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Continuous endocytic recycling of tight junction proteins: How and why?

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Abstract

Tight junctions consist of many proteins including transmembrane and associated cytoplasmic proteins, which act to provide a barrier regulating transport across epithelial and endothelial tissues. These junctions are dynamic structures that are able to maintain barrier function during tissue remodelling and rapidly alter it in response to extracellular signals. Individual components of tight junctions also show dynamic behaviour including migration within the junction and exchange in and out of the junctions. In addition, it is becoming clear that some tight junction proteins undergo continuous endocytosis and recycling back to the plasma membrane. Regulation of endocytic trafficking of junctional proteins may provide a way of rapidly remodelling junctions and will be the focus of this review.

An introduction to tight junctions

Tight junctions were first identified in electron micrographs showing close contacts linking adjacent epithelial cells [1]. These junctions that are also found in endothelial cells, provide a barrier that regulates diffusion through the paracellular space (Figure 1). The history, function and molecular components of the tight junctions has been recently reviewed [2-3] Here we will provide a brief introduction to tight junctions before focusing on their dynamic nature and in particular the endocytic recycling of individual tight junction proteins.

Although tight junctions provide a barrier to diffusion, this barrier is not absolute as it allows the passage of certain solutes [2]. In addition the permeability of tight junctions varies between different epithelial cells so that some epithelia form a “tight” barrier while others are “leaky”. Tight junctions not only provide a barrier but there is growing evidence they act as a platform for cell signalling molecules that regulate cellular behaviours including gene expression and cell proliferation rates [4].

Molecular studies have shown that tight junctions consist of a number of proteins, including members of the claudin, occludin and junctional adhesion molecule (JAM)

families of transmembrane proteins [2]. The claudin proteins, consisting of over 20 members in humans, are thought to be the main mediators of the epithelial permeability barrier [5-7]. There are many lines of evidence to support this hypothesis, for example claudins can promote tight junction strand formation when expressed in fibroblasts [8] and mouse knockout studies show alterations in barrier function in a wide range of tissues including the skin for claudin-1 knockouts [9] and kidney for claudin-2 knockouts [10]. Importantly, some claudins promote a high resistance barrier to diffusion, while others are thought to form pores that allow ionic diffusion [5]. Thus, variations in the expression profile of claudins is proposed to be a major determinant of tissue-specific variations in permeability [7]. The other transmembrane tight junction proteins appear to have a role in the regulation/modulation of this claudin based barrier [2-3]. A range of cytoplasmic proteins can associate with the transmembrane proteins, such as ZO-1 and cingulin, which indirectly link tight junctions to the actin cytoskeleton and various signalling proteins [11-13]. The formation of these large multi-subunit structures allows the tight junctions to fulfil the various functions associated with them, including barrier formation and regulation of cell signalling.

The dynamic nature of tight junctions

Tight junctions are found in tissues that show dynamic behaviours. For example, in the mammalian intestine, epithelial cells are replaced every 4/5 days [14] and during pregnancy there is a massive increase in mammary epithelial cells which are then removed at the end of lactation [15]. During the addition and removal of cells tight junctions must be able to maintain barrier function. In addition tight junctions must be remodelled during the transmigration of immune cells through epithelia and during wound healing [3]. Many stimuli, including inflammatory cytokines and bacterial infection, also rapidly modify the tight junction barrier [16]. These observations suggest that rapid alteration of tight junction composition occurs and there is general acceptance that tight junctions, like the epithelial tissues they are found in, are dynamic structures. The dynamic behaviour of the tight junctions includes movement of tight junction strands and of individual tight junction proteins, which can move within the tight junctions and in and out of the junctions [2-3]. There also appears to be continuous endocytosis and recycling back to the plasma membrane of tight

junction proteins in a range of epithelial cells types [17-19], an aspect to tight junction dynamics that will be the focus of this chapter.

Is continuous recycling of tight junction proteins a common feature of epithelial cells?

Following endocytosis from the plasma membrane into early endosomes, transmembrane cargo proteins can follow the degradative pathway to the lysosome or be recycled back to the plasma membrane. They can also undergo retrograde transport to the trans golgi network (TGN) where they may undergo further sorting [20]. In epithelial cells the picture is further complicated by endocytosis from distinct apical and basolateral membrane domains into apical or basolateral early endosomes and transcytosis between the two domains [4, 21] (Figure 2). There are also multiple independent pathways responsible for recycling proteins back to the plasma membrane [22] making endocytic protein trafficking, particularly in epithelial cells, an extremely complex process.

Identifying the trafficking fate of an endocytosed transmembrane tight junction protein can be achieved using a biotinylation assay that labels extracellular lysine residues of proteins allowing their trafficking to be monitored [23]. The biotinylation assay was used to provide the first evidence that tight junction proteins can be continuously recycled in confluent epithelial cells when occludin was found to be endocytosed and recycled back to the plasma membrane in a mouse mammary cell line (MTD1A) [19]. In contrast, claudin-1 was not endocytosed in these cells. However, a potential limitation of this study is that the rate of degradation of occludin was not measured. Due to the nature of the biotinylation assay employed it is possible that at least some of the occludin trafficking classified as recycling could have been a consequence of degradation.

Subsequent experiments with MDCK II cells, a canine kidney line [17] which is commonly used to study tight junctions, examined the trafficking of claudin-1 and occludin. Importantly, this work added an additional control so that degradation and recycling could be distinguished. Claudin-1 was found to be constantly endocytosed and recycled in MDCK II cells, with no detectable degradation observed over the time frame of these assays (20 minutes for degradation and recycling). Over longer time

periods (hours/days) claudin-1 is degraded [24] indicating that a small percentage of endocytosed claudin-1 is directed for degradation in MDCK II cells. This would produce a gradual turnover of claudin-1 protein. Subsequent work showed that, like claudin-1, claudin-2 is endocytosed and recycled in MDCK II cells [18]. In contrast, significant endocytosis of occludin [17] and claudin-4 [18] was not detected in MDCK II cells using the same assay and incubation time (1 hour), showing that tight junction proteins have different rates of flux through the endocytic system.

Analysis of additional epithelial cell lines showed that claudin-1 is constantly recycled in the human colon-cancer derived line CaCo-2 and the human lung derived line 16-HBE[17]. Interestingly, unlike in MDCK II cells, in these lines occludin was endocytosed. Its fate was then split with some protein being recycled and the rest degraded. Combining results from the four different epithelial cell lines currently analysed shows that recycling of at least a subset of tight junction proteins is a common feature of epithelial cells. In addition there is cell type specific variation in the rate that individual tight junction proteins are trafficked through the endocytic system. This data is summarised in Table 1. Most of this work is very recent and there are a number of issues that remain to be addressed. For example, it will be important to determine which other tight junction proteins are recycled and whether similar rates of recycling occur *in vivo*. It is also important to stress that the biotin assay does not label cytoplasmic proteins. This means it cannot be used to follow the fate of proteins such as ZO-1, which could be transported with the transmembrane tight junction proteins they associate with.

How is endocytosis of tight junction proteins mediated?

The first step in endocytic trafficking is the internalisation of transmembrane proteins from the plasma membrane. Endocytosis can occur via clathrin dependent [25] and clathrin independent mechanisms which include caveolae-driven endocytosis and macropinocytosis [26]. Endocytosis of tight junction proteins has been reported to occur by all of these mechanisms: for example calcium depletion, a non-physiological stimulus that induces tight junction breakdown, triggers endocytosis of occludin, claudin-1 and JAM-A by a clathrin-mediated pathway [27]. Studies on stimulus-induced remodelling have revealed clathrin-independent internalization of tight

junction proteins, For example, occludin endocytosis occurs via caveolae following *Escherichia coli* cytotoxic necrotizing factor-1 (CNF-1) stimulation [28] or TNF α stimulation [16]. Recent work also shows that the chloride channel CIC-2 acts to reduce caveolae mediated endocytosis of occludin in CaCo-2 cells [29]. Claudin-1, occludin and JAM-A are internalised via macropinocytosis in IFN- γ treated T84 cells [30]. Live imaging studies using fluorescent fusion proteins have revealed a further feature of endocytosis for claudin-3 [31]. This involves a peculiar mechanism whereby plasma membrane from two juxtaposed cells are internalised into one of the cells. This has been referred to as "eat-each-other" endocytosis.

It should also be noted that different tight junction proteins are not always endocytosed *en-masse* but can be endocytosed independently of one another. For example, EGF stimulation of MDCK II cells increases endocytosis and degradation of claudin-2 without affecting claudin-1 [32]. In addition, occludin endocytosis occurs in latrunculin A treated MDCK II cells [33] and *in vivo* in anti-CD3 treated mice [34] without concomitant internalisation of claudin proteins. The ability to independently endocytose proteins may allow cells to alter the repertoire of tight junction proteins at the plasma membrane and fine-tune paracellular permeability in response to changing conditions.

An interesting question is what determines the selection of tight junction proteins as endocytic cargo and one possibility is that post-translational modification of individual tight junction proteins is responsible. Ubiquitylation, phosphorylation and palmitoylation are reversible modifications that have all been linked to endocytosis [21]. Transmembrane tight junction proteins have also been shown to undergo these modifications [35-36]. Expression of the ubiquitin ligase LNX1p80 drives endocytosis of claudin-1 [24] while an alternative ubiquitin ligase Itch promotes endocytosis of occludin [37]. An attractive hypothesis is that low level activity of these, and possibly other ubiquitin ligases, promote continuous endocytosis of tight junction proteins. Regulation of specific ubiquitin ligase activity could also alter endocytic rates of individual tight junction components. However, knockdown or knockout experiments ablating the function of these proteins and analysis of the effect on endocytosis rates are required to establish if this is the case. Phosphorylation of

claudins, occludin and JAMs have all been reported, with the phosphorylation status having an affect on tight junction localisation [38-41]. For example phosphorylation of serine residue 490 of occludin is associated with its endocytosis and barrier loss in endothelial cells [42]. Finally, claudin family proteins contain conserved signature cysteine residues that are palmitoylated and are required for efficient localisation at tight junctions [43]. Palmitoylation is a reversible protein modification that has been linked to endocytosis [44] in addition to other protein trafficking events [21, 45-46] making it a potential regulator of claudin endocytosis.

Another important issue is where endocytosis of tight junction proteins occurs. It is plausible that tight junction proteins are not endocytosed from the junctions themselves but from adjacent (or even distant) regions of the plasma membrane. Live imaging and computer modelling experiments have been performed to investigate the dynamics of individual tight junction components in living cells [47]. These studies indicate that occludin within the tight junctions continuously exchanges with an extra-tight junction pool. Furthermore, claudin-1 is located in the lateral membrane in addition to the junctional complexes of polarised epithelial cells [48-50]. Cytoskeletal rearrangements at tight junctions are likely to be crucial in facilitating junctional remodelling and endocytosis. If endocytosis occurs away from the tight junctions, then regulation of the rate that tight junction proteins move out of junctions and into regions of the plasma membrane undergoing active endocytosis would provide another possible mechanism of regulating endocytic cargo selection. The mechanisms, pathways and modifications mentioned are not mutually exclusive and a complex interplay is likely to regulate selection for endocytosis.

Regulation of recycling and junctional remodelling

While many studies have considered stimulated endocytosis as an important process in tight junction re-modelling, relatively little attention has focussed on post-endocytic sorting of tight junction components. However, the continuous recycling of junctional proteins [17-19] raises the possibility that a reduction in the rate of recycling could achieve the same goal as increased endocytosis, that is, depleting tight junction proteins from the plasma membrane and causing either an accumulation of internal protein or increased degradation. The post-translational modifications, ubiquitylation, phosphorylation and palmitoylation, mentioned previously, all have

roles to play in post-endocytic sorting [21], in addition to endocytosis, and so provide potential signals to regulate the rate of recycling of tight junction components. This leads to the question of whether control of post-endocytic sorting, in particular recycling, participates in junctional re-modelling.

Evidence to suggest that control of post-endocytic sorting is important comes from studies on the fate of tight junction proteins in IFN γ treated epithelial cells [30, 51]. Occludin, claudin-1 and JAM-A all accumulate intracellularly, in compartments containing recycling endosome markers Rab4 and Rab11, following IFN γ treatment with no increase in degradation of these proteins. Removal of IFN γ results in release of the accumulated proteins back to tight junctions so it is reasonable to postulate that IFN γ treatment blocks recycling. Similarly, other manipulations of epithelial cells, such as removal of calcium [27] or incubation with CNF-1 [28] reversibly displace tight junction proteins to intracellular locations, indicating that regulation of recycling may be a common mechanism by which tight junction composition can be controlled. Intriguingly, following CNF-1 treatment, occludin does not co-localise intracellularly with claudin or JAM-A, providing evidence that these proteins could be recycled by distinct routes. Control of tight junction recycling is not limited to epithelial cells as claudin-5 and occludin recycle in endothelial cells following recovery from chemokine treatment [52].

Post-endocytic sorting and trafficking of tight junction proteins

In order to understand how stimuli might modify post-endocytic trafficking it is important to establish the endocytic routes taken by tight junction proteins and identify the proteins responsible for this trafficking. Following endocytosis the early endosomes are the primary destination for material removed from the plasma membrane. Proteins entering these endosomes must be sorted to degradative, recycling or retrograde endocytic trafficking pathways and this sorting is a major determinant of the fate of endocytosed proteins [21].

A group of proteins which are tightly linked to post-endocytic sorting is the Endosomal Sorting Complex Required for Transport (ESCRT). This complex has a well established role in the trafficking of ubiquitylated transmembrane proteins to

lysosomes for degradation [53-54] and is important for attenuating signalling from growth factor receptors such as the epidermal growth factor receptor (EGFR) [55]. However, in addition to blocking the degradative pathway, inhibiting ESCRT function can cause defects in the recycling of receptors such as those for EGF, transferrin, asialoglycoprotein and low-density lipoprotein [56-59]. Experiments using a dominant negative ESCRT protein showed that ESCRT function is required for the continuous recycling of claudin-1 and when ESCRT function is inhibited claudin-1 accumulates intracellularly [17]. Why is ESCRT function required for claudin-1 recycling? ESCRT proteins are known to interact with deubiquitylating enzymes and the timing of deubiquitylation is emerging as an important factor in modulating the fate of endocytosed proteins [60-61]. Therefore, it is possible that perturbations in the ESCRT machinery leads to mis-regulation of ESCRT associated deubiquitylating enzymes resulting in incorrect post-endocytic sorting and a failure in claudin-1 recycling.

Addition of YM201636, a small molecule inhibitor of the lipid kinase PIKfyve also inhibits claudin-1 and claudin-2 recycling [18]. This kinase is responsible for the synthesis of PtdIns(3,5)P₂ from PtdIns(3)P on early endosomes and its function has been linked to a number of endocytic sorting events [62-63]. It is unclear why PIKfyve function might be required for recycling of claudins, but as PtdIns(3,5)P₂ binds the ESCRT III component Vps24/CHMP3 *in vitro* [64], it is possible that recycling is blocked by inhibiting the recruitment of the ESCRT machinery to endosomes.

Members of the Rab family of small GTPases have well documented roles in vesicular trafficking [65] and experiments using a dominant-negative construct showed that Rab13 is required for the continuous recycling of occludin in MTD1A cells [19]. siRNA knockdown experiments have also shown that the trafficking of internalised claudin-1 to the plasma membrane after a calcium switch (calcium depletion and subsequent repletion) requires Rab13 and its binding protein JRAB/MICAL-L2 [66]. This identifies Rab13 as a key mediator of tight junction recycling although it also has an additional role in the biosynthetic delivery of cargoes from the TGN to endosomes in polarised epithelial cells [67]. Rab11 is well known to be involved in recycling, and tight junction proteins accumulate in Rab11 positive

compartments following stimulation with IFN γ or CNF-1 [28, 30], but we are not aware of any publications that have investigated the requirement of Rab11 in tight junction protein recycling. The ESCRT, PIKfyve and Rab13 studies described above have identified the first few proteins whose function is required for continuous tight junction recycling. However, it is clear that much more research is needed to provide a full description of the molecular basis for these events and to elucidate how specificity of recycling of individual tight junction proteins is achieved.

Why do cells continuously recycle tight junction proteins?

The final and perhaps most important question is why should cells expend the energy required to constantly move tight junction proteins into the cell and then back to the cell surface? A simple explanation is that no system is perfect and as some proteins may be internalised inappropriately, recycling would provide an efficient system to return these to the plasma membrane. Tight junction recycling may also have evolved as a consequence of the dynamic nature of tight junctions. If a cell needs to form more tight junctions it can reduce endocytosis and/or increase the rate of recycling to increase the amount of tight junctions at the cell surface. Conversely, if the area of tight junctions needs decreasing a cell can promote endocytosis and/or reduce recycling to remove excess tight junction proteins from the plasma membrane. This provides a flexible way to rapidly deal with physiological variations in tight junction size. It could also be used to fine-tune barrier function: for example, the composition of claudins at the plasma membrane could be altered to allow cells/tissues to regulate of paracellular permeability.

The recycling of tight junctions may also be important in pathological conditions. Alterations of tight junctions have been associated with tumour formation [68] and blocking recycling would reduce the amount of functional tight junction protein at the plasma membrane, perhaps reducing the stability of epithelial tissues. Modulation of tight junctions also occurs in inflammatory bowel disease and following bacterial infection [16, 28, 30, 51]. The evidence described above suggests that inflammatory cytokines and bacterial proteins may act in part by blocking tight junction recycling. These potential links to disease illustrate the importance of future work aimed at fully understanding this process.

Conclusions

The demonstration that tight junction recycling appears to be a common feature of epithelial cells throws up many questions such as how is it controlled and why is it important? Our understanding is still at a rudimentary level: tight junction recycling is known to occur in several different types of epithelial cells and the first few proteins which are required for this process have been identified, but many more experiments are required to provide a true mechanistic understanding. Key issues that need to be addressed include what proteins mediate trafficking through the endocytic system, is there more than one tight junction recycling pathway and whether this process is regulated. Rates of recycling *in vivo* also need to be determined and finally, the question of whether tight junction recycling is altered during pathological conditions such as cancer, inflammatory bowel diseases and bacterial infection needs to be addressed.

Summary

- Tight junctions provide a permeability barrier that is dynamic and constantly undergoing remodelling.
- A number of tight junction components, including claudin and occludin, are constitutively endocytosed and recycled in epithelial cells.
- There is cell type specific variation in the tight junction proteins that are recycled.
- ESCRT, PIKfyve, Rab13 are required for tight junction recycling.
- Tight junction recycling may be aberrantly regulated in disease.

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Biography of each author

Andrew D Chalmers

My research career started in 1996 as a Wellcome Prize PhD student at the University of Bath where I investigated the development of the *Xenopus* digestive system. I then moved to a postdoctoral position at the Gurdon Institute, Cambridge and worked on epithelial polarity and asymmetric division. In 2004 I was awarded an MRC Career Development Fellowship and in 2005 a RCUK Academic Fellowship at the University of Bath. My group investigates the relationship between epithelial cell polarity, junctions and proliferation. This has involved a key collaboration with Dr Paul Whitley investigating the role of endosomal trafficking in polarity.

Paul Whitley

I obtained my Ph.D on protein secretion in yeast at the University of Edinburgh in 1991. I then took up postdoctoral positions at the Karolinska Institute (Novum) in Stockholm and Stockholm University working on membrane protein assembly. I moved to the Department of Clinical Immunology at the Karolinska Hospital/Institute before returning to the UK and the University of Bath in 1999. I have been a lecturer in the Department of Biology and Biochemistry at the University of Bath since 2000. My group is interested in endosomal trafficking and recently in collaboration with Dr Andrew Chalmers we have been investigating its role in cell polarity.

Key words

Biotinylation assay, CaCo-2, claudin, endocytosis, endosomes, epithelia, ESCRT, JAM, MDCK II, occludin, PIKfyve, Rab13, recycling, tight junctions, ubiquitin.

	MDCK II	CaCo-2	16-HBE	MTD-1A
Tight junction proteins				
Claudin-1	Recycled [17]	Recycled [17]	Recycled [17]	Not endocytosed* [19]
Claudin-2	Recycled [18]	-	-	-
Claudin-4	Not endocytosed* [18]	-	-	-
Occludin	Not endocytosed* [17]	Degraded and recycled [17]	Degraded and recycled [17]	Recycled [19]

Table 1 Continuous recycling of tight junction proteins in polarised epithelial monolayers. The continuous recycling of tight junction proteins has been studied in the canine kidney line MDCK II, the human colon cancer line CaCo-2, the human lung epithelial line 16-HBE and the mouse mammary line MTD-1A. * There was no detectable endocytosis in the time frame of the biotinylation assays (often one hour) but there may be a slower rate of endocytosis which would produce a gradual turnover in protein.

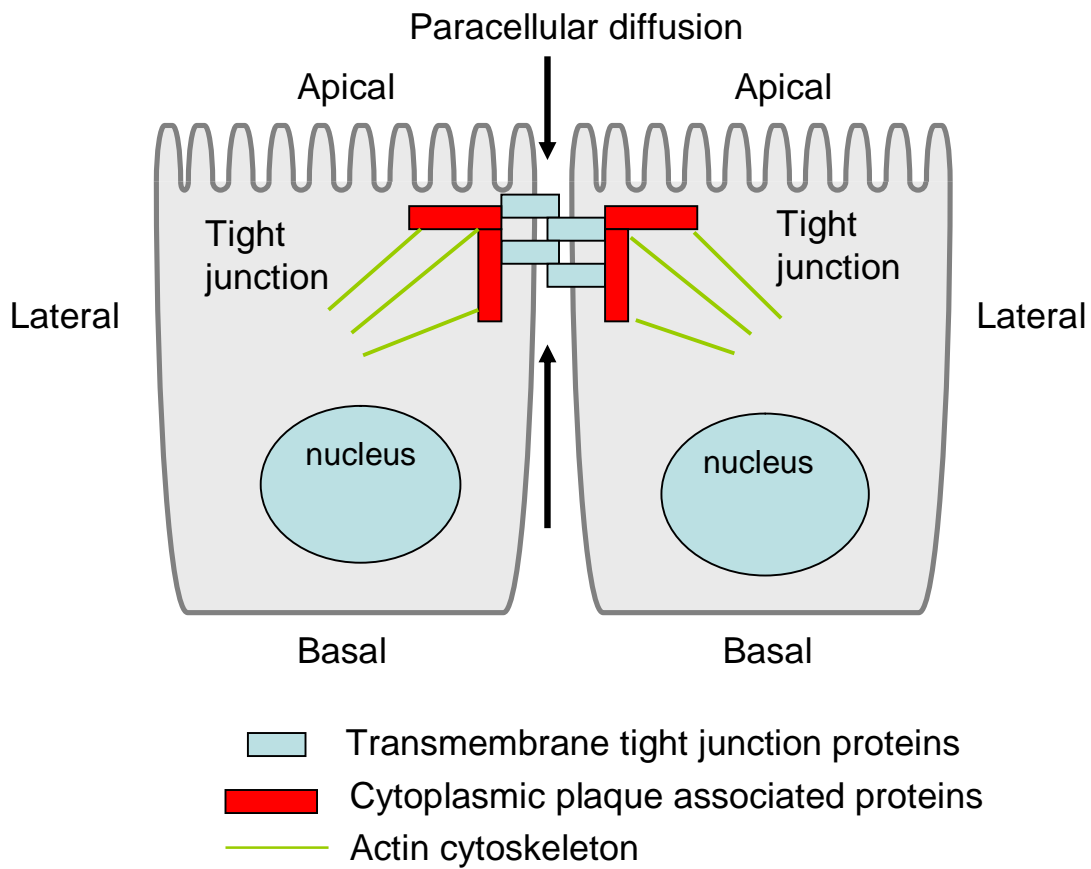


Figure 1. Tight junctions provide a barrier that regulates paracellular diffusion across epithelial sheets. They consist of many proteins including transmembrane proteins of the occludin, claudin and JAM families and associated cytoplasmic proteins including ZO-1 and cingulin. The transmembrane proteins are thought to mediate the permeability barrier while the adaptor proteins link the junctions to the cytoskeleton and recruit cell signalling proteins.

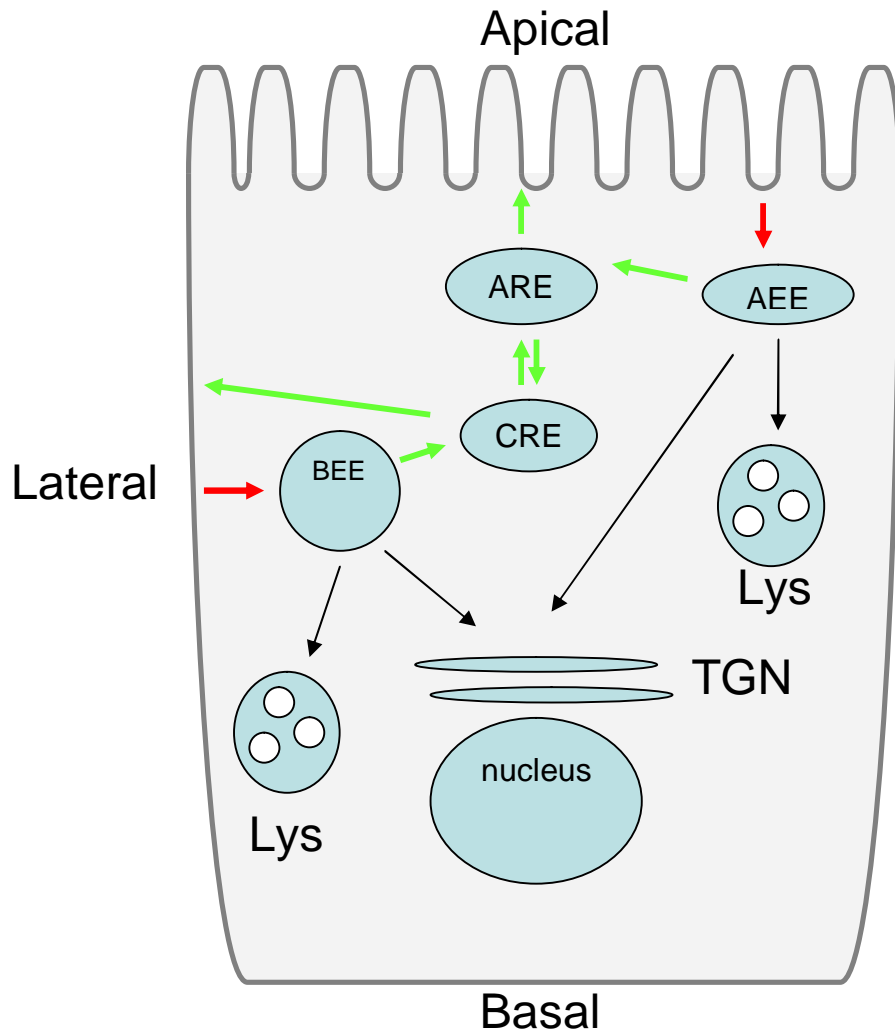


Figure 2 Endocytic trafficking routes in a polarised epithelial cell. This schematic diagram aims to provide an overview of endocytic trafficking routes in epithelial cells. However, characterisation of these routes is ongoing and endocytic trafficking is likely to be more complicated than presented here. There is also likely to be cell type specific and stimuli induced variations in trafficking. Abbreviations; AEE, Apical early endosome; ARE, apical recycling endosome; BEE, basal early endosome; CRE, common recycling endosome; LYS, lysosome; TGN, trans golgi network.