Functional ESCRT machinery is required for constitutive recycling of claudin-1 and maintenance of polarity in vertebrate epithelial cells

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Running Head: ESCRTs in vertebrate epithelial cells

Abbreviations; Endosomal Sorting Complex Required for Transport (ESCRT), epidermal growth factor receptor (EGFR), Mannose-6-phosphate receptor (M6PR), Transepithelial resistance (TER), transferrin receptor (TfR).

Highlight summary

Drosophila ESCRT mutants lose epithelial polarity and show increased proliferation, suggesting that ESCRT proteins act as tumour suppressors. Here we show for the first time that ESCRT proteins are required to maintain polarity in mammalian epithelial cells, supporting the idea that ESCRT proteins are tumour suppressors.

Abstract

Genetic screens in Drosophila have identified regulators of endocytic trafficking as neoplastic tumour suppressor genes. For example, Drosophila ESCRT mutants lose epithelial polarity and show increased cell proliferation, suggesting that ESCRT proteins could function as tumour suppressors. In this study we show for the first time that ESCRT proteins are required to maintain polarity in mammalian epithelial cells. Inhibition of ESCRT function caused the tight junction protein claudin-1 to accumulate in intracellular vesicles. In contrast E-cadherin and occludin localisation was unaffected. We investigated the cause of this accumulation and show that claudin-1 is constitutively recycled in kidney, colon, and lung epithelial cells, identifying claudin-1 recycling as a newly described feature of diverse epithelial cell types. This recycling requires ESCRT function, explaining the accumulation of intracellular claudin-1 when ESCRT function is inhibited. We further demonstrate that siRNA knockdown of the ESCRT protein Tsg101 causes epithelial monolayers to lose their polarised organisation and interferes with the establishment of a normal epithelial permeability barrier. ESCRT knockdown also reduces the formation of correctly polarised three dimensional cysts. Thus, in mammalian epithelial cells ESCRT function is required for claudin-1 trafficking and for epithelial cell polarity, supporting the hypothesis that ESCRT proteins function as tumour suppressors.
Introduction

Epithelial tissues are characterised by a polarised cellular architecture and specialised cell-cell junctions. These include desmosomes and adherens junctions which mediate cell adhesion and tight junctions that control paracellular movement of molecules across epithelial sheets (Getsios et al., 2004; Shin et al., 2006; Niessen and Gottardi, 2008). Each of these epithelial junctions consists of multiple proteins, for example tight junctions contain transmembrane proteins such as occludin and claudins and membrane-associated proteins such as ZO-1. The molecules responsible for recruiting these proteins into functional junctions and generating polarised tissues include a number of polarity complexes and signalling proteins (Suzuki and Ohno, 2006; Goldstein and Macara, 2007; Bryant and Mostov, 2008).

Epithelial junctions, once established, are dynamic structures that are constantly being remodelled (Shen et al., 2008; Steed et al., 2010). Understanding the mechanisms responsible for the formation, maintenance and remodelling of epithelial junctions is crucial, as alterations in cell junctions have been linked to a wide range of pathological conditions, such as cancer and inflammatory bowel diseases (Yang and Weinberg, 2008; Yu and Turner, 2008; Capaldo and Nusrat, 2009; Brennan et al., 2010).

There is growing evidence of links between endocytosis, regulation of epithelial junctions (Ivanov et al., 2005) and cell polarity (Shivas et al., 2010). For example, mutants in Drosophila Endosomal Sorting Complex Required for Transport (ESCRT) machinery components lose epithelial cell polarity and show a dramatic tissue overgrowth phenotype (Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005; Herz et al., 2006; Herz et al., 2009; Rodahl et al., 2009; Vaccari and Bilder, 2009; Vaccari et al., 2009). The ESCRT machinery is conserved from yeast to mammals and is made up of ESCRT-0, I, II and III sub-complexes, each consisting of multiple ESCRT proteins. These proteins have a well established role in the trafficking of ubiquitylated transmembrane proteins to lysosomes for degradation (Hurley and Emr, 2006; Raiborg and Stenmark, 2009). They are believed to function in the selection of cargo, invagination of endosome membranes and scission of the invaginations to form intraluminal vesicles (Hurley and Emr, 2006; Raiborg and Stenmark, 2009). This pathway is important for attenuating signalling from growth factor receptors such as the epidermal growth factor receptor (EGFR) (Malerod et al., 2007). In addition to blocking the degradative pathway, it has been reported that inhibiting ESCRT function can cause defects in the recycling of transmembrane proteins (Yoshimori et al., 2000; Fujita et al., 2003; Doyotte et al., 2005; Baldys and Raymond, 2009). These effects may be due to a loss of endosomal domain organisation, caused by inhibiting the degradative pathway (Woodman, 2009), and suggest an interdependence between different endocytic trafficking routes. However, a requirement for ESCRT proteins in recycling to the plasma membrane is controversial as other studies report that inhibiting ESCRT function causes an increase or has no effect on recycling (Babst et al., 2000; Razi and Futter, 2006; Raiborg et al., 2008). Finally, ESCRT proteins are involved in other processes that require membrane fission, such as cytokinesis, autophagy and viral budding (Garrus et al., 2001; Martin-Serrano et al., 2003; Zamborlini et al., 2006; Carlton and Martin-Serrano, 2007; Rusten et al., 2007; Dukes et al.,
The striking loss of polarity and overgrowth phenotypes seen in Drosophila ESCRT mutants suggests that ESCRT proteins could act as tumour suppressors. However, although the ESCRT pathway has been linked to stimulus-induced degradation of cell adhesion and junction proteins (Palacios et al., 2005; Leithe et al., 2009; Lobert et al., 2010) a role, if any, for the ESCRT proteins in maintaining epithelial polarity in vertebrates has not been determined.

In this study we investigate for the first time whether ESCRT protein function is required for epithelial cell polarity and junction formation in mammalian epithelial cells. We show that after inhibiting ESCRT function in epithelial cell lines, the tight junction protein claudin-1 accumulated on intracellular vesicles while the localisation of E-cadherin, occludin and ZO-1 appeared normal. We investigated the possible causes of the intracellular accumulation and found that claudin-1 is constantly endocytosed and recycled back to the plasma membrane in different epithelial cell types. This recycling requires ESCRT function, explaining the accumulation of intracellular claudin-1 when ESCRT function is inhibited. We further demonstrate that the ESCRT protein Tsg101 is required for maintaining a polarised monolayer in human epithelial cells and for the establishment of a normal epithelial permeability barrier. Finally we show that Tsg101 is required for correct formation of 3D cysts. Thus, ESCRT function is required for the recycling of claudin-1 and maintenance of polarity in mammalian epithelial cells.

Results

ESCRT function is required for the correct localisation of claudin-1 in epithelial cells

To investigate whether perturbation of ESCRT function affects cell polarity in vertebrate epithelial cells, two dominant negative ESCRT constructs were used. The first was a CHMP31-179GFP construct that has the C-terminal autoinhibitory domain removed (Whitley et al., 2003; Zamborlini et al., 2006; Shim et al., 2007). CHMP31-179GFP was expressed in MDCK cells, a canine kidney cell line commonly used to study epithelial polarity. CHMP31-179GFP accumulated on intracellular vesicular structures (Figure 1), a similar phenotype to that observed in other cell lines (Dukes et al., 2008). The tight junction proteins occludin and ZO-1 and the adherens junction protein E-cadherin showed the same localisation in CHMP31-179GFP expressing cells as control cells (Figure 1A-C). The apical marker GP135/podocalyxin and the basolateral marker numb were also correctly localised (Figure S1A). However, the localisation of claudin-1 was markedly different in CHMP31-179GFP expressing cells as compared to non-transfected cells (Figure 1D). Control cells contained small amounts of internal claudin-1, but levels of intracellular claudin-1 were greatly increased in CHMP31-179GFP expressing cells (Figure 1D, arrowhead in z projection). A similar phenotype was also seen with claudin-2 (Figure S7A). Despite the intracellular accumulation of claudin-1, some remained at the junctions and along the lateral membrane (Figure 1D), as seen in non-transfected cells. The additional intracellular claudin-1 accumulated in large vesicular structures and there was extensive colocalisation between the internal claudin-1 and the dominant negative
CHMP3\textsuperscript{1-179}GFP compartment (Figure 1D and Figure S1B). Claudin-1 distribution was not affected by the expression of GFP alone (Figure 1E).

The second dominant negative mutant used was a GFP tagged ATPase-defective Vps4 construct, GFP-Vps4\textsuperscript{E235Q} (Bishop and Woodman, 2000; Whitley et al., 2003). Similar to the phenotype produced by expressing CHMP3\textsuperscript{1-179}GFP, when expressed in MDCK cells this construct resulted in the accumulation of GFP-Vps4\textsuperscript{E235Q} and claudin-1 on large vesicular compartments (Figure S2B). The cellular distribution of ZO-1 was indistinguishable from non-expressing cells, localising almost exclusively to cell surface junctions (Figure S2A). Claudin-1 distribution was unchanged after expression of a GFP-Vps4\textsuperscript{wt} control construct that does not alter endosomal trafficking (Figure S2C).

To assess whether the requirement of ESCRT function for the junctional localisation of claudin-1 is specific to kidney derived MDCK cells we investigated the distribution of junction proteins in the human colon cancer derived CaCo-2 cell line. Inhibition of ESCRT function with CHMP3\textsuperscript{1-179}GFP produced the same phenotype as seen in MDCK cells, an increase in intracellular claudin-1 (Figure S3A) and normal occludin, E-cadherin and ZO-1 localisation (Figure S3B-D).

Internal claudin-1 and CHMP3\textsuperscript{1-179}GFP partially colocalise with ubiquitin and the transferrin receptor

To establish the identity of the CHMP3\textsuperscript{1-179}GFP/claudin-1 positive intracellular vesicles the localisation of ubiquitin and a range of markers for endomembrane compartments were examined. There was a dramatic increase in the amount of ubiquitin-positive structures in CHMP3\textsuperscript{1-179}GFP transfected cells in comparison to non-transfected cells (Figure 2A) consistent with our previous work in Cos-7 and HeLa cells (Dukes et al., 2008). Accumulation of ubiquitin is also seen in Drosophila ESCRT mutants (Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005; Herz et al., 2006; Herz et al., 2009; Rodahl et al., 2009; Vaccari and Bilder, 2009; Vaccari et al., 2009). The ubiquitin partially colocalised with the CHMP3\textsuperscript{1-179}GFP (Figure 2A) and triple labelling showed that there was partial colocalisation between CHMP3\textsuperscript{1-179}GFP, claudin-1 and ubiquitin (Figure 3A arrow). This suggests that the dominant negative mutant is altering the flux of ubiquitylated proteins through the endocytic system. Intriguingly, the dominant negative did not markedly alter the distribution of early endosomal marker EEA1, the late endosomal marker Mannose-6-phosphate receptor (M6PR), the trans golgi network marker furin convertase, the lysosomal marker LAMP1 or the apical recycling endosome marker Rab11 (Figure 2B-F). There was also no significant colocalisation between the dominant negative compartment and these markers. This is in contrast to previous work in non-polarised Cos-7 and HeLa cells where early and late endosomal markers colocalised to the same compartment as CHMP3\textsuperscript{1-179}GFP (Dukes et al., 2008). In polarised MDCK cells transferrin is proposed to traffic through common recycling endosomes (Leung et al., 2000) and interestingly the transferrin receptor (TfR) accumulated in CHMP3\textsuperscript{1-179}GFP expressing cells (Figure 2G). Low levels of CHMP3\textsuperscript{1-179}GFP, that still caused claudin-1 accumulation, did not cause TfR accumulation (Figure 3B), but cells with higher levels of CHMP3\textsuperscript{1-179}GFP showed accumulation of TfR (Figure 3C+D). The intracellular TfR partially
colocalised with CHMP3<sup>1-179</sup>GFP and the intracellular claudin-1 (Figure 3D arrow). These results show that inhibiting ESCRT function in vertebrate epithelial cells does not alter the localisation of several markers of endomembranes, but does cause the accumulation of intracellular ubiquitin and TfR, both of which partially colocalise with the intracellular claudin-1.

**Claudin-1 is continually endocytosed and recycled to the cell surface in diverse epithelial cell types**

Inhibition of ESCRT function leads to intracellular accumulation of claudin-1 (Figure 1+3) and ESCRT function is required for the degradation of selected membrane proteins (Raiborg and Stenmark, 2009). Therefore, an inability of claudin-1 to be trafficked to lysosomes for degradation could explain its intracellular accumulation. However, perturbation of ESCRT function may also inhibit the recycling of transmembrane proteins and other endosomal sorting events (Yoshimori et al., 2000; Fujita et al., 2003; Doyotte et al., 2005; Baldys and Raymond, 2009). The intracellular claudin-1 also partially colocalises with TfR, which is a recycled cargo. Thus, intracellular accumulation of claudin-1 could be a result of inhibiting endocytic recycling.

To investigate the trafficking of claudin-1 and occludin in MDCK cells, a surface biotinylation assay was used. Surface claudin-1 and occludin were detected after labelling with a membrane-impermeant biotin label (Figure S4A, surface biotin) but not in pull-downs from mock treated cells, demonstrating that the pull-down is specific to biotinylated proteins (Figure S4A, non-specific). The biotin was also efficiently removed from labelled proteins by surface stripping (Figure S4A, strip control). For the endocytosis assay (Figure S4B) surface proteins on MDCK cells were labelled at 4°C. Following this, the cells were placed at 37°C to allow trafficking, biotin was stripped from surface proteins and analysis of the remaining biotinylated proteins was performed. Only those proteins that are surface labelled and then endocytosed will be resistant to surface stripping. Following a 60 minute incubation at 37°C approximately 35% of the biotinylated claudin-1 was resistant to surface stripping, showing that a significant amount of claudin-1 is endocytosed (Figure S4B, Endocytosis 60 min). The endocytosis is rapid as the amount of internal claudin-1 reached a plateau by 30 minutes (Figure S4C). Biotinylated occludin that was resistant to surface stripping could not be detected (Figure S4B, Endocytosis 60 min), suggesting that in MDCK cells occludin is not endocytosed in this time frame.

The fate of the endocytosed claudin-1 was then investigated. MDCK cells were labelled as described above and incubated at 37°C for 1 hour to allow endocytosis. Cells were surface stripped of biotin and then incubated again at 37°C for 20 minutes. Following this second 37°C incubation, cells were surface stripped for a second time or mock treated. The second stripping removes biotin from endocytosed proteins that have returned to the cell surface so recycling would be shown by a reduction in signal in the recycling lane relative to the degradation control. This revealed that the majority of internalised claudin-1 returns to the cell surface (Figure 4A, recycling 20 min). When cells were incubated for the second time but not stripped there was no reduction in levels of biotinylated claudin-1 (Figure 4A, degradation control). This is an important result which demonstrates that the loss of biotinylated claudin-1 is not due to it being targeted for degradation. The biotinylation
assays demonstrate that claudin-1, but not occludin, is constantly endocytosed and then recycled back to the plasma membrane in MDCK cells. It is possible that this dynamic endocytosis and recycling of claudin-1 occurs in cells that have just reached confluency but not in more mature monolayers. Recycling of claudin-1 was therefore investigated in MDCK cells that had been confluent for 10 days rather than 3 days. No difference in the amount of endocytosis or recycling was observed in MDCK cells with the more mature junctions (Figure S4D). The surface biotinylation assay also confirmed that the TfR is endocytosed and recycled (Figure S4E) as expected (Leung et al., 2000).

To establish whether claudin-1 recycling is a common feature of epithelial cells, the biotinylation assay was performed on CaCo-2 cells and 16-HBE cells, a human bronchial epithelial cell line (Figure 4B+C). The results show that claudin-1 is continuously endocytosed and recycled in both of these cell types, with only a very small proportion of claudin-1 being targeted for degradation (Figure 4B+C). However, whereas in the MDCK cells endocytosis of occludin was not observed, in CaCo-2 and 16-HBE cells approximately 40% of surface labelled occludin was internal following 60 minutes of incubation at 37°C. In these cell lines the fate of internalised occludin was split between degradation and recycling back to the plasma membrane (Figure 4B+C). To summarise, claudin-1 is endocytosed and recycled in all cell lines tested. This is in contrast to occludin that is endocytosed, then degraded or recycled in CaCo-2 and 16-HBE cells but not endocytosed in MDCK cells.

**ESCRT function is required for the continuous recycling of claudin-1**

The demonstration that claudin-1 is continually recycled suggests that a block in recycling could cause the intracellular accumulation of claudin-1 seen in cells expressing dominant negative ESCRT proteins. Testing this hypothesis with the biochemical assay requires that the dominant negative be expressed in a high percentage of cells. To achieve this, an adenovirus system was used, which gave expression in virtually all cells (Figure 5A). Expressing the CHMP3\(^{1-179}\)-GFP protein caused a striking inhibition of claudin-1 recycling, from 65% of the endocytosed protein in controls to just 4% in CHMP3\(^{1-179}\)-GFP adenovirus infected cells (Figure 5B+C). There was also an increase in the amount of endocytosed protein and a reduction in surface claudin-1 (Figure 5C+D). Consistent with the accumulation of TfR seen after expressing CHMP3\(^{1-179}\)-GFP, the recycling of TfR was also inhibited (Figure S5). This shows for the first time that ESCRT function is required for the recycling of claudin-1.

**Tsg101 is required to maintain a polarised single layered epithelial barrier**

The dominant negative ESCRT experiments show that ESCRT function is required for the recycling of claudin-1 but it remained to be established if ESCRT function is required to maintain epithelial polarity in vertebrates as it is in Drosophila. To study the requirement of ESCRT proteins in maintaining polarity siRNA knockdown of the ESCRT-I protein Tsg101 was used. This protein was chosen as it was initially identified as a potential tumour suppressor in mammalian cells (Li and Cohen, 1996) and it was one of the first ESCRT proteins shown to be required for polarity in Drosophila (Moberg...
et al., 2005). CaCo-2 cells rather than MDCK cells were used due to the availability of siRNA reagents for human genes.

Efficient knockdown of Tsg101 was confirmed by immunoblotting (Figure 6A) and analysis of the resulting phenotype demonstrated that there were large areas where the cellular architecture appeared disrupted (Figure 6B, arrow). In these regions the normal single layered organisation was lost and multilayered stacks of cells formed (Figure 6C). In other areas the cells appeared to maintain their normal monolayered organisation (Figure 6B, arrowhead).

Analysis of junction and polarity markers in Tsg101 knockdown cells showed that, in regions which maintained monolayer organisation accumulation of internal claudin-1 was observed, which partially colocalised with ubiquitin (Figure 6D). Intracellular claudin-4 also accumulated (Figure S7B). In contrast, there did not appear to be any internal accumulation or disruption of desmoglein 2 (a desmosome marker), occludin, E-cadherin or ZO-1 (Figure S6). This phenotype is consistent with results using the dominant negative ESCRT constructs. However, in the multilayered regions formed after Tsg101 knockdown a much more pronounced polarity defect was observed. In these regions, correct apicobasal polarity was disrupted and many cells were incorrectly oriented relative to the epithelial sheet (Figure 7). To establish if Tsg101 knockdown had a functional impact on epithelial barrier formation transepithelial resistance (TER) was monitored. Tsg101 knockdown significantly reduced TER compared to control at all time points measured (Figure 8A), demonstrating that Tsg101 is required for epithelial cells to form a tight diffusion barrier across an epithelial sheet.

**Tsg101 knockdown interferes with the polarisation of 3D cysts**

3D cultures are considered a more physiologically relevant way of investigating cell polarity in vitro (Martin-Belmonte and Mostov, 2008). We therefore employed a 3D epithelial cyst formation assay to further investigate the role of ESCRT proteins in regulating epithelial cell polarity. CaCo-2 cells treated with non-targeting siRNA developed into cysts with a single central lumen in 64% of cases (Figure 8B). Remaining cysts failed to develop a lumen or formed epithelial balls containing multiple small cavities. However, upon depletion of Tsg101 CaCo-2 cells formed cysts with a single lumen in only 42% of cases and the proportion of multi-lumen cysts doubled (Figure 8B). In summary, these results show that Tsg101 is required for correct epithelial polarity in both 2D cysts and 3D CaCo-2 cell cultures.

**Discussion**

In this study the consequences of perturbing ESCRT function on the polarity of vertebrate epithelial cells has been investigated for the first time. Our data demonstrate that inhibiting ESCRT function with dominant negative constructs or siRNA knockdown causes accumulation of internal claudin-1. The biotinylation assays show that claudin-1 is constitutively recycled in epithelial cells and that ESCRT function is required for this recycling. Therefore, the accumulation of claudin-1 in ESCRT deficient cells can be attributed to a block in this recycling pathway. Finally, the ESCRT protein Tsg101 is shown to be required for maintenance of polarity and establishment of a permeability barrier in epithelial sheets, as well as for epithelial cells to
correctly polarise in 3D cultures. Thus, ESCRT function is required for the recycling of a key junction protein and for the correct polarisation of vertebrate epithelial cells.

**Claudin-1 recycling is a feature of diverse epithelial cell types**

Claudins are crucial for producing a tight junction permeability barrier (Van Itallie and Anderson, 2006), which makes understanding the processes that regulate localisation of claudin proteins central to tight junction biology. Endocytosis of claudins can occur in epithelial cells by a peculiar mechanism involving internalisation of plasma membrane from juxtaposed cells (Matsuda et al., 2004) and expression of the ubiquitin ligase LNX1p80 drives endocytosis and degradation of claudin-1 (Takahashi et al., 2009). Our data demonstrates that in unstimulated epithelial monolayers internalised claudin-1 is constitutively and rapidly recycled back to the plasma membrane. We did not detect significant degradation of claudin-1 over the time frame of our assays (minutes), but previous work has shown that over longer time periods (hours/days) claudin-1 is degraded (Takahashi et al., 2009). We propose that, while the majority of endocytosed claudin-1 is recycled, a small percentage is directed for degradation producing a gradual turnover of claudin-1 in MDCK cells.

Claudin-1 is located in the lateral membrane in addition to the junctional complexes of polarised epithelial cells (Rahner et al., 2001; Van Itallie et al., 2003; Vogelmann and Nelson, 2005). Both of these pools are still present in cells expressing CHMP3\(^{1-179}\)GFP and we see no evidence of selective depletion of a particular pool. However, as there is a mobile fraction of claudin-1 in tight junctions (Shen et al., 2008) there may well be exchange between lateral and junctional pools of claudin-1. Depletion of one would then cause an indirect depletion of the other pool. This means an effect on a particular pool is likely to be difficult to observe.

This work focuses on claudin-1 but in addition to the accumulation of claudin-1 there was accumulation of claudin-2 in CHMP3\(^{1-179}\)GFP expressing MDCK cells and claudin-4 in Tsg101 knockdown CaCo-2 cells (Figure S7) suggesting that these claudins may also show ESCRT dependent recycling. However, there are many examples of individual claudins showing different behaviours, for example occludin dephosphorylation enhances exchange of junctional claudin-1 and claudin-2 but not claudin-4 (Raleigh et al., 2011), while EGF stimulation induces degradation of claudin-2 but not claudin-1 (Ikari et al., 2010), so a thorough analysis of the trafficking of different claudin family members is required to establish if they undergo ESCRT dependent recycling.

The continuous recycling of claudin-1 adds an important element to our understanding of claudin trafficking and supports a model whereby stability of cell contacts in epithelial monolayers is maintained by a balance between disassembly and assembly of cell junctions (Shen and Turner, 2008). Disruption of tight junction protein recycling would tend to lead to weakening or perhaps disassembly of tight junctions. Therefore, it is tempting to speculate that claudin recycling might be altered in the wide range of pathogenic states associated with altered epithelial junctions, such as cancer and inflammatory bowel diseases (Yang and Weinberg, 2008; Yu and Turner, 2008; Capaldo and Nusrat, 2009). This raises the issue of how this recycling is regulated. Our data shows that ESCRT function is required for claudin-1
This is consistent with previous work showing that ESCRT function is required for the recycling of EGFR and LDLR (Yoshimori et al., 2000; Fujita et al., 2003; Doyotte et al., 2005; Baldys and Raymond, 2009). The trafficking of internalised claudin-1 to the plasma membrane after a calcium switch requires Rab13 and its binding protein JRAB/MICAL-L2 (Yamamura et al., 2008). Rab13 is also required for the continuous recycling of occludin in the mouse mammary epithelial line (MTD1a) (Morimoto et al., 2005) making it possible that Rab13 will be required for the continuous recycling of claudin-1 described here. Future work will need to study ESCRTs, Rabs and other regulators of endocytic trafficking to establish how the recycling of claudin-1 is mediated.

Variation between the recycling of claudin-1 and occludin

A feature of this study is the differences seen between the trafficking of claudin-1 and occludin. Claudin-1 was recycled in kidney (MDCK), colon (CaCo-2) and lung (16-HBE) cell lines. In contrast occludin was recycled and degraded in CaCo-2 and 16-HBE cells, whilst in MDCK cells it was not endocytosed over the time frame analysed. Previous work has shown that, in MTD1a cells, occludin is rapidly endocytosed and recycled to the plasma membrane, whereas claudin-1 is not (Morimoto et al., 2005). It appears that there is cell type specific variation in the trafficking of claudin-1 and occludin, so that in some cells types they are both undergoing rapid trafficking while in others trafficking is restricted to one of these junction proteins.

Perturbing ESCRT function caused intracellular accumulation of claudin-1 but not occludin. In MDCK cells low levels of occludin endocytosis may explain this difference. However, in CaCo-2 cells claudin-1 and occludin are both endocytosed, but only claudin-1 accumulated. There are a number of independent recycling pathways (Grant and Donaldson, 2009) and our data may indicate that claudin-1 is returned to the plasma membrane by a pathway requiring ESCRT function, which is distinct from that used to recycle occludin. Consistent with junctional proteins following independent endocytic trafficking routes is data showing that selective internalisation of tight junction proteins occurs (Hopkins et al., 2003; Yu and Turner, 2008). This internalisation may be mediated by specific ubiquitin ligases such as LNX1p80 for claudin-1 (Takahashi et al., 2009), Itch for occludin (Traweger et al., 2002) and Hakai for E-cadherin (Fujita et al., 2002). Furthermore, in MDCK cells, E-cadherin returns to the plasma membrane via a Rab8 dependent route and tight junction proteins via a Rab13 dependent pathway (Yamamura et al., 2008). It will be interesting to establish the mechanism underlying potential differences in trafficking routes of junctional proteins and why there is variation across different cell types.

Junction recycling and loss of polarity

Individual ESCRT proteins are required to maintain polarity and prevent tissue overgrowth in Drosophila (Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005; Herz et al., 2006; Herz et al., 2009; Rodahl et al., 2009; Vaccari and Bilder, 2009; Vaccari et al., 2009) and our data shows that this requirement is conserved in vertebrate epithelial cells. It is currently not clear why ESCRT proteins are required to maintain polarity, but the widespread role of endocytic traffic in regulating signalling suggests
that altered polarity signalling may be responsible (Vaccari and Bilder, 2009). Based on our results we propose that a failure to return junctional proteins to the cell surface, where they normally function, provides an additional mechanism that might destabilise epithelial junctions and contribute to the loss of polarity. Simply inhibiting claudin-1 recycling is unlikely to explain the loss of polarity as claudin-1 knockout mice have an epidermal defect but apparently not a large scale loss of epithelial polarity (Furuse et al., 2002). However, epithelial cells contain many junctional proteins and it seems possible the recycling of a number of these could require ESCRT function, so a loss of recycling combined with alterations in signalling might cause the loss of epithelial polarity.

ESCRTs as tumour suppressors
The reduced ability of ESCRT knockdown cells to polarise supports the hypothesis that ESCRT proteins could function as tumour suppressors in mammals. Previous work has shown that the ESCRT-I component VPS37A has reduced expression in hepatocellular carcinoma (Xu et al., 2003), the ESCRT-III component CHMP1A has lower expression in pancreatic tumours (Li et al., 2008) and knocking down the expression of CHMP1A in human pancreatic ductal tumour cells increases cell growth (Li et al., 2008). However, mouse knockout studies have not supported a tumour suppressor function of ESCRT proteins (Ruland et al., 2001; Wagner et al., 2003), although it may be that apoptosis masks the tumourigenic potential, as is the case in Drosophila (Thompson et al., 2005). There are also reports of increased expression of ESCRT proteins in tumours (Liu et al., 2002; Oh et al., 2007; Toyoshima et al., 2007; Young et al., 2007) and it has been suggested that the overexpressed protein may have a dominant negative effect (Vaccari and Bilder, 2009). In cancers with reduced ESCRT function, the cells may have increased signalling from growth factor receptors such as the EGFR (Malerod et al., 2007). Our work shows that, in vertebrate epithelial cells, a reduction in ESCRT function promotes a loss of the normal polarised epithelial monolayer. Therefore, loss of ESCRT function in cancer cells could cause increased proliferation together with less stable tissue architecture, two of the key features acquired by tumour cells (Hanahan and Weinberg, 2000).

Materials and Methods
Antibodies, DNA constructs — The DNA constructs CHMP3\textsuperscript{1-175}GFP, GFP-Vps\textsubscript{4}\textsuperscript{E235Q} and GFP-Vps\textsubscript{4}\textsuperscript{WT} have been described previously (Whitley et al., 2003; Dukes et al., 2008) and the GFP construct used was pCS2-GFP (Chalmers et al., 2006). Rabbit anti-claudin-1* (59-9000), mouse anti-claudin-2* (32-5600), mouse anti-claudin-4* (32-9400), rabbit anti-occludin (71-1500), mouse anti-occludin* (33-1500), mouse anti-ZO-1 (33-9100), mouse anti-E-cadherin (33-4000), mouse anti-TfR (13-6800) and rabbit anti-Rab11 (71-5300) were all purchased from Zymed (San Fransico, CA, USA), and all used at a dilution of 1:25 for immunofluorescence and 1:1000 for Western blotting (anti-claudin-1, anti-occludin and anti-TfR). Rabbit anti-PKCζ (C-20; sc-216; 1:75) and mouse anti-CD63 (sc5275; 1:1000) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-ubiquitin (recognises mono- and polyubiquitinylated conjugates; clone FK2; 1:50) and rabbit anti-furin convertase (canine; 1:300) were purchased from Enzo Life Sciences
(Plymouth Meeting, PA, USA). Mouse anti-Tsg101 (4A10; ab83; 1:1000), rabbit anti-NUMB* (ab14140; 1:50), mouse anti-desmoglein-2 (ab14415; 1:25) and mouse anti-M6PR (ab2733; 1:200) were purchased from AbCam (Cambridge, UK). Mouse anti-EEA1 (1:100) was purchased from BD biosciences (610457). Mouse anti-β-tubulin (T4026; 1:5000) was purchased from Sigma. Mouse anti-GP135 (Ojakian and Schwimmer, 1988) was a kind gift from Professor George Ojakian (SUNY Downstate Medical Centre, New York, USA) and used at a dilution of 1:10. Mouse anti-LAMP1* (Developmental Studies Hybridoma Bank, University of Iowa, Iowa, USA) was a kind gift from Dr Scott Lawrence (UCL, London, UK) and used at 1:500. Species-specific fluorophore (Alexa Fluor® 488, 546 and 633 nm)-conjugated anti-IgG secondary antibodies were all purchased from Molecular Probes and used at dilutions of 1:200. Goat anti-mouse-HRP conjugated secondary antibody was obtained from Sigma and goat anti-rabbit-HRP was purchased from Pierce (Rockford, IL, USA) and used at a 1:5000 dilution.

Generation of adenovirus — The generation of high-titre, purified adenoviruses using the Iowa RapAd system has been described previously (Anderson et al., 2000). Briefly, Ad-expressing CHMP31-179GFP (Ad-CHMP31-179GFP) was prepared by subcloning the DNA construct into pacAd5 CMV K-N pA shuttle vector (a kind gift from Professor Beverly Davidson, University of Iowa). This was then digested with PacI alongside pacAd5 9.2-100 sub360 backbone vector and then mixed and transfected according to manufacturer’s instructions, into low passage HEK293 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were left for 10 days to allow recombination between digested shuttle and backbone vectors and for visible cytopathic effects to be observed. Lysates were then collected and used for further bulking of the virus. Ad vectors were then grown to high titre and purified using CsCl gradient methods, as previously described (Anderson et al., 2000; Caunt and McArdle, 2010).

Cell culture, transfections and transduction — MDCKII cells (Madin-Darby canine kidney cells; purchased from ECACC, cat no: 00062107) and 16HBE14o- (Cozens et al., 1994) (human bronchial epithelial cells; a kind gift from Professor Dieter Gruenert) were maintained at 37°C and 5% CO₂ in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% (v/v) FBS (fetal bovine serum), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. CaCo-2 cells (ECACC, cat no: 86010202) were similarly maintained except for the addition of 20% (v/v) FBS, 1x non-essential amino acids and 10 mM HEPES. All cell media and supplements were purchased from Lonza (Basel, Switzerland). Cells were tested for mycoplasma contamination using MycoAlert (Lonza). Cells were plated onto 13 mm coverslips in 24-well plates (Nunc) and grown until approx. 80-90% confluent when they were transfections with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Immunofluorescence — At 24 h post-transfection, cells were fixed with 4% (w/v) PFA in PBS for 20 min and permeabilised in methanol cooled to -20°C for 5 min. Cells stained with antibodies indicated by a * (see ‘Antibodies and DNA constructs’) were fixed and permeabilised with methanol cooled to -20°C
for 10 min. Cells were then blocked with 10% (v/v) FBS in PBS for 30 min. Primary and secondary antibodies were diluted in 2% FBS-PBS (2% FBS in PBS) and cells were incubated with primary antibodies for 2 h at 18°C and 1 h for secondary antibodies. Cells were washed five times for 5 min with 2% FBS-PBS following all antibody incubations. Stained cells were then mounted in Mowiol (Calbiochem) containing DAPI (Sigma) and examined on a Zeiss LSM510META laser-scanning confocal microscope and images taken.

Small interfering RNA (siRNA) knockdowns — siRNA oligonucleotides and reagents were purchased from Dharmacon (Thermo Fisher Scientific). Tsg101 was depleted using ON-TARGETplus individual siRNA duplex (CCGUUUAGAUCAAGAAGUA; J-003549-06). As a control, ON-TARGETplus Non Targeting siRNA was used. CaCo-2 cells were plated at high density into either 6 well plates (for western blotting; Nunc), or onto 13 mm glass coverslips (for immunofluorescence) and incubated with complete CaCo-2 growth media (lacking antibiotics) at 37°C, 5% CO₂ for approximately 4 h to adhere. Cells were transfected with 100nM of siRNA using DharmaFECT 1 transfection reagent according to the manufacturer's instructions and incubated at 37°C, 5% CO₂ for the desired time period, changing media after 3 d.

Endocytosis and recycling biotin assays
The biotinylation assay to study endocytosis and recycling of tight junction proteins was modified from a method described previously (Nishimura and Sasaki, 2008). Confluent cells plated on to 35 mm dishes were transferred to ice and washed with phosphate buffered saline supplemented with 0.9 mM calcium chloride and 0.33 mM magnesium chloride (PBS-CM). Cells were then incubated with the cleavable non-membrane permeable sulfo-NHS-SS-biotin (Pierce; in PBS-CM) at a concentration of 0.5 mg/ml for 30 min on ice. Free biotin was then quenched using 50 mM NH₄Cl (in PBS-CM) for 15 min (4°C). For the endocytosis assay, pre-warmed complete growth medium was added and cells returned to 37°C for indicated times. Cells were then transferred to ice to stop endocytosis, and surface (non-endocytosed) biotin was stripped by reduction with 100 mM 2-mercaptoethanesulphonate (MESNA) (in tris-buffered saline supplemented with calcium and magnesium; TBS-CM) for 3 x 10 min treatments (on ice). Internalised biotinylated cargo was protected from biotin stripping with MESNA by an intact membrane. Free – SH groups were then quenched by incubating cells with 5 mg/ml iodoacetamide (in PBS-CM) for 3 x 5 min.

For the recycling assay this process was repeated with 20 min incubations at 37°C in complete growth medium. To control for potential loss of biotinylated cargo via degradation, a recycling condition was included that lacked the second MESNA treatment. Thus any loss in biotinylated cargo at this step, relative to the endocytosis step, would indicate a loss in signal due to degradation of cargo instead of recycling. Cells were lysed (1.25% (v/v) Triton X-100, 0.25% (w/v) SDS, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 5 mg/ml iodoacetamide, 10 µg/ml APMSF) on ice, pulse sonicated, and centrifuged at 15,000g to remove large/nuclear debris. An equal volume of the post-nuclear supernatant was taken from each sample for use as a loading control. Biotinylated proteins were collected by incubation
with Neutravidin beads (Pierce), rotating overnight at 4°C. Beads were then washed by centrifuging at 1000g, 5 times with wash buffer (0.5% (v/v) Triton X-100, 0.1% (w/v) SDS, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA) and 3 times with PBS-CM. Reducing sample buffer was then added to each sample to release biotinylated proteins from the beads and following boiling, samples were loaded onto a 15% tris-glycine SDS-PAGE gel. Separated proteins were transferred to nitrocellulose and immunoblotted for the protein indicated. Signals were detected by ECL® (enhanced chemiluminescence) or Chemiglow West Chemiluminescence Substrate (Alpha Innotech, San Leandro, CA, USA) and quantified using an Optichem detector with associated software (Ultra Violet Products). For quantification of the biotinylated proteins, the amount of claudin-1 and occludin was normalized to their respective total protein bands determined from non-isolated lysate samples. Where results were plotted graphically (Graphpad Prism), values were expressed as a percentage of the total claudin-1 or occludin biotinylated at the surface. For recycling assays using Ad-CHMP3-H179GFP infections, MDCKII cells were allowed to grow past confluency on 35 mm dishes, then infected with Ad-CHMP3-H179GFP for 16 hours and subsequently surface biotinylated. The recycling assay was then performed as described.

_Caco-2 TER measurements_ – CaCo-2 cells were plated into 6 well Nunc plates and transfected with Non Targeting or Tsg101 siRNA as detailed previously. Cells were incubated at 37°C, 5% CO₂ for 3 d. Cells were washed in PBS, trypsinised and resuspended in CaCo-2 growth media. Cells were plated in triplicate on Transwell® (Corning, Corning, NY, USA; cat no. 3470) permeable polyester filters (0.4µm pore size, 0.33cm² surface area) at 2.5 x 10⁵ cells/filter and incubated at 37°C, 5% CO₂. TER was measured every 24 h for 4 d using a EVOM TER machine with an Endohm-6™ chamber (World Precision Instruments, Sarasota, FL, U.S.A.), changing media after every reading.

_Caco-2 3D cyst culture and immunofluorescence_ - CaCo-2 cells were plated into 6 well Nunc plates and transfected with Non Targeting or Tsg101 siRNA as detailed previously. Cells were incubated at 37°C, 5% CO₂ for 3 d. Cells were washed in PBS, trypsinised and resuspended in antibiotic-free CaCo-2 growth media. A cell:matrix mix was prepared containing 5.8 x 10⁴ CaCo-2 cells/ml, 0.02 M HEPES, pH 7.4, 1 mg/ml Collagen I (Inamed Biomaterials, Fremont, CA, USA,) and 40% Growth Factor Reduced BD Matrigel™ Matrix (BD Biosciences, Franklin Lakes, NY, USA). The cell:matrix mix was plated into an 8 chamber slide, incubated at 37°C, 5% CO₂ for 1 h to solidify and overlaid with antibiotic-free CaCo-2 growth media. Cysts were allowed to develop for 10 d at 37°C, 5% CO₂, changing media every 3-4 d. After 10 d culture, cysts were washed in PBS, treated with 5U/ml Collagenase VII (Sigma-Aldrich) for 15 min and fixed in 4% (w/v) PFA for 30 min at room temperature. Cysts were washed three times for 20 min with 1X wash buffer (10X stock, pH 7.4: 1.3M sodium chloride, 70 mM dibasic heptahydrate sodium phosphate, 30 mM monobasic monohydrate sodium phosphate, 77 mM sodium azide, 1% (w/v) BSA, 2% (v/v) Triton-X 100, 0.4% (v/v) Tween-20; all Sigma). Cysts were incubated with blocking buffer (10% (v/v) FBS in
1X wash buffer) for 1 h at room temperature followed by incubation with primary antibodies diluted in blocking buffer overnight at 4°C. Cysts were washed three times for 20 min with 1X wash buffer and incubated with secondary antibodies diluted in blocking buffer for 1 h at room temperature. After a further three 20 min washes with 1X wash buffer, cysts were rinsed twice in PBS and post-fixed in 4% (w/v) PFA for 30 min at room temperature. Cysts were washed three times for 5 min with PBS and incubated with DAPI (4 μg/ml) for 30 min at room temperature to stain the nuclei. After a final rinse in PBS, cysts were mounted in Mowiol (Calbiochem) and examined on a Zeiss LSM510 META laser-scanning confocal microscope.

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References


Figure 1. Perturbing ESCRT function in MDCK cells results in an intracellular accumulation of claudin-1. CHMP3<sup>1-179</sup>GFP expressing MDCK cells were stained with antibodies against; (A) occludin, (B) ZO-1, (C) E-cadherin and (D) claudin-1. Claudin-1 accumulates intracellularly and extensively overlaps with the CHMP3<sup>1-179</sup>GFP compartment. Z-sections are displayed below each of the panels and arrows indicate junctional region of CHMP3<sup>1-179</sup>GFP expressing cells (marked with a *). Arrowheads show internal overlap of claudin-1 and CHMP3<sup>1-179</sup>GFP. Antibody staining (red) and CHMP3<sup>1-179</sup>GFP (green) are shown together with merged images and DAPI (blue; right panels). (E) GFP (green) expressing cells (marked with a *) were stained with antibodies against claudin-1 (red). Dotted lines show position of Z projections. Bar 10µm.
Figure 2. Perturbing ESCRT function in MDCK cells results in ubiquitin and TfR accumulation. CHMP3^{1-179}GFP expressing MDCK cells were stained with antibodies against; (A) ubiquitin, (B) EEA-1 (early endosomes), (C) M6PR (late endosomes), (D) Furin convertase (TGN), (E) LAMP-1 (lysosomes), (F) Rab11 (recycling endosomes), (G) TfR (a recycled protein). Expression of CHMP3^{1-179}GFP caused accumulation of ubiquitin and TfR. The distribution of the other markers was unchanged. Antibody staining (red) CHMP3^{1-179}GFP (green) and nuclei stained with DAPI (blue) are shown together with merged images (right panels). Bar 10µm.
Figure 3. The accumulated intracellular claudin-1 partially colocalises with ubiquitin and TfR. CHMP3^{1-179}GFP expressing MDCK cells were stained with antibodies against; (A) Ubiquitin and (B-D) TfR. CHMP3^{1-179}GFP, claudin-1 and ubiquitin/TfR show an overlapping distribution in some regions (arrows), but co-localisation is not complete (arrowheads). The TfR distribution seems to be dependent on the level of expression of CHMP3^{1-179}GFP as illustrated by the differing phenotypes. CHMP3^{1-179}GFP (green) claudin-1 (red) and ubiquitin/TfR (blue) are shown with merged images (far right panels). Bar 10µm.
Figure 4. Claudin-1 is constitutively endocytosed and recycled back to the plasma membrane in different epithelial cell types. The surface biotinylation, endocytosis and recycling assay was performed on; (A) MDCK cells. (B) CaCo-2 cells. (C) 16-HBE cells. Lanes marked ‘Surface biotinylated’ represent the initial biotinylated protein at the cell surface and ‘Endocytosis 60 min’ is the internal biotinylated protein that is resistant to surface stripping. Degradation is shown by a reduction of signal in the ‘Degradation control’ lane in comparison to the ‘Endocytosis 60 min’ lane. Recycling is the reduction of signal in the ‘Recycling 20 min’ lane relative to the ‘Degradation control’ lane. The data shown graphically are the means +/- standard deviation from four independent experiments.
Figure 5. ESCRT function is required for the normal recycling of claudin-1 in MDCK cells. (A) Infection of MDCK cells with Ad-CHMP3$^{1-179}$GFP resulted in most cells expressing the protein. CHMP3$^{1-179}$GFP (green), nuclei (blue). (B+C) Cells were either mock treated (left blot) or infected with Ad-CHMP3$^{1-179}$GFP (right blot) for 16 h and the biotin endocytosis and recycling assay performed. The data shown graphically are the means +/- standard deviation from three independent experiments. In Ad-CHMP3$^{1-179}$GFP treated cells there is no significant reduction in the ‘Recycling 20 min’ lane indicating that claudin-1 is not recycled. (D) Quantification of surface biotinylated claudin-1 showed a reduction in Ad-CHMP3$^{1-179}$GFP infected cells.
Figure 6. siRNA knockdown of Tsg101 disrupts epithelial monolayer organisation and causes internal accumulation of claudin-1 and ubiquitin in CaCo-2 cells. (A) CaCo-2 cells were transfected with either Non-Targeting Control siRNA ‘C’ or Tsg101 siRNA ‘T’, incubated for 3, 7 and 10 days and lysates immunoblotted for Tsg101 and β-tubulin as a loading control. (B) CaCo-2 cells were transfected with either Non-Targeting Control siRNA or Tsg101 siRNA and analysed via light microscopy after 7 days. Tsg101 knockdown cells form monolayers in some areas (arrowheads) but many regions are observed where epithelial organisation appears disrupted.
(arrows). (C) Nuclei of knockdown cells stained with DAPI. Tsg101 knockdown disrupts cellular architecture and regions of cells show a multilayered organisation. (D) After 7 day knockdown, cells were stained for claudin-1 (green) and ubiquitin (red). Knockdown of Tsg101 resulted in increased levels of internal claudin-1 and ubiquitin. There was some overlap between them (arrows). Images in D are from regions of the knockdown where monolayered organisation was maintained, a more striking polarity phenotype is seen in the multilayered regions (Figure 7). Merged images with nuclei stained with DAPI (blue) are shown. Bar 10µm.
Figure 7. Tsg101 is required to maintain a polarized epithelial monolayer in CaCo-2 cells. CaCo-2 cells were transfected with either Non-Targeting Control siRNA or Tsg101 siRNA, incubated for 7 days and stained for E-cadherin (green) and aPKC (red) to visualise basolateral and apical surfaces, respectively. Tsg101 knockdown cells form multilayered regions with compromised apico-basal polarity. Nuclei were stained with DAPI (blue). Apical confocal slices and corresponding z-sections (indicated by dotted line) are displayed. Bar 10µm.
Figure 8. Tsg101 siRNA knockdown decreases transepithelial resistance (TER) and impairs formation of CaCo-2 3D cysts. (A) CaCo-2 cells were transfected with either Non-Targeting Control siRNA or Tsg101 siRNA, replated on day 3 to transwell filters and TER was measured between day 4 and day 7. (B) CaCo-2 cells were transfected with either Non-Targeting Control siRNA or Tsg101 siRNA, incubated for 3 days and replated into a Matrigel suspension. Following incubation for 10 days, cysts were stained for aPKC (red) and E-cadherin (green) to mark the apical and basolateral membranes respectively. Nuclei were stained with DAPI (blue). Cysts display either a single lumen, multiple lumen or no lumen. Z-sections are displayed for each cyst along with a confocal plane through the centre. Bar 10µm. The number of cysts showing either single, multiple or no lumen was quantified (bar graph). Data shown is the mean from three independent experiments, >100 cysts were analysed per condition for each experiment. Error bars represent standard deviation. Results were analysed using a t-test, *** p<= 0.001 * p<= 0.05.