

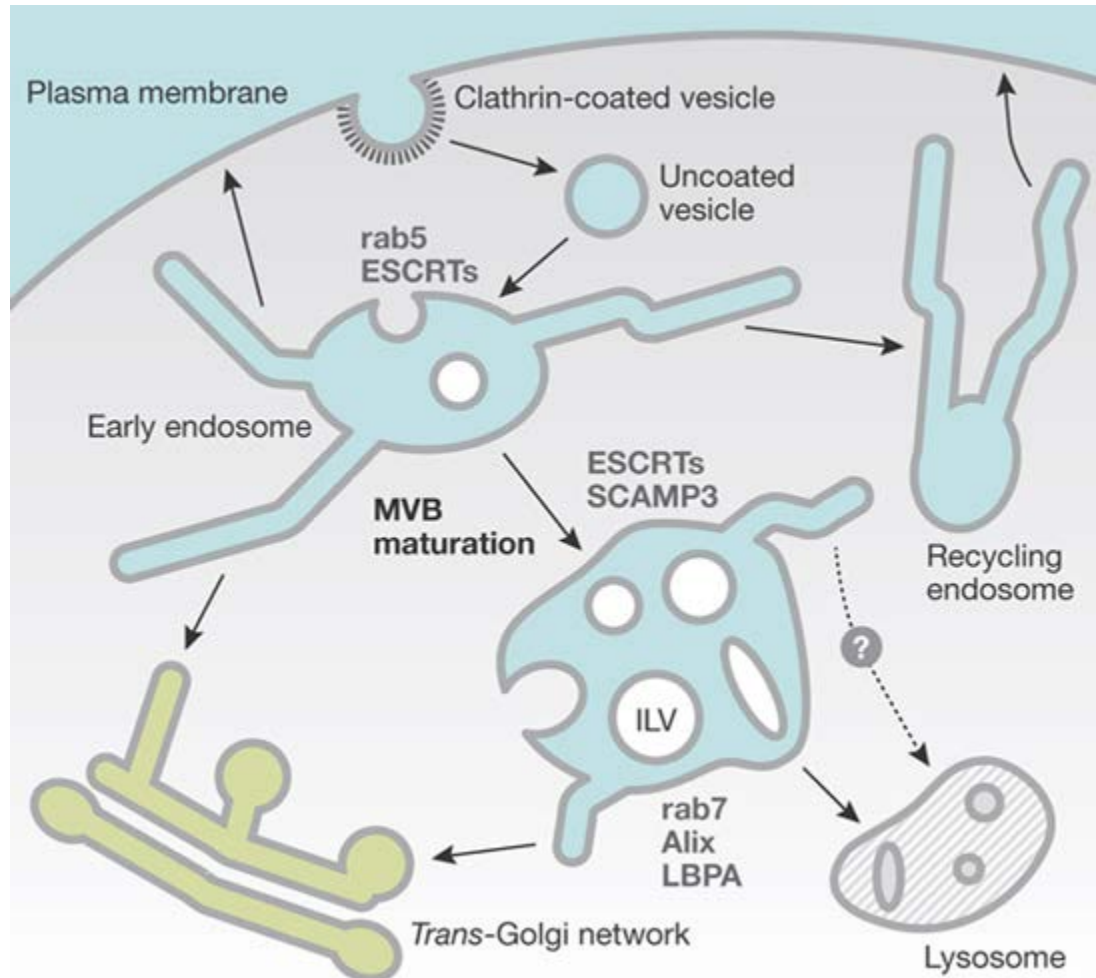


Regulation of tight junction trafficking



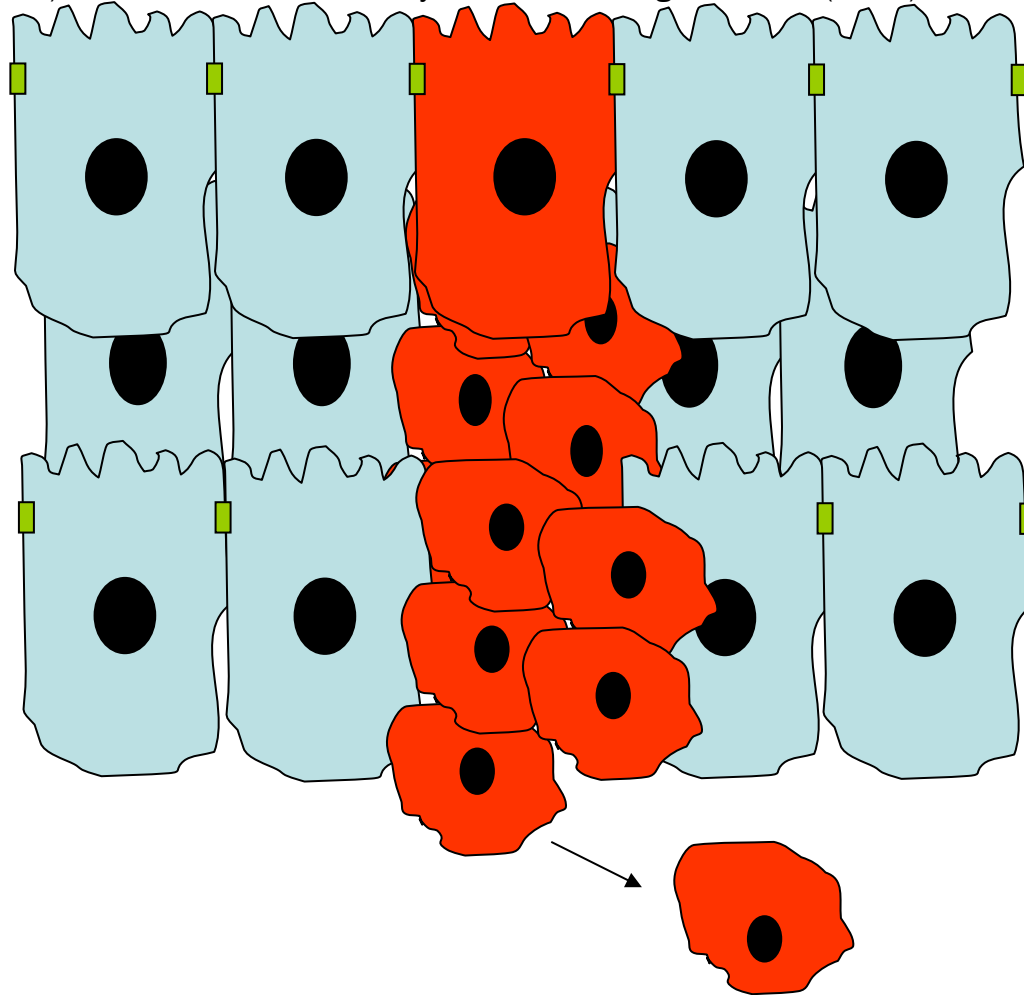
Paul Whitley

Endosomal sorting



Drosophila ESCRT mutants

Vaccari and Bilder; Dev Cell (2005); Moberg, Schelble, Burdick and Hariharan; Dev Cell (2005); Thompson, Mathieu, Sung, Loeser, Rorth and Cohen; Dev Cell (2005); Herz, Chen, Scherr, Lackey, Bolduc and Bergmann; Dev (2006)



ESCRT's and Cancer

- Mutations/misregulation of Tsg101 associated with tumorigenesis
- Vps37A associated with hepatocellular carcinomas
- Chmp1A silencing also linked to tumour formation

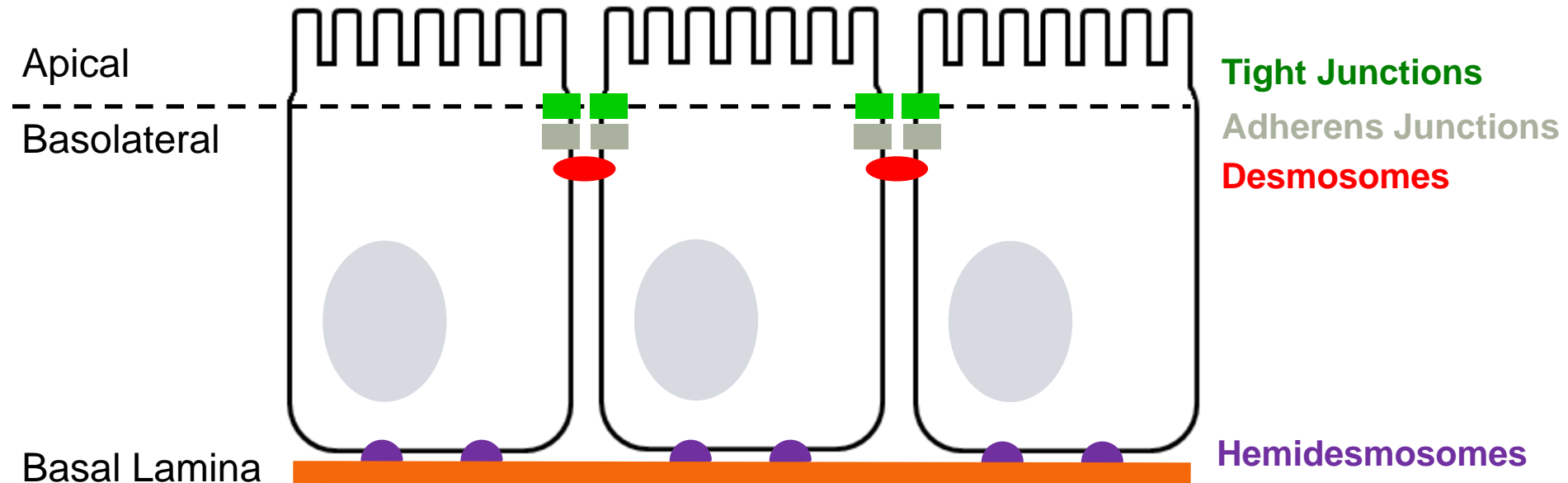
∞ BUT...

∞ In mammalian cells, the relationship between ESCRT proteins and cell polarity had not been studied

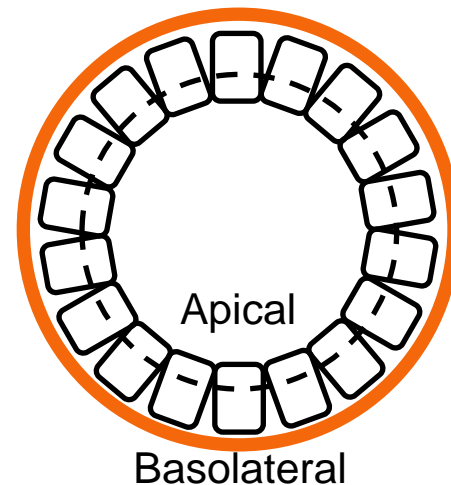
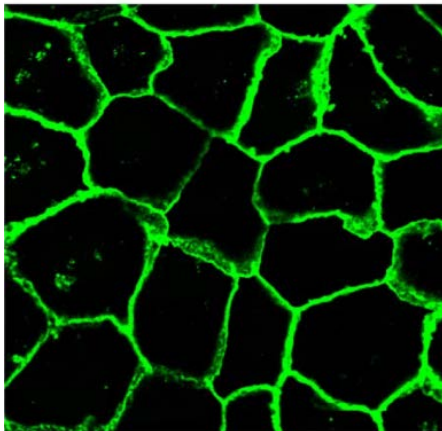
AIM

**Does disrupting ESCRT function
affect cell polarity in mammalian cells?**

Epithelial Cells

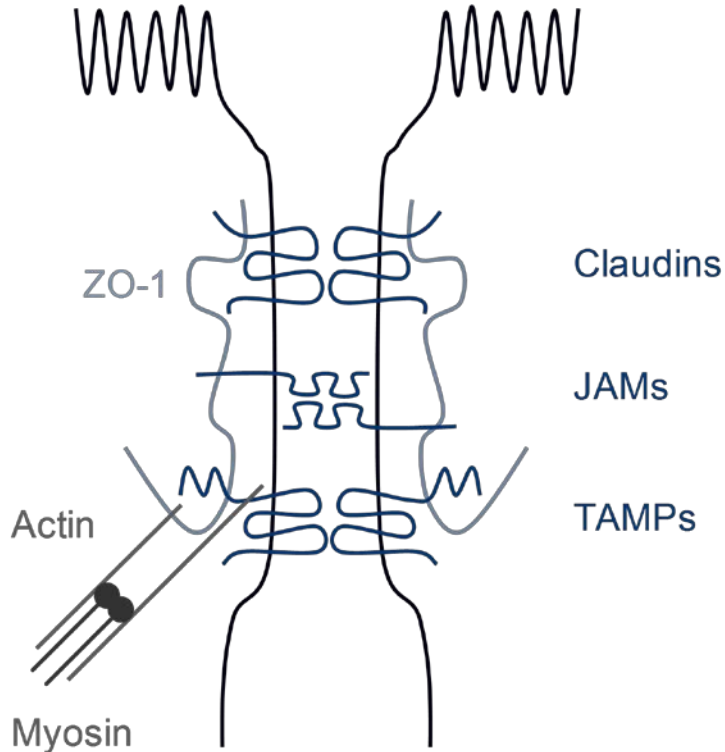


Claudin-1



Dukes et al, 2011.
*Molecular Biology of
The Cell*, 22 (17).

Tight junctions



Freeze–fracture replicas of the epithelium of mouse intestine



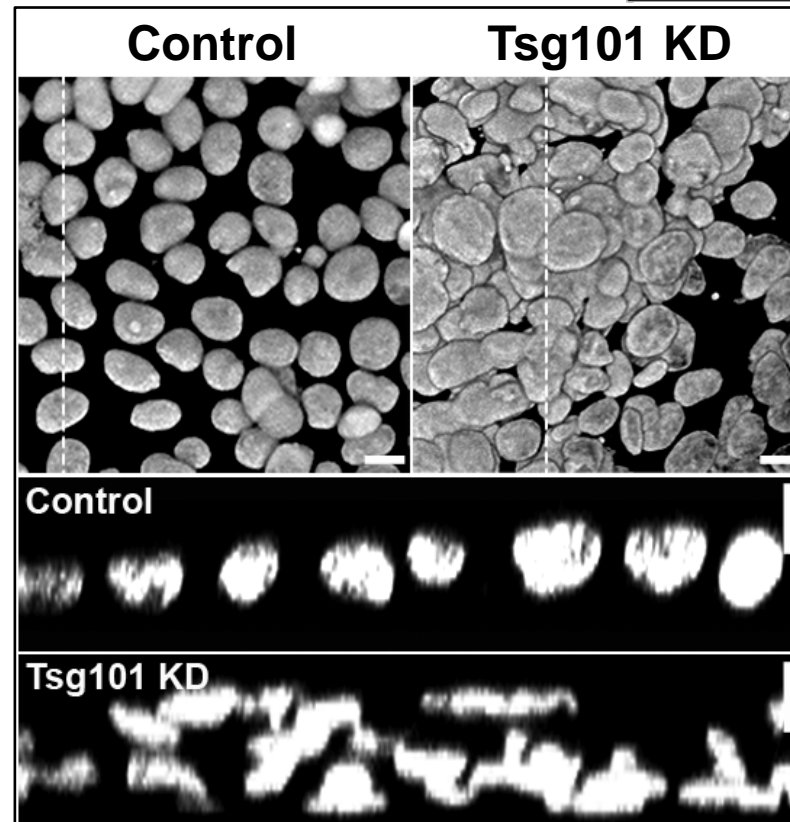
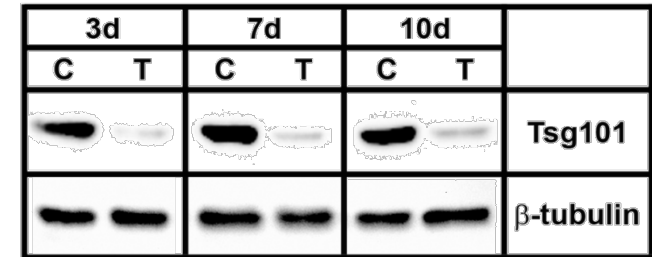
Adapted from González-Mariscal (2007) *Progress in histochemistry and cytochemistry*, 42(1), 1–57.

Adapted from: Shen (2011) *Annual review of physiology*, 73, 283–309.

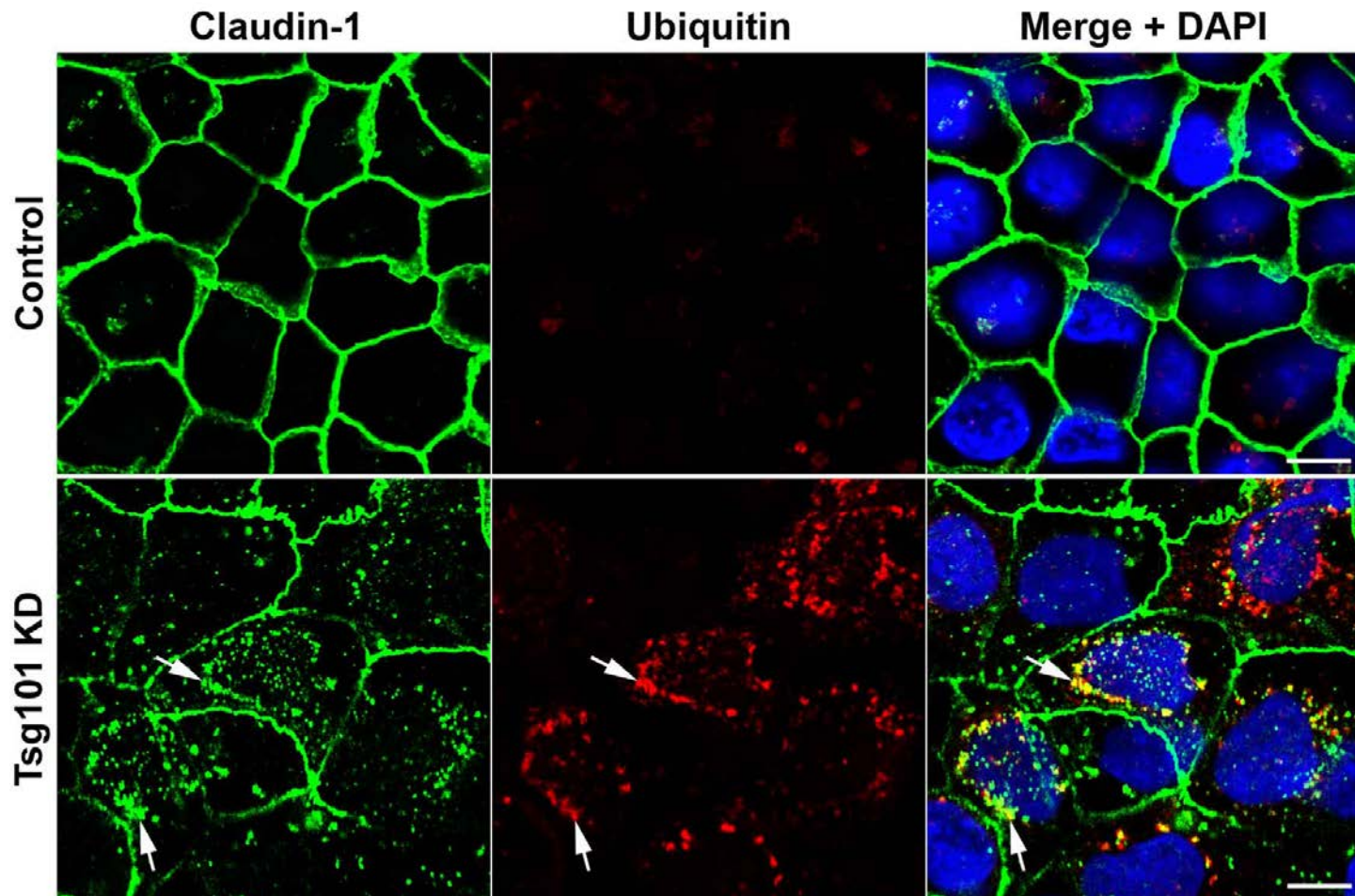
Loss of epithelial organisation upon ESCRT knockdown

Experimental Design:

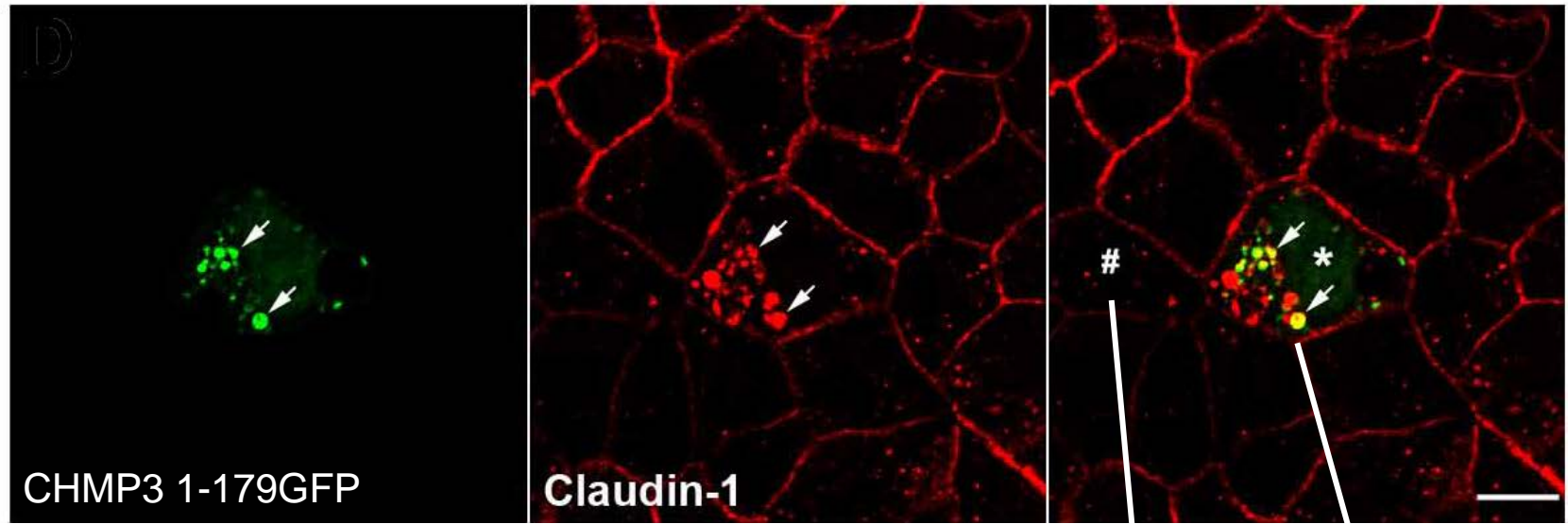
- Use siRNA to deplete Tsg101 (ESCRT-I) protein in human polarised epithelial Caco-2 cell line



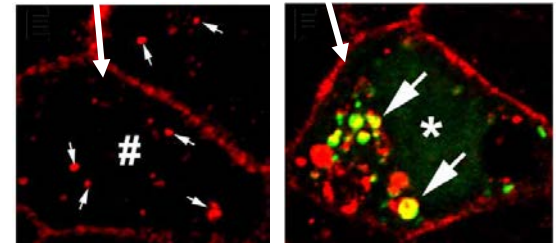
Tsg101 knockdown causes accumulation of internal claudin-1 and ubiquitin



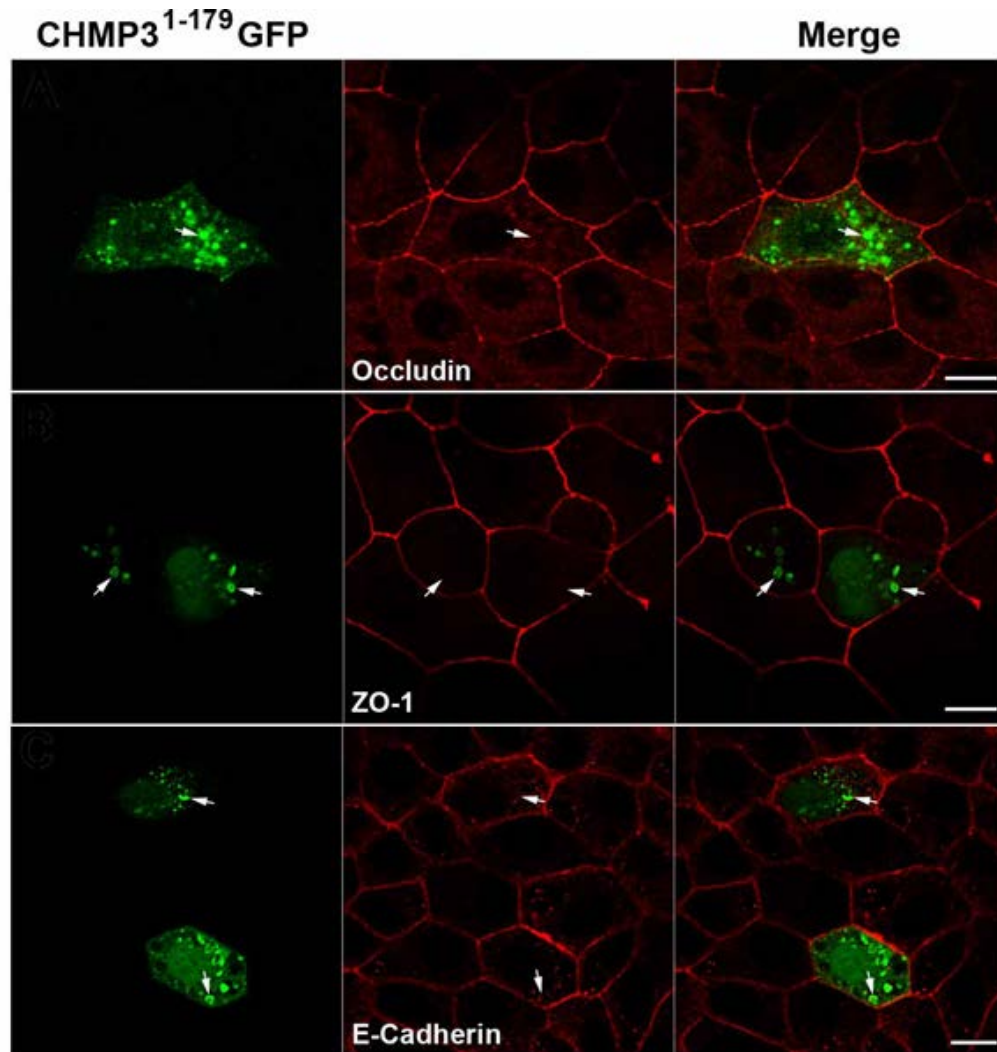
Dominant negative ESCRT proteins cause accumulation of internal Claudin-1



MDCK cells

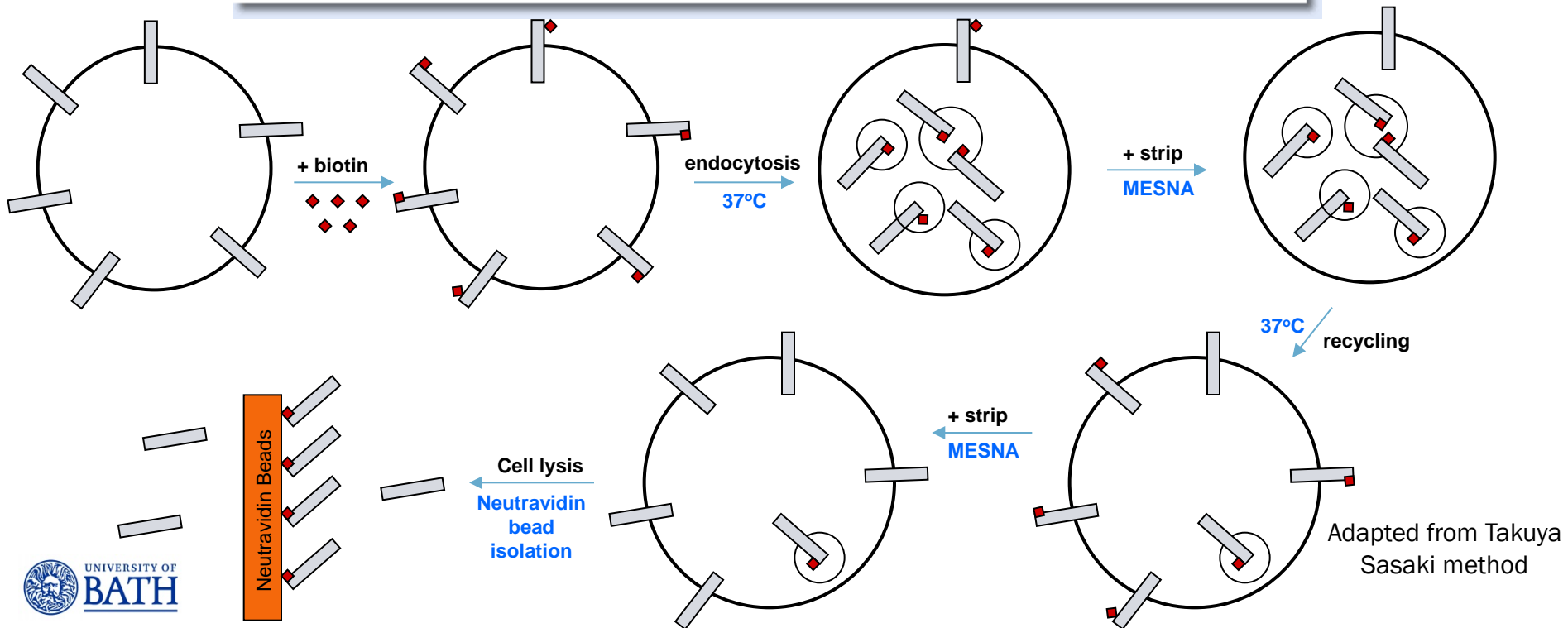
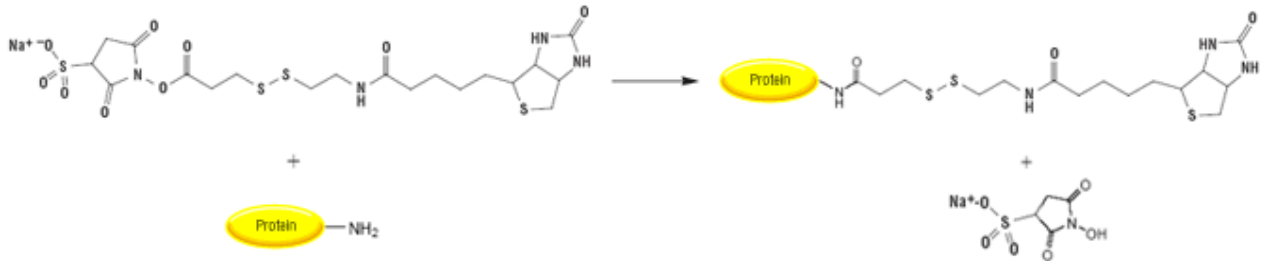


Other junctional proteins are not mis-localised upon ESCRT perturbation

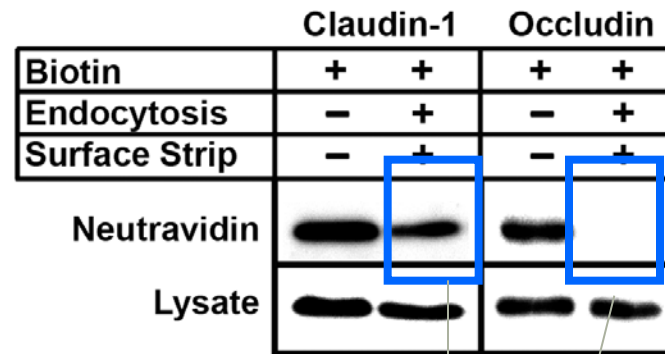


Biochemical analysis of tight junction trafficking

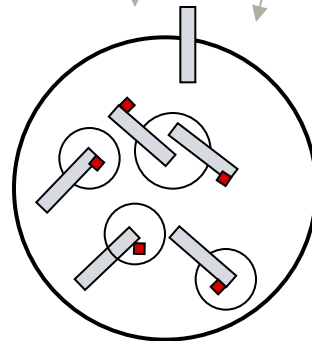
Sulfo-NHS-SS-biotin – allows for cleavage of the biotin from the protein



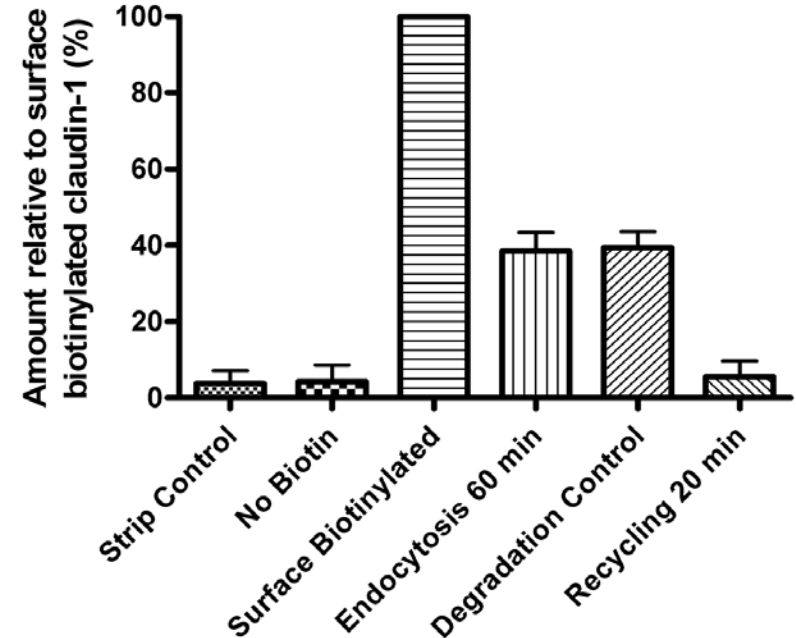
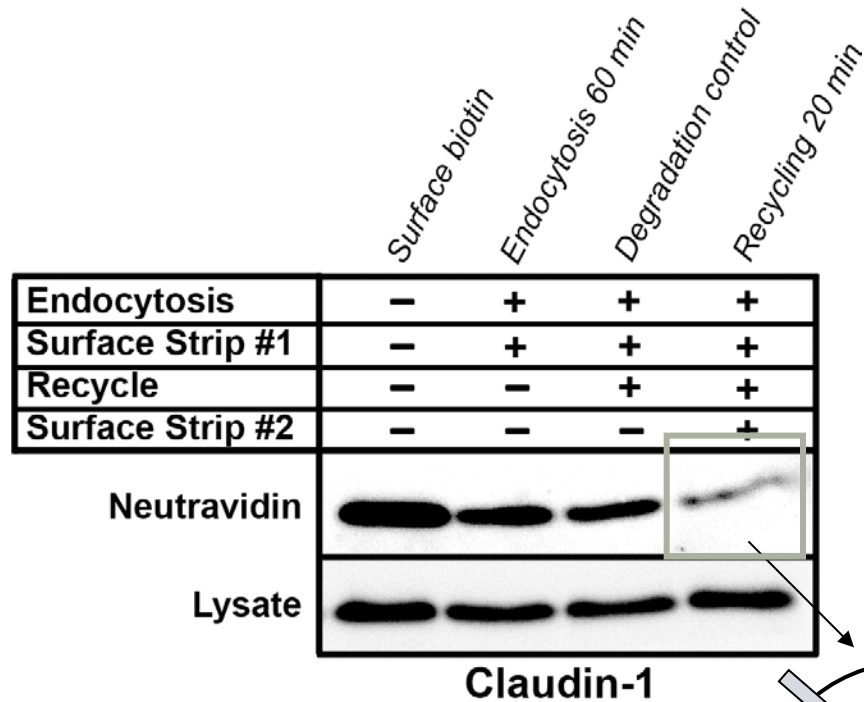
Claudin-1 but not occludin is endocytosed in MDCK cells



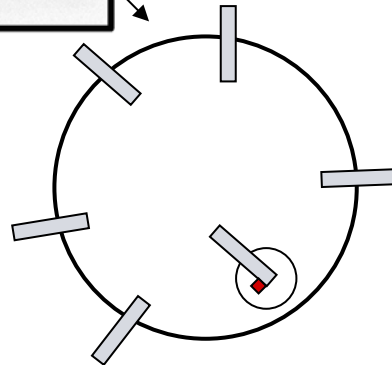
Endocytosis of
biotinylated
protein (60 min)



Claudin-1 undergoes rapid recycling to the surface



- ~35% of biotinylated surface Cla-1 is internalised in 60 min
- ~85% of internalised Cla-1 is recycled back to the PM in 20 min
- No evidence for degradation after 20 minutes but would expect degradation over longer time frames (hours).

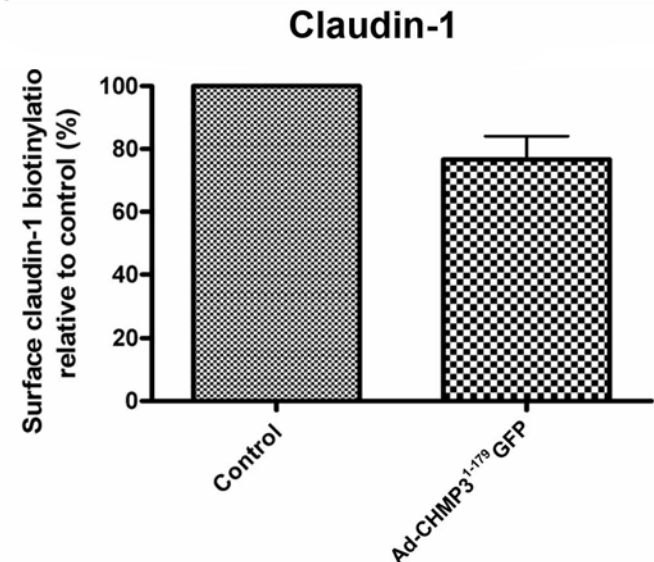
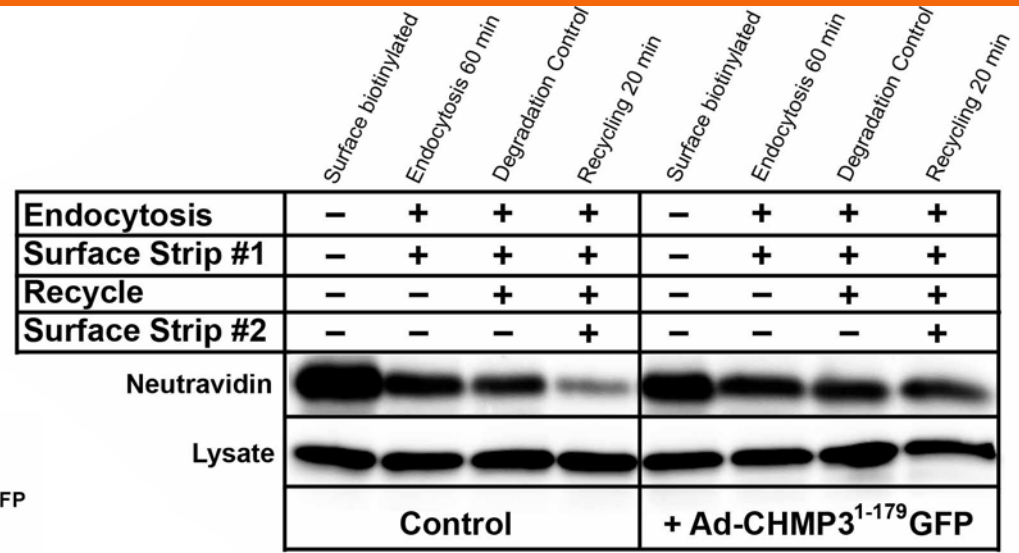
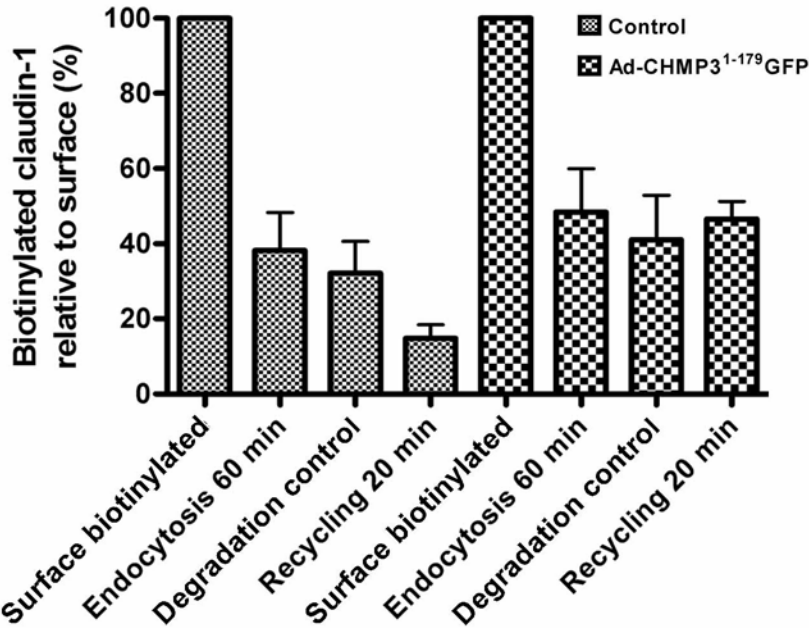
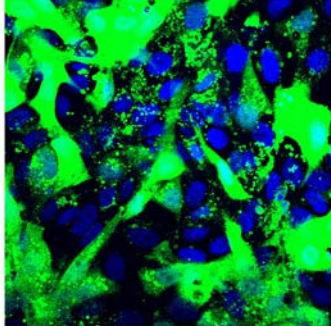


Cell type differences

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

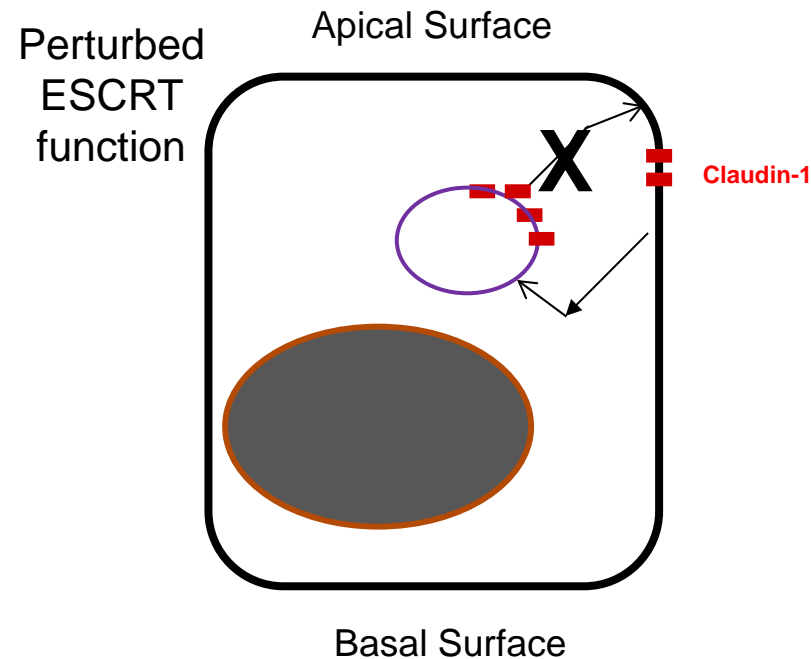
ESCRT function is required for Claudin-1 recycling

MDCKII + Ad-CHMP3¹⁻¹⁷⁹ GFP



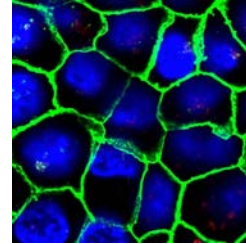
ESCRT function is required for epithelial polarity and claudin-1 recycling

- ESCRT function is required to maintain polarity in mammalian epithelial cells
- Claudin-1 (and 2) is constitutively recycled in mammalian epithelial cells
- Defects in recycling of junction proteins, combined with altered signalling, may drive the phenotype.
- This supports the hypothesis that ESCRT proteins could function as tumour suppressors.



Why are tight junction proteins continuously recycled?

∞ Junctions are not static structures



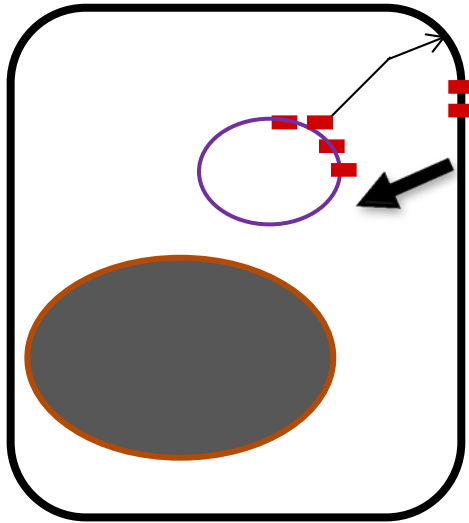
∞ Junctions need to be dynamic in remodelling of epithelial tissues:

- Lactation there is a massive increase in epithelial cell number
- Mammalian intestinal epithelia replaced every 4-5 days
- Extrusion (dead cells), incorporation (new cells), migration (within/through epithelium).

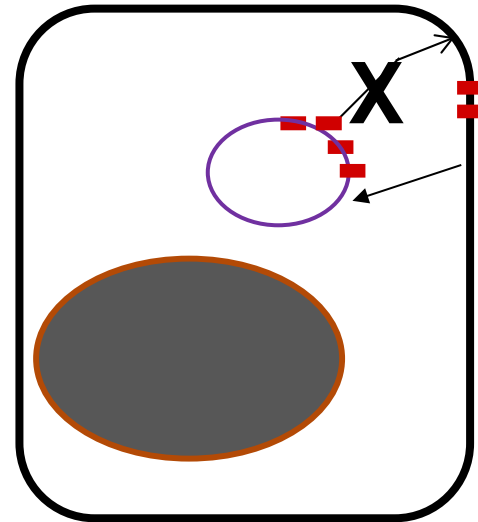
∞ Many stimuli regulate tight junction barrier:

- Cytokines ($\text{TNF}\alpha$, $\text{IFN}\gamma$)
- Bacteria

Is recycling regulated?



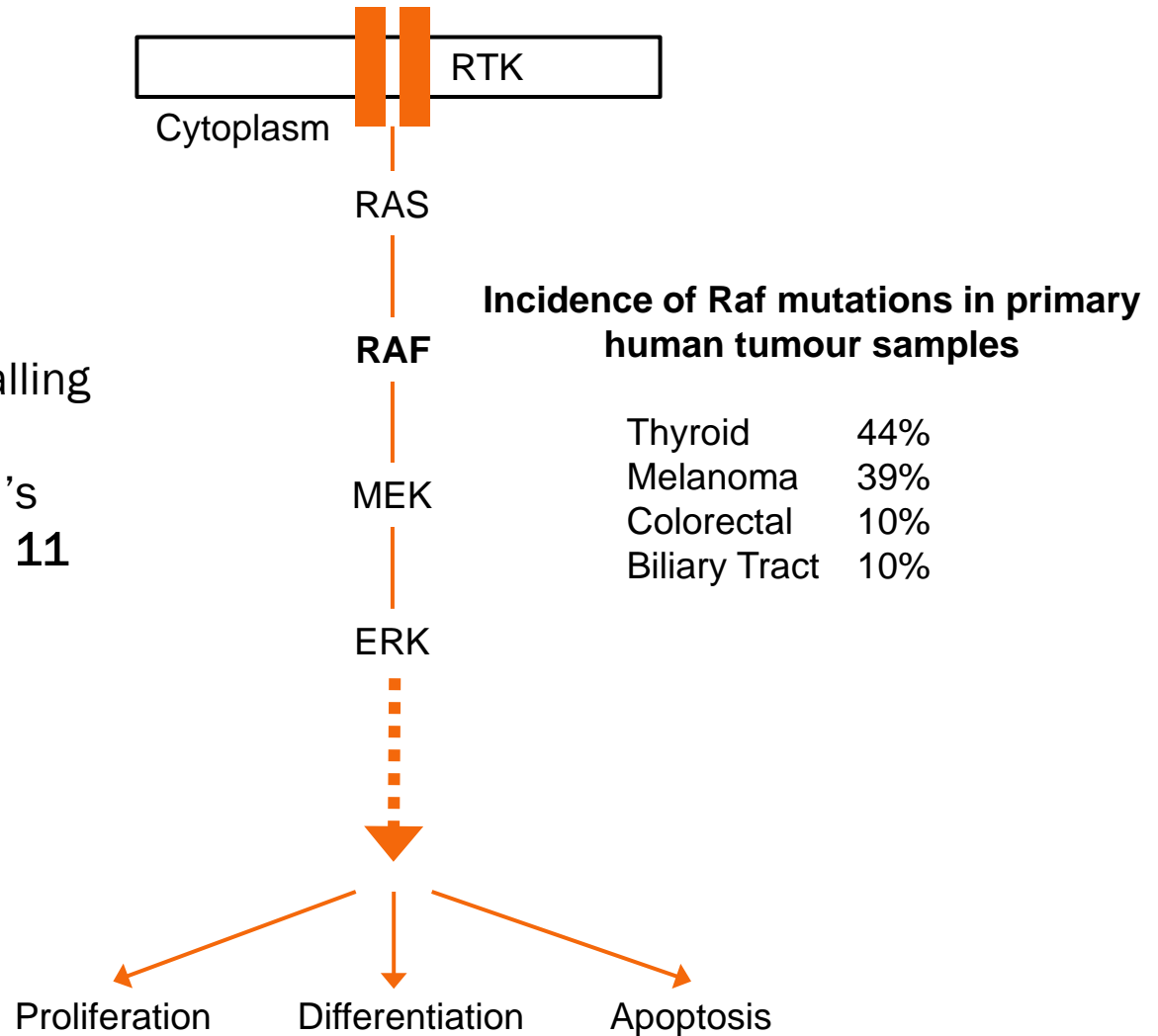
Increased endocytosis



Decreased recycling

MAPK signalling

Down regulation of MAPK signalling shown to restore epithelial cell morphology and assembly of TJ's (Chen et al 2000 Mol. Biol. Cell **11** (3): 849-862)



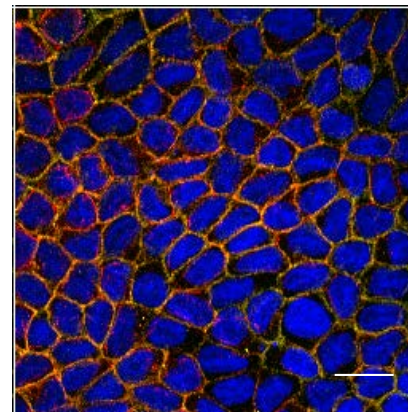
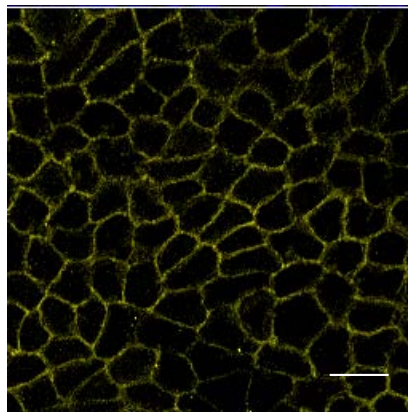
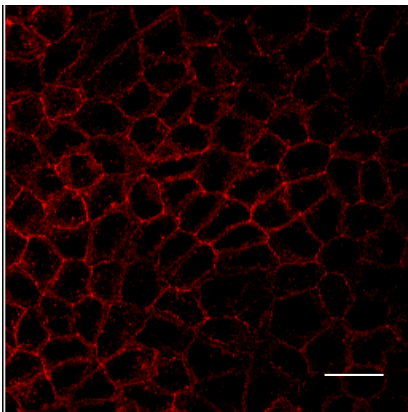
Ad BRAF^{WT} and junction protein localisation

Claudin-2 (546nm)

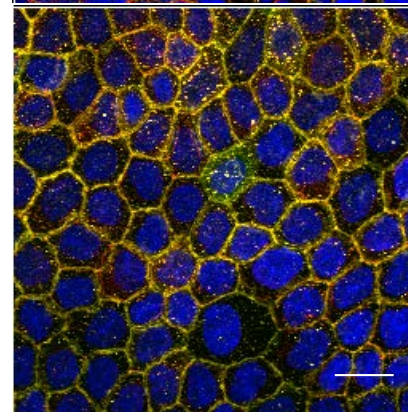
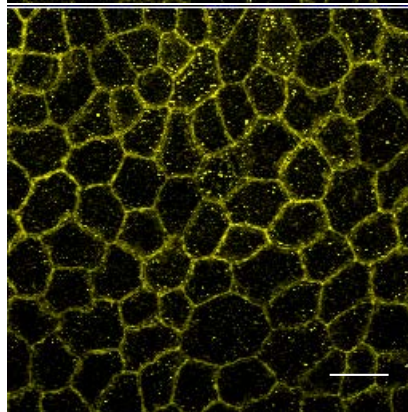
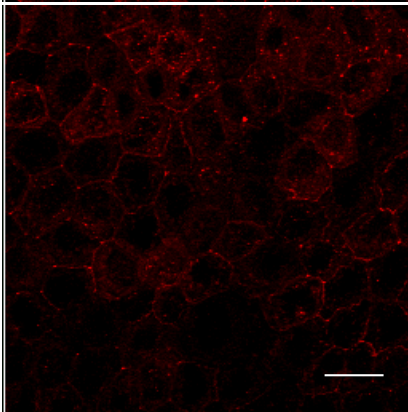
Claudin-1 (647nm)

Merge

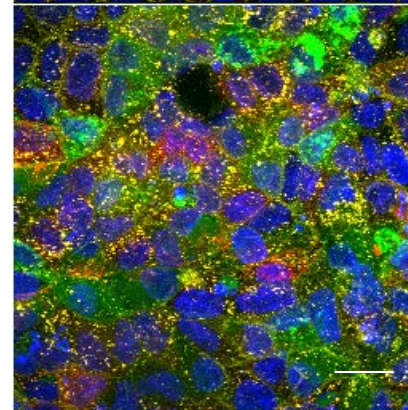
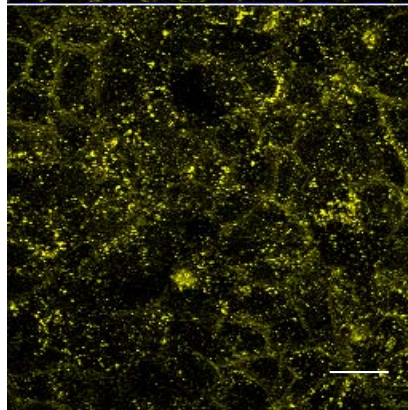
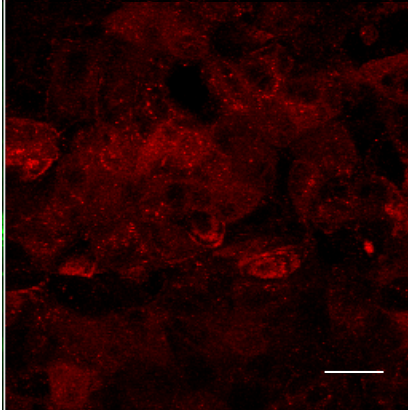
Uninfected
Control



Ad BRAF^{WT}
10pfu/nl



Ad BRAF^{WT}
30pfu/nl



Projections of individual 1 μ m Z slices of MDCKII cells imaged on LSM 510 META confocal microscope. Scale bar = 20 μ m

Preliminary conclusions

Recombinant adenoviral BRAF constructs provide powerful molecular tools

BRAF induces:

- Downregulation of claudin-2 at the total protein level
- Relocalisation of claudin-1 and -2 although claudin-1 levels are not altered

Junction markers are differentially sensitive to ERK signalling

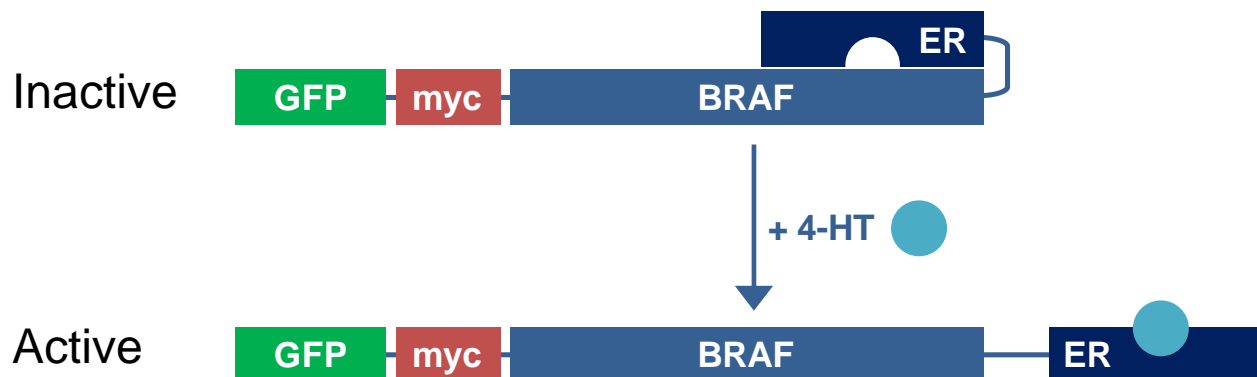
Future studies

BRAF-ER advantages:

Regulation of strength and duration of signalling (varying 4-HT dose/time)

Separation of expression and activation allows study over shorter time points:

- amenable to recycling assay, events prior to degradation and morphology changes, more amenable to drug treatments (synthesis, degradation, endocytosis, etc)

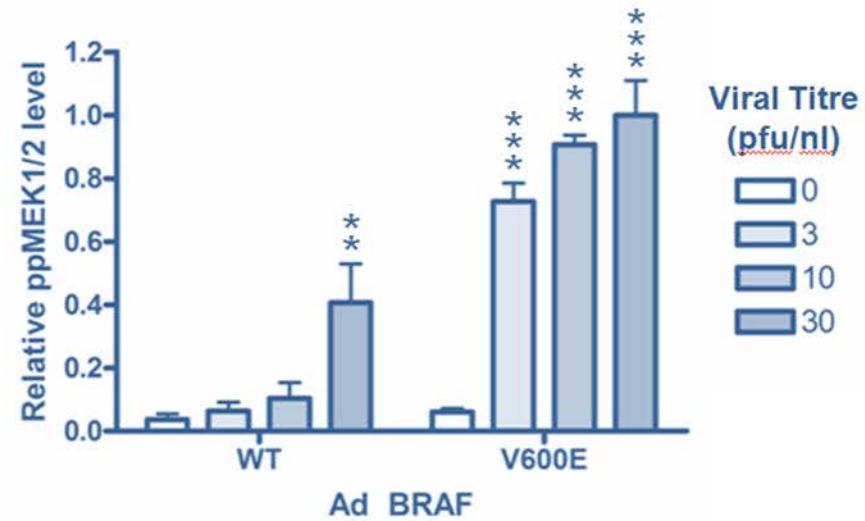
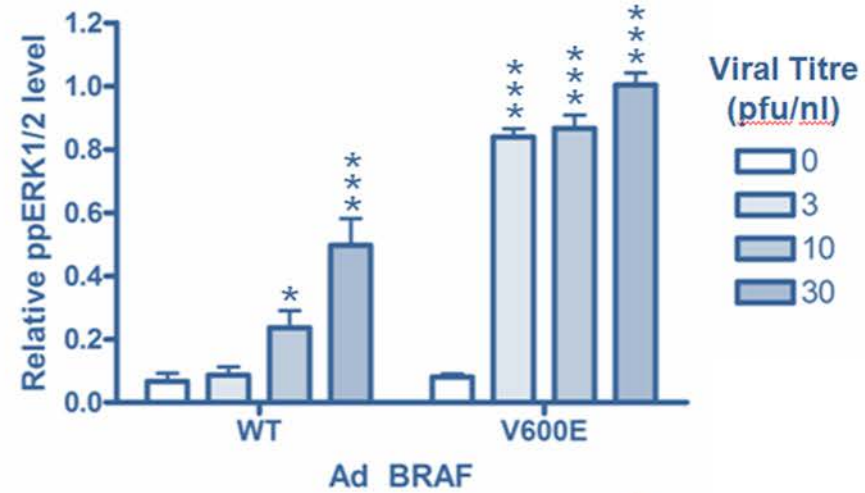
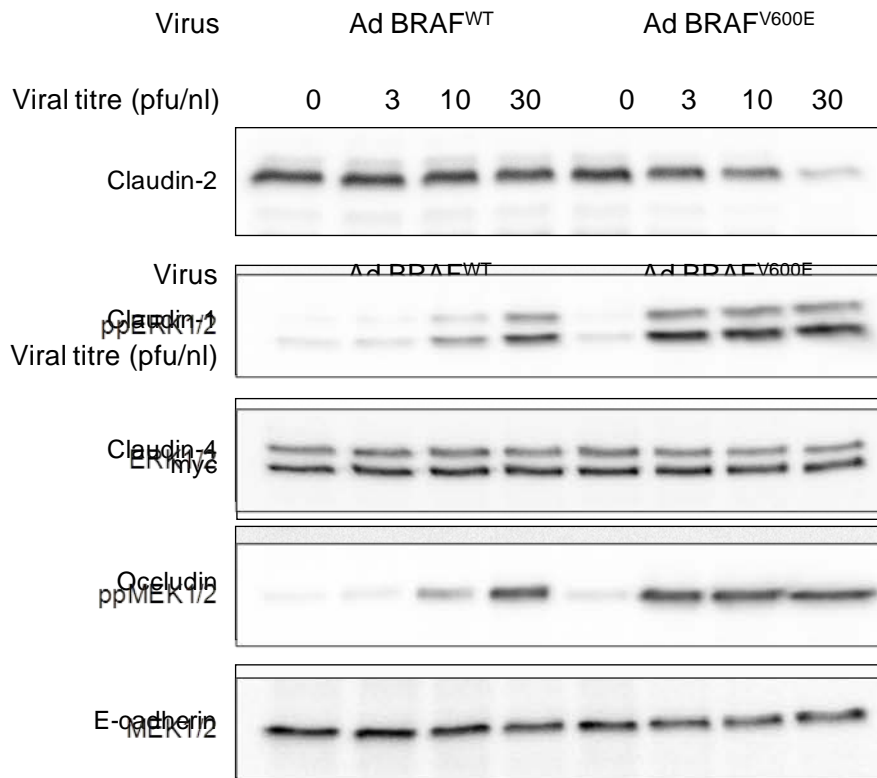


Acknowledgements

- ☞ Dr Andrew Chalmers
- ☞ Dr Jim Caunt
- ☞ Dr Joe Dukes
- ☞ Dr Laura Fish
- ☞ Mr Chris Bryant



Ad BRAF construct characterisation



Two-way ANOVA with Bonferroni post-test; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ comparing each treatment with lysates from uninfected control cells, $n = 3$, error bars represent + SEM

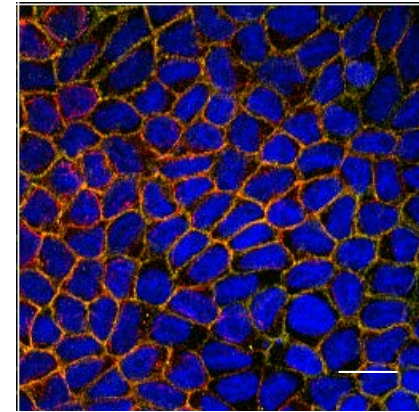
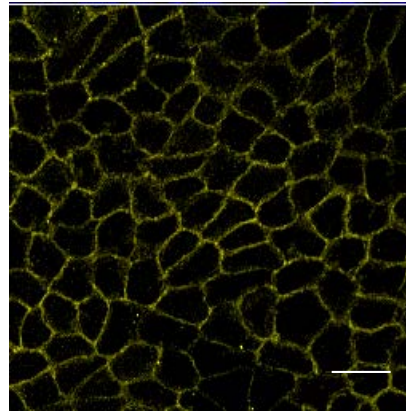
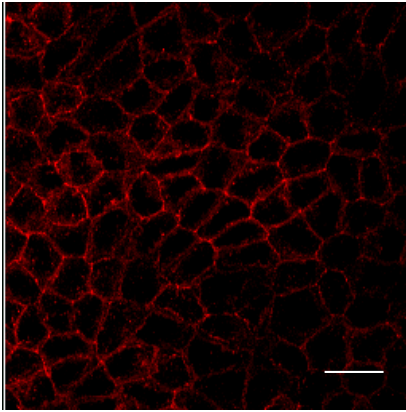
Ad BRAF^{V600E} and junction protein localisation

Claudin-2 (546nm)

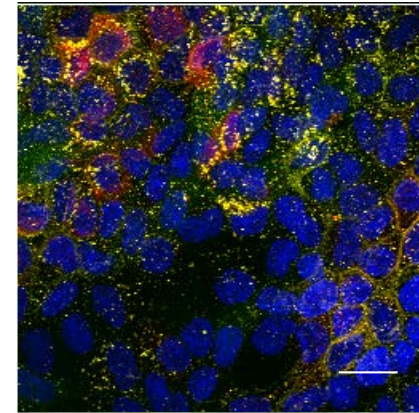
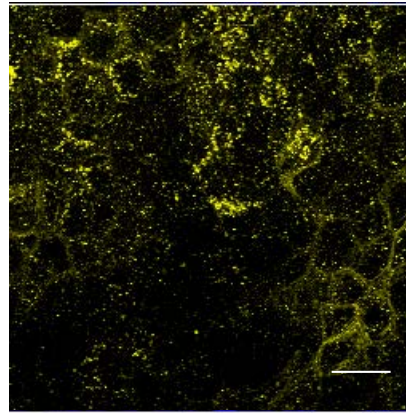
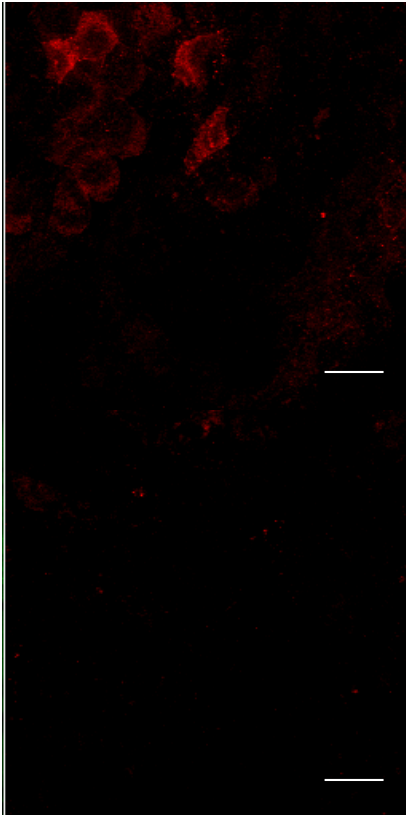
Claudin-1 (647nm)

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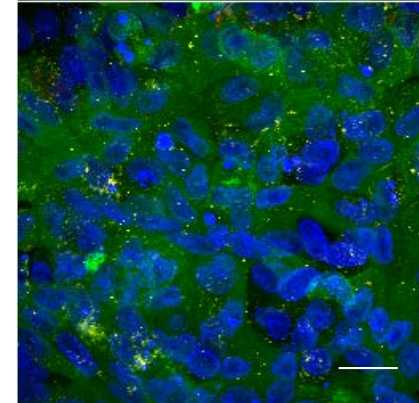
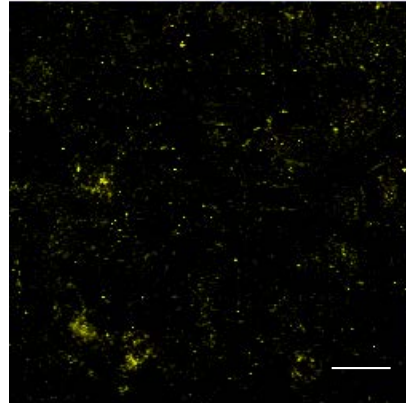
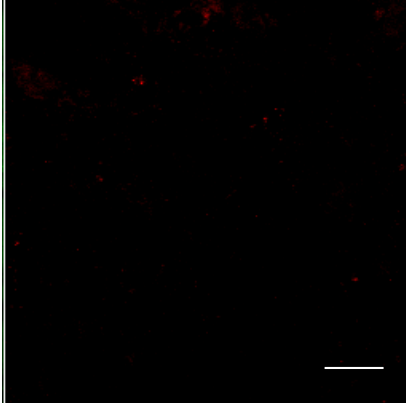
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Control



Ad BRAF^{V600E}
10pfu/nl



Ad BRAF^{V600E}
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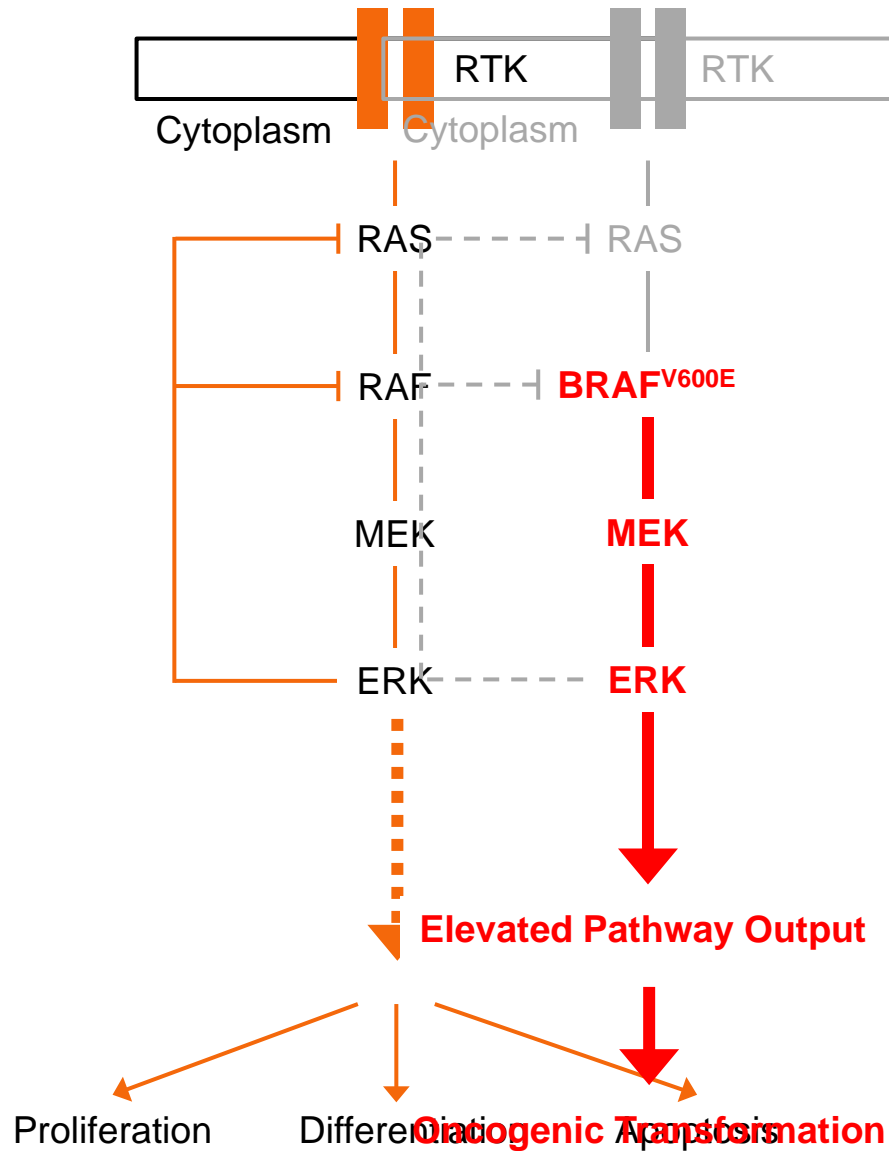
Projections of individual 1µm Z slices of MDCKII cells imaged on LSM 510 META confocal microscope. Scale bar = 20µm

MAPK signalling and tight junctions

- ⌘ Down regulation of MAPK signalling shown to restore epithelial cell morphology and assembly of TJ's (Chen et al 2000 Mol. Biol. Cell **11**. Li and Mrsny 2000, J. Cell. Biol. **148**)
- ⌘ MAPK pathway activated in many tumours

MAP kinase signalling and BRAF^{V600E}

Physiolo Oncogenic BRAF^{V600E} MAPK signalling

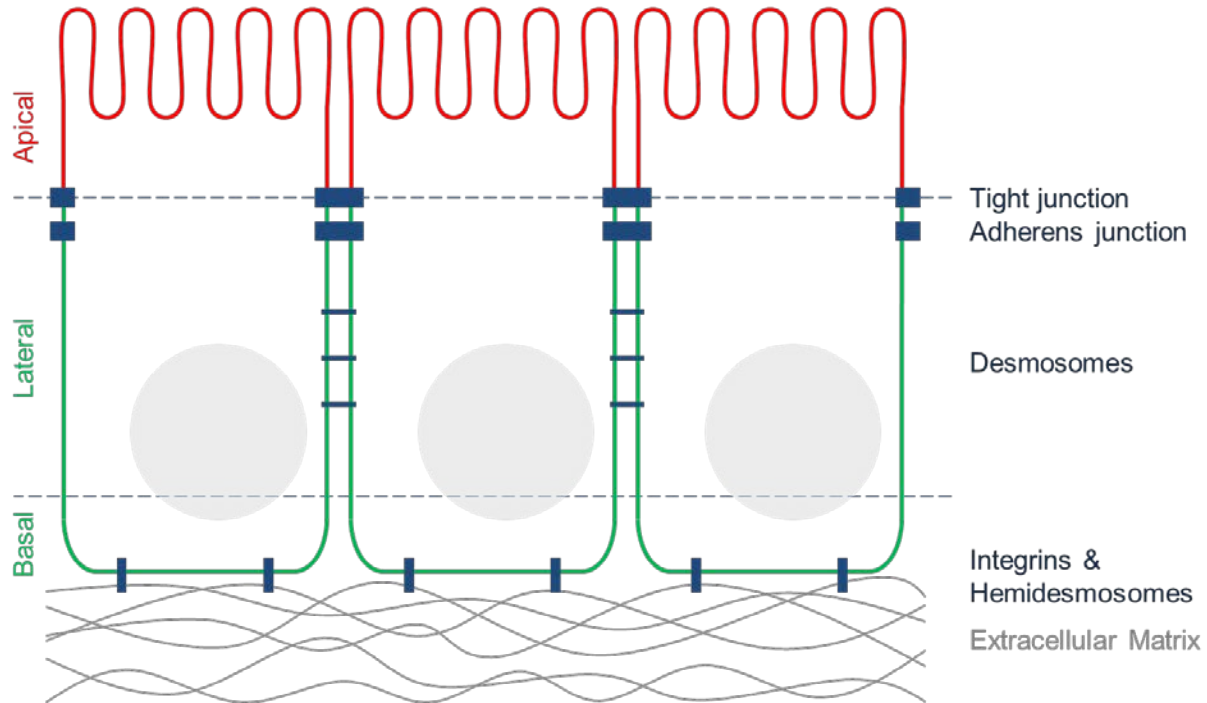


Incidence in primary human tumour samples

Thyroid	44%
Melanoma	39%
Colorectal	10%
Biliary Tract	10%

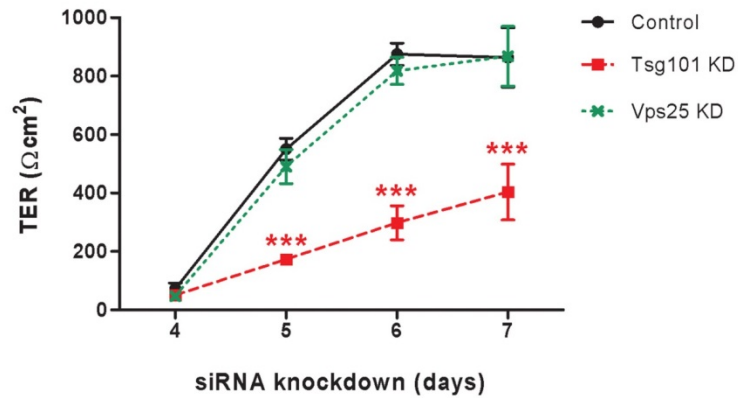
Adapted from:
Pratilas (2009)
PNAS, 106(11)

Epithelial cell polarity

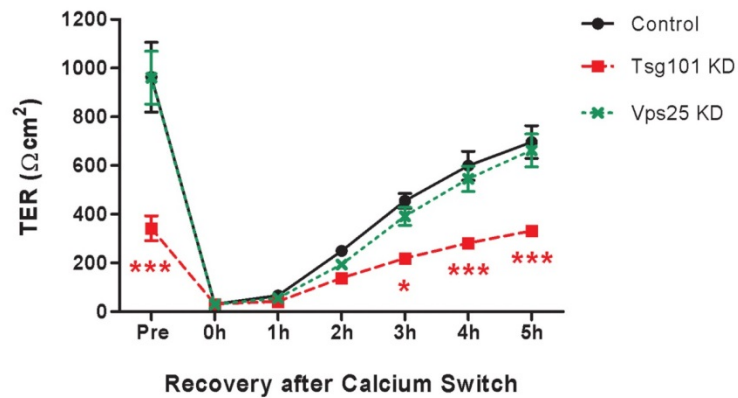


Effect of knockdown on TER

A.

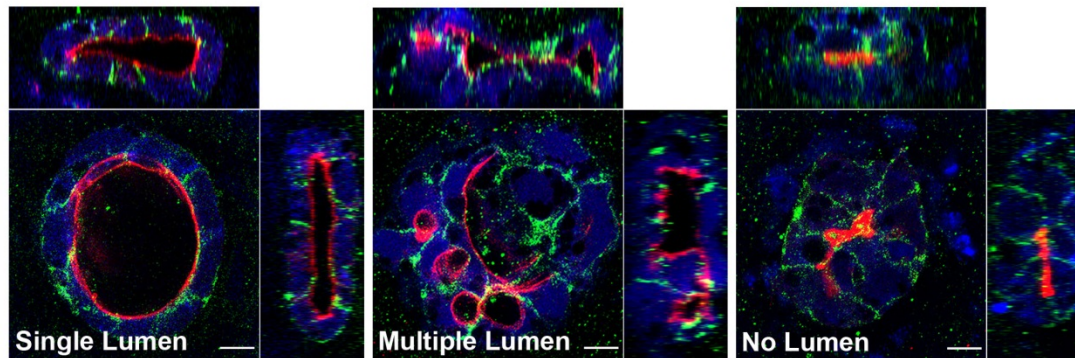


B.

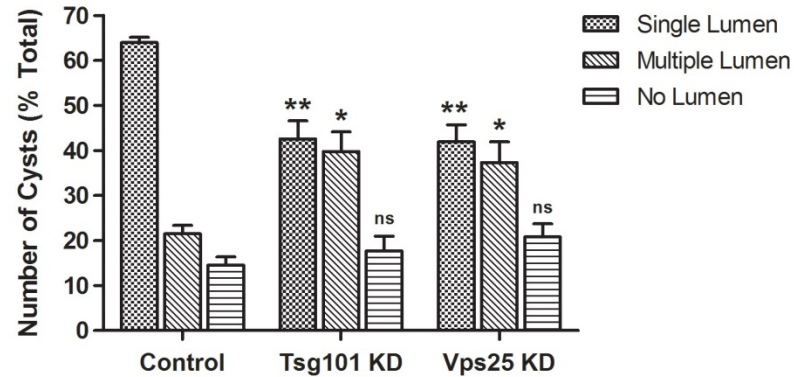


Formation of Caco-2 cysts is compromised upon ESCRT knockdown

A.



B.



Claudin-1 and occludin can be surface biotinylated

