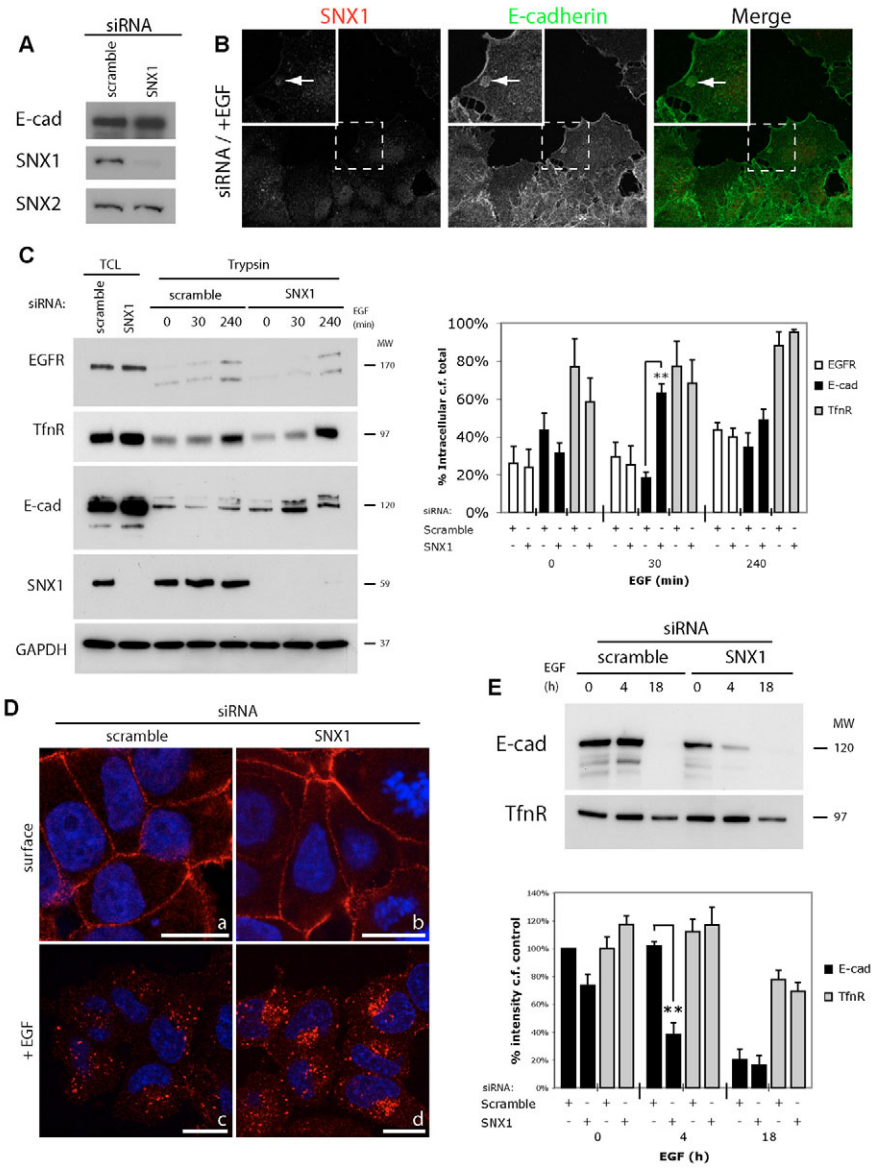


**Fig. 7.** SNX1 modulates E-cadherin intracellular trafficking and turnover. (A) MCF-7 cells were treated with either SNX1 siRNA or scrambled siRNA for 72 hours, and blotted for E-cadherin, SNX1 and SNX2. Notice the marked depletion of SNX1 levels in SNX1-siRNA-treated cells. (B) SNX1-depleted cells were stimulated with EGF for 10 minutes, fixed and stained for SNX1 (red) and E-cadherin (green). Inset depicts higher magnification of boxed regions of interest. Notice formation of an E-cadherin-containing macropinosome (arrows) in SNX1-depleted cells. (C) Lysates of SNX1-depleted or scramble siRNA-treated (control) cell monolayers were taken at either steady-state (total cell lysate, TCL) or following incubation with EGF for 0, 20 or 240 minutes, followed by trypsin-mediated cleavage of cell surface proteins. Total SNX1 and GAPDH levels were analyzed to ensure SNX1 knockdown and equivalent protein loading, respectively. Histograms were constructed from densitometry of immunoblots of intracellular (i.e. trypsin-protected) protein at relevant EGF treatment time points, and expressed as the percentage of total cellular protein (from TCL; $^{**}P>0.001$, $n=3$, independent experiments). Notice a 45% increase in the intracellular pool of E-cadherin at 30 minutes of EGF stimulation in SNX1-depleted cells, but not for EGFR or TfnR. (D) hECD1 antibody was bound to surface E-cadherin in (b) SNX1-depleted and (a) scramble-siRNA-treated (control), followed by incubation with EGF for 30 minutes (c,d). Surface-antibody labeling of E-cadherin was stripped by mild-acid wash (c,d). Notice the relative increase in intracellular juxtanuclear E-cadherin labeling in SNX1-depleted cells. (E) Surface proteins in SNX1-depleted or scramble-siRNA-treated cell monolayers were biotinylated, and E-cadherin levels were analyzed without activation of internalization (0 hours), or after 4 hours or 18 hours of EGF stimulation. Histograms represent immunoblot intensity over various conditions as a percentage of the total level in control cells (scramble siRNA, 0 hours; $^{**}P>0.001$, $n=3$, independent experiments). Bars, 20 μm.

examination of EGFR and TfnR trafficking in SNX1-depleted cells revealed an occasional increase in surface EGFR levels at steady-state but, most prominently, revealed a similar deficiency in EGFR recycling, but not for TfnR (Fig. 8B). Taken together, these data reveal a role for SNX1 in the intracellular sorting and recycling of membranes containing E-cadherin and, thus, identify SNX1 as a novel regulator of epithelial polarity.

## Discussion

In this study we have examined EGF-induced internalization and intracellular trafficking of E-cadherin. EGF induced the macropinocytosis of the E-cadherin-catenin complex from ruffling cell membranes, whereafter it colocalized with SNX1 in intracellular compartments, including macropinosomes and typical smaller endosomes. Interestingly, although a fraction of internalized E-cadherin was delivered to a late-endosomal compartment after EGF stimulation, there was no significant loss of stable cell-cell adhesion or total cadherin levels, suggesting that the majority of internalized E-cadherin is being recycled to the surface to maintain adhesion. siRNA studies revealed two prominent, complementary effects after depletion of cellular SNX1 levels – increased turnover of E-cadherin from the cell surface and the inability to recycle internalized E-cadherin to reform cell-cell junctions in the $Ca^{2+}$-switch assay – both of which indicate a modulation in the trafficking of E-cadherin within the endosome. These data reveal EGF-induced macropinocytosis of E-cadherin and SNX1-dependent recycling as mechanisms for transient modulation of cell-cell adhesion in epithelial cells. Such mechanisms may operate in instances where total loss of stable cell-cell contacts is not required, such as during proliferation, in accord with the predominantly mitogenic action of EGF in these cells (Fitzpatrick et al., 1984; Sarkar et al., 2005).

We describe macropinocytosis of E-cadherin. Four criteria were used to characterize the E-cadherin-containing macropinosomes. (1) The labeled macropinosomes were consistently seen near regions of actin rearrangements (ruffles) at the plasma membrane (Schnatwinkel et al., 2004). (2) Their appearance was stimulated by and accessible to growth factors (EGF) and their receptors (Haigler et al., 1979; Schnatwinkel et al., 2004; West et al., 1989). (3) The structures were labeled by rabankyrin-5 (Schnatwinkel et al., 2004). (4) They were consistently large (2-2.5 μm in diameter), and tubulated or lobulated (Conner and Schmid, 2003; Schnatwinkel et al., 2004). Macropinocytosis of E-cadherin occurred at regions of the plasma membrane that were not actively engaged in cell-cell junctions and that underwent ruffling specifically activated by EGF or GTP-Rac1 in semi-polarized MCF-7 cells. Interestingly, stimulation with HGF, FGF-1 or FGF-2 does not notably induce macropinocytosis of E-cadherin in these cells (Bryant et al., 2005) (and our unpublished observations). E-cadherin-positive macropinosomes have also been noticed in SCC12f keratinocytes in response to low-$Ca^{2+}$ medium or expression of CA-Rac1 (Akhtar and Hotchin, 2001), and when E-cadherin is overexpressed in 3T3 fibroblasts, which undergo constitutive macropinocytosis (L.A.H. and J.L.S., unpublished observations). Thus, the internalization of E-cadherin into macropinosomes appears to be linked to the ability of soluble factors or signaling events to activate cell surface membrane ruffling in semi-polarized epithelia.

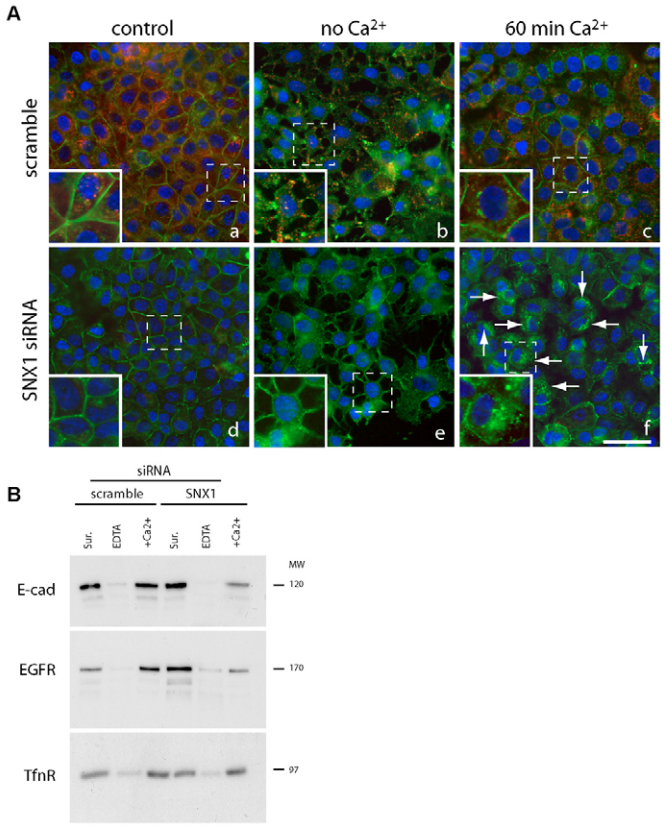


**Fig. 8.** SNX1 modulates E-cadherin recycling. (A) SNX1-depleted cells and scramble-siRNA-treated cells (control) were fixed and stained for SNX1 (red), E-cadherin (green) and nuclei (DAPI, blue) at steady-state (a,d), after chelation of extracellular $Ca^{2+}$ (b,e) or following restoration of $Ca^{2+}$ levels for 60 minutes (c,f). Notice the retarded reformation of cell-cell junctions and the intracellular accumulation of E-cadherin (arrows) in SNX1-depleted cells. Insets depict higher magnification images of boxed regions. (B) Surface proteins in SNX1-depleted cell monolayers or scramble-siRNA-treated cell monolayers were biotinylated before $Ca^{2+}$ chelation (Surface, Sur.), after chelation (EDTA) and after restoration of $Ca^{2+}$ levels for 60 minutes (+Ca2+), and levels of surface E-cadherin, EGFR and TfnR were analyzed. Bar, 20 μm.

In live cells, fluorescent E-cadherin was internalized into macropinosomes from non-contact cell edges, but then it was often seen being recycled to regions of cell-cell contact. Macropinocytosis might then also be a mechanism for redeploying E-cadherin on the cell surface to modify regions of cell-cell contact in response to growth factors. Macropinocytosis and recycling of other adhesion proteins, such as the tight-junction proteins occludin and JAM-A occurs in response to IFNγ stimulation without protein downregulation, to facilitate temporary trans-epithelial permeability (Bruewer et al., 2005; Utech et al., 2005). Our results here show too that EGF induces trafficking of E-cadherin without overt loss of the total protein levels. However, biotinylation experiments revealed that there is an increase in the turnover of E-cadherin in cells silenced for SNX1 expression, suggesting a role for SNX1 in sorting (for either recycling or degradation) of endocytic membrane containing internalized E-cadherin. Surprisingly, cells grown as monolayers and then depleted of SNX1 protein still maintained a polarized phenotype, and had slightly increased total cellular E-cadherin levels at steady-state. This may represent an increase in biosynthetic delivery of E-cadherin to compensate for a reduced recycling kinetic and increased turnover. To this end, inhibition of biosynthesis using cycloheximide resulted in a pronounced reduction in total E-cadherin levels in SNX1-depleted cells compared with control cells, suggesting that upregulated biosynthesis contributes to maintaining E-cadherin levels (D.M.B. and J.L.S., unpublished observations).

Interestingly, EGF stimulation induced macropinocytosis of β-catenin and p120ctn in addition to its cognate EGF receptor. β-catenin and p120ctn are generally considered to be removed from E-cadherin prior to or during classical endocytosis (Davis et al., 2003; Le et al., 1999; Miranda et al., 2003), and were not seen here on early endosomal structures. As β-catenin and p120ctn each have additional signaling roles, separate from E-cadherin, it will be of interest to determine whether, and at which point, β-catenin and p120ctn are removed from macropinocytosed E-cadherin. Similarly, we and others have shown that the E-cadherin–p120ctn complex regulates signaling and internalization of FGFR1, as well as EGFR and Met (Bryant and Stow, 2004; Bryant and Stow, 2005; Bryant et al., 2005; Qian et al., 2004). The impact of macropinocytosis of E-cadherin upon signaling from these receptors remains to be explored.

Classical clathrin-dependent endocytosis and its associated molecular machinery is increasingly being defined as a bona fide pathway for the internalization of E-cadherin (Le et al., 1999; Palacios et al., 2002; Izumi et al., 2004; Bryant and Stow, 2004; Kimura et al., 2006). However, a number of reports also implicate additional, non-clathrin-dependent pathways for E-cadherin internalization, such as macropinocytosis or pathways involving caveolin-1 (Akhtar

and Hotchin, 2001; Lu et al., 2003; Paterson et al., 2003). Rab GTPases, Rac-1 and Arf-6 have all been implicated in the endocytosis of E-cadherin, and perhaps act in multiple pathways. In MDCK cells, activation of the Met tyrosine kinase recruits the GEF for Rab5, RIN2, which induces Rab5-GTP activity during endocytosis (Kimura et al., 2006). Concomitantly, GTP-ARF6 levels are increased, which recruits its effector protein Nm23-H1 to sites of cadherin internalization, dually providing a source of GTP for dynamin-dependent endocytosis and promoting the conversion of Rac1-GTP to its GDP form (Palacios et al., 2002). Izumi et al. (Izumi et al., 2004) report that the Rac1-GTP-rich environment of the adherens junction inhibits the clathrin-dependent endocytosis of E-cadherin, and Rac1-GTP must be downregulated to facilitate this endocytosis. By contrast, our studies here, and also those of others (Akhtar and Hotchin, 2001), revealed that increased Rac1-GTP levels were required for macropinocytosis. We have previously demonstrated a dynamin2-dependent, Eps15-independent internalization process, suggesting a clathrin-independent internalization route for E-cadherin in MCF-7 cells (Paterson et al., 2003). Interestingly, macropinocytosis can be induced by chemical or GEF-induced sustained activation of ARF6 (Brown et al., 2001; Radhakrishna et al., 1996). A cellular environment of constant ARF6-GTP signaling and increased Rac1-GTP levels may favor macropinocytosis of E-cadherin, such as from non-junctional regions. Alternately, the clathrin-dependent endocytosis of E-cadherin from the lateral membrane of polarized monolayers of cells instead requires decreased Rac1-GTP levels, coupled to elevated ARF6-GTP signaling (Palacios et al., 2002). Such differences in localized signaling microenvironments may help to explain why multiple pathways have been implicated in E-cadherin internalization. Whereas in the current study we focused on macropinocytosis of E-cadherin, it is possible that classical endocytosis of E-cadherin also occurs in response to EGF stimulation. Given that SNX1 also localized to early endosomes with E-cadherin, it is possible that SNX1 operates to recycle E-cadherin from multiple endosomal membrane compartments. Whether E-cadherin recycles directly from macropinosomes (as suggested by live imaging; Fig. 1) or via another compartment, such as the recycling endosome (Langevin et al., 2005; Lock and Stow, 2005), is not yet clear.

In our system, SNX1 was not required for the internalization of surface E-cadherin following EGF stimulation or depletion of extracellular $Ca^{2+}$ levels. Rather, depletion of cellular SNX1 levels by siRNA resulted in accumulation of intracellular E-cadherin derived from the cell surface, coupled to enhanced turnover of E-cadherin protein. $Ca^{2+}$-switch experiments revealed that SNX1 was required to re-establish cell-cell adhesion and polarity, and also to recycle internalized E-cadherin. But how does SNX1 accomplish this? SNX1 possesses a PX domain that binds PtdIns(3)*P*, thereby facilitating its localization to endosomal membranes (Carlton et al., 2005; Carlton and Cullen, 2005). In addition, it has a Bin/amphiphysin/Rvs (BAR) domain, which binds to highly curved membranes and can further influence their deformation (Carlton and Cullen, 2005), for example, into the tubular E-cadherin-containing endosomal intermediates observed into this study. SNX1 may mediate a direct exchange of membranes that contain E-cadherin between endocytic and recycling endosomal compartments, for example through tubular intermediates or by sorting E-cadherin into subdomains of endosomes destined for retrieval away from the degradative pathway. Similarly, SNX1 may couple E-cadherin to the SNX-associated mammalian retromer complex or to other sorting nexins to facilitate retromer-mediated receptor recycling, in a fashion similar to EGFR or pIgR (Seaman, 2005; Verges et al., 2004). However, we have not been able to detect a direct interaction between E-cadherin and SNX1, suggesting that SNX1 acts indirectly – perhaps via intervening proteins – to marshall E-cadherin during its recycling (our unpublished data). Some level of specificity for E-cadherin is suggested by the differential effect on EGFR and TfnR trafficking compared with that of E-cadherin observed in SNX1-depleted cells. Taken together, our results reveal that the trafficking function of SNX1 is required for recycling of membrane containing internalized E-cadherin and the detailed molecular mechanisms responsible now await further study.

Overall, this study details EGF-induced macropinocytosis as an additional pathway for E-cadherin endocytosis that operates for temporary internalization of E-cadherin without overt loss of cell-cell adhesion. These findings demonstrate for the first time the participation of a sorting nexin protein in E-cadherin intracellular trafficking, thus highlighting SNX1 as a regulator of cell-cell adhesion and of epithelial integrity.

## Materials and Methods

### Cell culture and treatments

Cell lines were passaged as previously described (Bryant et al., 2005). Briefly, MCF-7 cells and MCF-7 cells stably expressing GFP-tagged human E-cadherin (hE-GFP-MCF-7) (Bryant et al., 2005) were passaged in minimal essential medium (Earle's Salts) supplemented with 10% FCS (Gibco, San Diego, CA), 20 mM Hepes, 2 g/l sodium bicarbonate, 4 mM L-glutamine, 1% non-essential amino acids, and 5 mg/ml insulin at 37°C in an atmosphere of 5% $CO_2$ and 95% air. For immunofluorescence experiments, cells were plated onto glass coverslips. EGF (Invitrogen, Carlsbad, CA) was used at a standard concentration of 100 ng/ml in either complete medium or after overnight serum-starvation.

### Antibodies and reagents

E-cadherin was detected by mouse monoclonal (hECD-1) and rabbit polyclonal antibodies against human E-cadherin (Alpha Yap, University of Queensland, Australia) for immunofluorescence, or by a mouse monoclonal antibody (BD Transduction Laboratories, Lexington, CA) for western blotting. Mouse monoclonal antibodies against β-catenin, $p120^{ctn}$, Rac1, EEA1, SNX1 and SNX2 were all from BD Transduction Laboratories. Mouse anti-human TfnR antibody was obtained from Zymed (Invitrogen), mouse anti-human GAPDH was from Chemicon (Temecula, CA). Rabbit polyclonal cathepsin D antibody was obtained from Chemicon (Boronia, Australia), rabbit polyclonal EGFR was from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse and rabbit secondary antibodies conjugated to Alexa Fluor-647, Cy3 or Alexa Fluor-488, DAPI (used to label nuclei), mouse monoclonal anti-tubulin and Alexa Fluor-488-conjugated phalloidin (to label F-actin) were all from Molecular Probes (Eugene, OR). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were obtained from Zymed (Invitrogen). Plasmids used are as previously described and include human EGFR-GFP (John Hancock, University of Queensland), pGex-PAK1-CRIB, wt-Rac1-GFP, CA-Rac1-GFP, DN-Cdc42-GFP (Alpha Yap, University of Queensland), YFP-rabankyrin-5 (Schnatwinkel et al., 2004) (obtained from R. Parton, University of Queensland) and human E-cadherin-GFP (Miranda et al., 2003).

### Immunofluorescence and live imaging

For immunofluorescence, cells were either fixed in 4% or 0.9% paraformaldehyde (to visualize tubules) (Kerr et al., 2006) in PBS for 60 minutes, permeabilized using 0.1% Triton X-100 for 10 minutes and then stained as previously described (Bryant et al., 2005). Fixed and live confocal imaging, including 4D imaging was performed using an LSM 510 META confocal microscope (Carl Zeiss Microscope Systems, Jena, Germany), whereas additional live cell video microscopy was performed on cells grown on 25-mm round coverslips using a TILLvision imaging system (TILL Photonics, Munich, Germany), all as previously described (Lock et al., 2005; Lock and Stow, 2005). Images were analyzed and adjusted using Adobe Photoshop 7 and ImageJ (NIH).

### Immunoblotting and quantification

Cells were solubilized in ice-cold extraction buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5 mM $MgCl_2$, 0.2 mM EGTA, and 1% Triton X-100 plus 50 mM NaF, 1 mM $Na_3VO_4$ and complete protease inhibitor cocktail tablet (Roche, Mannheim, Germany)] on ice for 5 minutes and then extracted at room temperature for 25 minutes. Post-nuclear supernatants were obtained by centrifugation at 14,000 ***g*** for 10 minutes. Samples were separated by SDS-PAGE, transferred to PVDF membranes and analyzed by immunoblotting using chemiluminescence (SuperSignal Chemiluminescence Kit; Pierce, Rockford, IL) (Miranda et al., 2001). Protein concentrations were determined using a Pierce BCA Protein Assay Reagent kit (Pierce); protein transfer and loading were assessed by staining with 0.1% Coomassie Brilliant Blue. Immunolabeling was assessed by densitometry using a Biorad GS-800 Densitometer and Quantity One software (BioRad). Statistical analysis of western blots and generation of accompanying graphs were performed using Microsoft Excel.

### Rac activation assay

GTP-loading of Rac1 was determined using pulldown of the Rac/Cdc42-binding CRIB-domain of p21-activated kinase (PAK1) and blotting for Rac1 as previously described (Goodwin et al., 2003). Briefly, MCF-7 cells were serum-starved overnight, pre-bound with EGF on ice for 1 hour then returned to 37°C for 10 minutes to initiate signaling. Cells were washed in PBS containing 1 mM $Na_3VO_4$, lysed, and equivalent concentrations of cell extracts were incubated with GST-PAK1-CRIB (1 mg/ml) pre-bound to glutathione sepharose beads. Cell extracts (5 μg) were blotted to determine total Rac1 levels, whereas GTP-Rac1 levels were blotted through association with sepharose bead pulldown.

### Transfection and siRNA

siRNA against human SNX1 (5′-AAGAACAAGACCAAGAGCCAC-3′) and corresponding scrambled control siRNA (5′-AAGACAAGAACCAGAACGCCA-3′) (Dharmacon, Lafeyette, CO) are as previously described (Carlton et al., 2004; Kerr et al., 2006). Transfection of siRNA duplexes was performed using HiPerFect reagent (Qiagen, Valencia, CA) over 72 hours, according to the manufacturer's instructions. Briefly, siRNA were diluted to a final concentration of 5 nM in serum-free medium containing appropriate levels of HiPerFect reagent, incubated at room temperature for 10 minutes and added to cells in full growth medium. Cells were used and assayed for knockdown after 72 hours of siRNA treatment. Transection of plasmid cDNA was performed using the LipofectAMINE PLUS system (Invitrogen) as previously described (Bryant et al., 2005).

### Trypsin protection assay

Intracellular levels of proteins were gauged by trypsin protection assay (Gavard and Gutkind, 2006; Xiao et al., 2003). Briefly, confluent monolayers of MCF-7 cells pre-treated with either SNX1 or SNX1-scrambled siRNA were serum-starved overnight. Cells were either left untreated (0 hours) or stimulated with EGF (100 ng/ml) for 30 minutes or 240 minutes at 37°C in medium containing 100 μM chloroquine. Medium was then removed from cells, which were washed twice with PBS (without $Ca^{2+}$ or $Mg^{2+}$ salts), and incubated with 0.25% trypsin (UCSF Cell Culture Facility) for 5 minutes at 37°C. An equivalent volume of growth medium was added to neutralize the trypsin reaction. Cells were centrifuged for 5 minutes at 1000 rpm, and cell pellets were lysed using extraction buffer. Samples of total cell lysate (TCL) without trypsin treatment were taken for comparison. Equivalent concentrations of protein were analyzed by SDS-PAGE. In instances of EGF treatment, cells were concomitantly treated with siRNAs to inhibit any translation of new SNX1 protein during incubation periods.

### Antibody-uptake experiments

Antibody uptake experiments were performed as previously described (Gavard and Gutkind, 2006; Paterson et al., 2003; Xiao et al., 2003) with some modifications. Briefly, MCF-7 cells pre-treated for 72 hours with appropriate siRNAs were equilibrated in antibody-binding medium (serum-free medium containing 50 mg/ml BSA and 5 mM CaCl) for 1 hour at 37°C. Antibody was then bound to surface E-cadherin by washing twice with ice-cold binding buffer, followed by incubation (on ice, 1 hour) in binding buffer containing HECD-1 antibody against the extracellular region of human E-cadherin. Cells were washed twice in ice-cold PBS, before being returned to pre-warmed binding medium supplemented with EGF (100 ng/ml; to initiate antibody uptake) and chloroquine (100 μM; to inhibit degradation of internalized protein). Following uptake, cells were washed twice with ice-cold PBS and, where appropriate, cell surface antibody was stripped using a mild acid wash (three washes of 5 minutes; 0.5 M acetic acid, 0.5 M NaCl, in PBS). Cells were washed once more in PBS before fixation, immunolabeling and imaging by confocal microscopy. For quantification, cells were also labeled for β-catenin to delineate cell-cell boundaries, and the number of E-cadherin-positive vesicles were manually counted from confocal images.

### $Ca^{2+}$-switch experiments

Internalization and recycling of E-cadherin was induced by $Ca^{2+}$-switch experiments (Le et al., 1999), with some modifications. For immunofluorescence, confluent monolayers of MCF-7 cells pre-treated for 72 hours with appropriate siRNAs were washed twice in HBSS and either fixed immediately or treated with 2.5 mM EDTA to induce cadherin internalization before fixation. For some conditions, EDTA-containing HBSS was removed and replaced with HBSS containing 2 mM CaCl, to initiate cadherin recycling and restoration of cell-cell junctions before fixation. For biotinylation experiments cell surface E-cadherin was biotinylated at 4°C at each step instead of fixing the cells (see below).

### Surface biotinylation

The fate of proteins on the cell surface was traced using biotinylation. Briefly, confluent monolayers of cells on ice were washed thrice with ice-cold Hanks' balanced salt solution (HBSS; Gibco) containing 20 mM Hepes. Cells were then incubated with HBSS-Hepes containing EZ-link Sulfo-NHS-LC-Biotin (0.5 mg/ml) twice for 15 minutes. The biotinylation reaction was quenched by the addition of ice-cold serum-free medium, twice quickly, then once for 10 minutes. For turnover experiments, cells were incubated at 37°C for appropriate times in medium containing EGF (100 ng/ml) and fresh siRNAs (to inhibit new SNX1 protein production). Following incubation periods, cells were returned to ice, washed twice in ice-cold PBS, lysed using extraction buffer (see above). Cell lysates were recovered by centrifugation in a table-top centrifuge at 14,000 rpm at 4°C and biotinylated proteins were recovered by incubation with streptavidin (Pierce), for 1 hour at 4°C, before SDS-PAGE analysis. For biotinylation of surface proteins during $Ca^{2+}$-switch experiments, cells were instead incubated with biotin once for 30 minutes, to avoid loss of cells upon $Ca^{2+}$ chelation.

We acknowledge J. Hancock, R. Parton and A. Yap (University of Queensland) for generous gifts of plasmids. We thank J. Venturato, T. Khromykh, T. Su and K. Tang for expert technical assistance. D.M.B. was supported by a Young Researcher of the Year award from Cure Cancer Australia, and a Graduate School Research Travel Award from the University of Queensland. Both D.M.C. and M.C.K. were supported by an ARC Australian Postgraduate Award. This work was supported by a grant and fellowships (to R.D.T. and J.L.S.) from the National Health and Medical Research Council of Australia.

## References

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

**Brown, F. D., Rozelle, A. L., Yin, H. L., Balla, T. and Donaldson, J. G.** (2001). Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic. *J. Cell Biol.* **154**, 1007-1017.

**Bruewer, M., Utech, M., Ivanov, A. I., Hopkins, A. M., Parkos, C. A. and Nusrat, A.** (2005). Interferon-gamma induces internalization of epithelial tight junction proteins via a macropinocytosis-like process. *FASEB J.* **19**, 923-933.

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

**Bryant, D. M. and Stow, J. L.** (2005). Nuclear translocation of cell-surface receptors: lessons from fibroblast growth factor. *Traffic* **6**, 947-954.

**Bryant, D. M., Wylie, F. G. and Stow, J. L.** (2005). Regulation of endocytosis, nuclear translocation, and signaling of fibroblast growth factor receptor 1 by e-cadherin. *Mol. Biol. Cell* **16**, 14-23.

**Calkins, C. C., Setzer, S. V., Jennings, J. M., Summers, S., Tsunoda, K., Amagai, M. and Kowalczyk, A. P.** (2006). Desmoglein endocytosis and desmosome disassembly are coordinated responses to pemphigus autoantibodies. *J. Biol. Chem.* **281**, 7623-7634.

**Carlton, J. G. and Cullen, P. J.** (2005). Sorting nexins. *Curr. Biol.* **15**, R819-R820.

**Carlton, J., Bujny, M., Peter, B. J., Oorschot, V. M., Rutherford, A., Mellor, H., Klumperman, J., McMahon, H. T. and Cullen, P. J.** (2004). Sorting nexin-1 mediates tubular endosome-to-TGN transport through coincidence sensing of high-curvature membranes and 3-phosphoinositides. *Curr. Biol.* **14**, 1791-1800.

**Carlton, J., Bujny, M., Rutherford, A. and Cullen, P.** (2005). Sorting nexins – unifying trends and new perspectives. *Traffic* **6**, 75-82.

**Conner, S. D. and Schmid, S. L.** (2003). Regulated portals of entry into the cell. *Nature* **422**, 37-44.

**D'Souza-Schorey, C.** (2005). Disassembling adherens junctions: breaking up is hard to do. *Trends Cell Biol.* **15**, 19-26.

**Davis, M. A., Ireton, R. C. and Reynolds, A. B.** (2003). A core function for p120-catenin in cadherin turnover. *J. Cell Biol.* **163**, 525-534.

**Drees, F., Pokutta, S., Yamada, S., Nelson, W. J. and Weis, W. I.** (2005). Alpha-catenin is a molecular switch that binds E-cadherin-beta-catenin and regulates actin-filament assembly. *Cell* **123**, 903-915.

**Fitzpatrick, S. L., LaChance, M. P. and Schultz, G. S.** (1984). Characterization of epidermal growth factor receptor and action on human breast cancer cells in culture. *Cancer Res.* **44**, 3442-3447.

**Fujita, Y., Krause, G., Scheffner, M., Zechner, D., Leddy, H. E., Behrens, J., Sommer, T. and Birchmeier, W.** (2002). Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex. *Nat. Cell Biol.* **4**, 222-231.

**Gavard, J. and Gutkind, J. S.** (2006). VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin. *Nat. Cell Biol.* **8**, 1223-1234.

**Goodwin, M., Kovacs, E. M., Thoreson, M. A., Reynolds, A. B. and Yap, A. S.** (2003). Minimal mutation of the cytoplasmic tail inhibits the ability of E-cadherin to activate Rac but not phosphatidylinositol 3-kinase: direct evidence of a role for cadherin-activated Rac signaling in adhesion and contact formation. *J. Biol. Chem.* **278**, 20533-20539.

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

**Haigler, H. T., McKanna, J. A. and Cohen, S.** (1979). Rapid stimulation of pinocytosis in human carcinoma cells A-431 by epidermal growth factor. *J. Cell Biol.* **83**, 82-90.

**Hamasaki, M., Araki, N. and Hatae, T.** (2004). Association of early endosomal autoantigen 1 with macropinocytosis in EGF-stimulated A431 cells. *Anat. Rec. A Discov. Mol. Cell. Evol. Biol.* **277**, 298-306.

**Ivanov, A. I., Nusrat, A. and Parkos, C. A.** (2005). Endocytosis of the apical junctional complex: mechanisms and possible roles in regulation of epithelial barriers. *BioEssays* **27**, 356-365.

**Izumi, G., Sakisaka, T., Baba, T., Tanaka, S., Morimoto, K. and Takai, Y.** (2004). Endocytosis of E-cadherin regulated by Rac and Cdc42 small G proteins through IQGAP1 and actin filaments. *J. Cell Biol.* **166**, 237-248.

**Kerr, M. C., Lindsay, M. R., Luetterforst, R., Hamilton, N., Simpson, F., Parton, R. G., Gleeson, P. A. and Teasdale, R. D.** (2006). Visualisation of macropinosome maturation by the recruitment of sorting nexins. *J. Cell Sci.* **119**, 3967-3980.

**Kimura, T., Sakisaka, T., Baba, T., Yamada, T. and Takai, Y.** (2006). Involvement of the Ras-Ras-activated Rab5 guanine nucleotide exchange factor RIN2-Rab5 pathway in the hepatocyte growth factor-induced endocytosis of E-cadherin. *J. Biol. Chem.* **281**, 10598-10609.

**Kurten, R. C., Cadena, D. L. and Gill, G. N.** (1996). Enhanced degradation of EGF receptors by a sorting nexin, SNX1. *Science* **272**, 1008-1010.

**Langevin, J., Morgan, M. J., Sibarita, J. B., Aresta, S., Murthy, M., Schwarz, T., Camonis, J. and Bellaiche, Y.** (2005). Drosophila exocyst components Sec5, Sec6, and Sec15 regulate DE-Cadherin trafficking from recycling endosomes to the plasma membrane. *Dev. Cell* **9**, 355-376.

**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.

**Le, T. L., Joseph, S. R., Yap, A. S. and Stow, J. L.** (2002). Protein kinase C regulates endocytosis and recycling of E-cadherin. *Am. J. Physiol. Cell Physiol.* **283**, C489-C499.

**Lock, J. G. and Stow, J. L.** (2005). Rab11 in recycling endosomes regulates the sorting and basolateral transport of E-cadherin. *Mol. Biol. Cell* **16**, 1744-1755.

**Lock, J. G., Hammond, L. A., Houghton, F., Gleeson, P. A. and Stow, J. L.** (2005). E-cadherin transport from the trans-Golgi network in tubulovesicular carriers is selectively regulated by golgin-97. *Traffic* **6**, 1142-1156.

**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.

**Miranda, K. C., Khromykh, T., Christy, P., Le, T. L., Gottardi, C. J., Yap, A. S., Stow, J. L. and Teasdale, R. D.** (2001). A dileucine motif targets E-cadherin to the basolateral cell surface in Madin-Darby canine kidney and LLC-PK1 epithelial cells. *J. Biol. Chem.* **276**, 22565-22572.

**Miranda, K. C., Joseph, S. R., Yap, A. S., Teasdale, R. D. and Stow, J. L.** (2003). Contextual binding of p120ctn to E-cadherin at the basolateral plasma membrane in polarized epithelia. *J. Biol. Chem.* **278**, 43480-43488.

**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.

**Qian, X., Karpova, T., Sheppard, A. M., McNally, J. and Lowy, D. R.** (2004). E-cadherin-mediated adhesion inhibits ligand-dependent activation of diverse receptor tyrosine kinases. *EMBO J.* **23**, 1739-1748.

**Radhakrishna, H., Klausner, R. D. and Donaldson, J. G.** (1996). Aluminum fluoride stimulates surface protrusions in cells overexpressing the ARF6 GTPase. *J. Cell Biol.* **134**, 935-947.

**Sarkar, S., Maceyka, M., Hait, N. C., Paugh, S. W., Sankala, H., Milstien, S. and Spiegel, S.** (2005). Sphingosine kinase 1 is required for migration, proliferation and survival of MCF-7 human breast cancer cells. *FEBS Lett.* **579**, 5313-5317.

**Schnatwinkel, C., Christoforidis, S., Lindsay, M. R., Uttenweiler-Joseph, S., Wilm, M., Parton, R. G. and Zerial, M.** (2004). The Rab5 effector Rabankyrin-5 regulates and coordinates different endocytic mechanisms. *PLoS Biol.* **2**, E261.

**Seaman, M. N.** (2005). Recycle your receptors with retromer. *Trends Cell Biol.* **15**, 68-75.

**Thiery, J. P.** (2002). Epithelial-mesenchymal transitions in tumour progression. *Nat. Rev. Cancer* **2**, 442-454.

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

**Utech, M., Ivanov, A. I., Samarin, S. N., Bruewer, M., Turner, J. R., Mrsny, R. J., Parkos, C. A. and Nusrat, A.** (2005). Mechanism of IFN-gamma-induced endocytosis of tight junction proteins: myosin II-dependent vacuolarization of the apical plasma membrane. *Mol. Biol. Cell* **16**, 5040-5052.

**Verges, M., Luton, F., Gruber, C., Tiemann, F., Reinders, L. G., Huang, L., Burlingame, A. L., Haft, C. R. and Mostov, K. E.** (2004). The mammalian retromer regulates transcytosis of the polymeric immunoglobulin receptor. *Nat. Cell Biol.* **6**, 763-769.

**West, M. A., Bretscher, M. S. and Watts, C.** (1989). Distinct endocytotic pathways in epidermal growth factor-stimulated human carcinoma A431 cells. *J. Cell Biol.* **109**, 2731-2739.

**Worby, C. A. and Dixon, J. E.** (2002). Sorting out the cellular functions of sorting nexins. *Nat. Rev. Mol. Cell Biol.* **3**, 919-931.

**Xiao, K., Allison, D. F., Buckley, K. M., Kottke, M. D., Vincent, P. A., Faundez, V. and Kowalczyk, A. P.** (2003). Cellular levels of p120 catenin function as a set point for cadherin expression levels in microvascular endothelial cells. *J. Cell Biol.* **163**, 535-545.

**Xu, Y., Hortsman, H., Seet, L., Wong, S. H. and Hong, W.** (2001). SNX3 regulates endosomal function through its PX-domain-mediated interaction with PtdIns(3)P. *Nat. Cell Biol.* **3**, 658-666.

**Yamada, S., Pokutta, S., Drees, F., Weis, W. I. and Nelson, W. J.** (2005). Deconstructing the cadherin-catenin-actin complex. *Cell* **123**, 889-901.

**Yap, A. S.** (1998). The morphogenetic role of cadherin cell adhesion molecules in human cancer: a thematic review. *Cancer Invest.* **16**, 252-261.

**Yap, A. S., Brieher, W. M. and Gumbiner, B. M.** (1997). Molecular and functional analysis of cadherin-based adherens junctions. *Annu. Rev. Cell Dev. Biol.* **13**, 119-146.

**Yap, A. S., Niessen, C. M. and Gumbiner, B. M.** (1998). The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120ctn. *J. Cell Biol.* **141**, 779-789.