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Title

The FAM deubiquitylating enzyme localises to multiple points of protein trafficking in epithelia, where it associates with E-cadherin and β-catenin.

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Running title

DUBs in adhesion protein trafficking

Key words

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Abstract

Ubiquitylation is a necessary step in the endocytosis and lysosomal trafficking of many plasma membrane proteins and can also influence protein trafficking in the biosynthetic pathway. Although a molecular understanding of ubiquitylation in these processes is beginning to emerge very little is known about the role deubiquitylation may play. Fat Facets in mouse (FAM) is substrate-specific deubiquitylating enzyme highly expressed in epithelia where it interacts with its substrate, β-catenin. Here we show, in the polarised intestinal epithelial cell line T84, FAM localised to multiple points of protein trafficking. FAM interacted with β-catenin and E-cadherin in T84 cells but only in sub-confluent cultures. FAM extensively co-localised with β-catenin in cytoplasmic puncta but not at sites of cell-cell contact as well as immunoprecipitating with β-catenin and E-cadherin from a higher molecular weight complex (approximately 500kDa). At confluence FAM neither colocalised with, nor immunoprecipitated, β-catenin or E-cadherin which were predominantly in a larger molecular weight complex (approximately 2MDa) at the cell surface. Over-expression of FAM in MCF-7 epithelial cells resulted in increased β-catenin levels which localised to the plasma membrane. Expression of E-cadherin in L cell fibroblasts resulted in the re-localisation of FAM from the Golgi to cytoplasmic puncta. These data strongly suggest that FAM associates with E-cadherin and β-catenin during trafficking to the plasma membrane.

Introduction

Correct sorting of the E-cadherin cell-cell adhesion protein to the basolateral plasma membranes of epithelial cells is essential for polarisation, maintenance of cell integrity and function. Sorting primarily occurs at the trans-Golgi network (TGN) and the route taken to the plasma membrane can either be direct, as observed in MDCKII cells, or indirect as in hepatocytes (Maurice et al., 1994) and intestinal epithelia (Le Bivic et al., 1990; Matter et al., 1990). The indirect route entails initial transport to either the basolateral or apical surface, before internalisation and sorting via endosomes and reinsertion into the lateral membrane. A dileucine repeat in the cytoplasmic domain of E-cadherin is necessary and sufficient for targeting E-cadherin to the basolateral membrane (Miranda et al., 2001). However, increased efficiency of E-cadherin delivery to the cell surface can be influenced by trans-acting factors, including the presence of the E-cadherin binding protein β-catenin (Chen et al., 1999).

Post-translational ubiquitylation can also influence protein trafficking (Katzmann et al., 2002; Schnell and Hicke, 2003). In the yeast *Saccharomyces cerevisiea,* mono-ubiquitylation of cytoplasmic domains is necessary and sufficient for the internalisation of plasma membrane proteins (Hicke, 1996; Shih et al., 2000) and has been suggested to be the primary endocytic signal for most, if not all, yeast proteins (Hicke, 2001). In cultured mammalian cells ligand-dependent ubiquitylation of cell surface receptors triggers their internalisation and is a major mechanism of their down-regulation (Katzmann et al., 2002). In addition to regulating the initial internalisation of membrane proteins, ubiquitin also determines sorting and hence protein fate at a number of points along the endocytic pathway such that if a protein remains ubiquitylated it is sorted to internal vesicles of the multi-vesicular bodies (MVB) and ultimately degraded in the lysosome (Katzmann et al., 2002). Not surprisingly a number of ubiquitin ligases and cargo-sorting proteins containing ubiquitin binding or interacting motifs

localise and function at these routing points of the endocytic pathway (Katzmann et al., 2001; Reggiori and Pelham, 2002; Wang et al., 2001) Katzmann et al., 2002).

Ubiquitylation can be reversed by deubiquitylating enzymes (DUBs) some of which display substrate-specificity (Wilkinson, 2000). However, very little is known about the possible role of DUBs in protein trafficking. The *Saccharomyces cerevisiae* DOA4 gene encodes a DUB which deubiquitylates several endocytosed membrane proteins (Chen and Davis, 2002; Dupre and Haguenauer-Tsapis, 2001; Springael et al., 2002). DOA4 acts at the late endosome/prevacuolar compartment removing ubiquitin from its substrates prior to their entrance into the lysosome and is necessary for the recycling of ubiquitin from this pool (Amerik et al., 2000). Recently, the yeast Ubp3p DUB has been shown to be necessary for deubiquitylation of the COPII protein Sec23, to facilitate transport between the endoplasmic reticulum and the cis-Golgi (Cohen et al., 2003). There is very little information however about the involvement of DUBs in protein trafficking in mammalian cells. The murine mUBPY deubiquitylating enzyme, interacts with the Hrs-binding protein Hbp which, together with Hrs, is thought to play a regulatory role in endocytic trafficking of growth factor-receptor complexes through early endosomes. This suggests that mUBPY may play a regulatory role in growth factor-receptor complex degradation but this has not been directly tested (Kato et al., 2000).

Fat facets in mouse (FAM, also known as Usp9X) is a substrate-specific, developmentally regulated UBP (Kanai-Azuma et al., 2000; Noma et al., 2002; Pantaleon et al., 2001; Taya et al., 1999; Taya et al., 1998). Two FAM substrates have been identified, both of which are involved in the establishment and maintenance of epithelial cell adhesion and polarity and cell signalling. FAM interacts with and stabilises both AF-6 (Taya et al., 1998) and β-catenin

(Taya et al., 1999) in-vitro and in-vivo. AF-6 is a peripheral component of tight junctions that binds to ZO-1 (Yamamoto et al., 1997), and also binds the adherens junction proteins nectin and ponsin as well as activated Ras and Rap1a (Boettner et al., 2000; Takahashi et al., 1999; Yamamoto et al., 1997). β-catenin, a member of the 'armadillo' repeat family of proteins, has two essential roles in the epithelial cells. It is required for the maintenance of adherens junctions where it binds E-cadherin connecting it to the actin cytoskeleton via α-catenin (Conacci-Sorrell et al., 2002). β-catenin is also found in the nucleus where it acts as a transcriptional co-activator of Wnt signalling genes through its interaction with members of the LEF/TCF family of transcription factors (Conacci-Sorrell et al., 2002). When not bound to either E-cadherin or LEF/TCF the free cytoplasmic pool of β-catenin is rapidly degraded by the ubiquitin-proteasome pathway (Conacci-Sorrell et al., 2002). In vivo depletion of FAM in pre-implantation mouse embryos, by addition of antisense oligonucleotides, resulted in a parallel decrease in β-catenin. AF-6 levels were also initially reduced but returned to normal, however the nascent protein was mis-localised to the apical surface of blastomeres (Pantaleon et al., 2001).

Given the high levels of FAM expression in epithelia during development (Kanai-Azuma et al., 2000; Wood et al., 1997), the identification of β-catenin and AF-6 as substrates (Taya et al., 1999; Taya et al., 1998) and the loss of blastomere adhesion following depletion of FAM (Pantaleon et al., 2001) we decided to further investigate FAM function in epithelia.

Materials and Methods

Cell Culture

L-cells and MCF-7 cells were grown in DMEM (GIBCO) supplemented with 10 % (v/v) heat inactivated fetal calf serum in a humidified atmosphere of 5 % (v/v) CO_2/air. T84 cells were cultured in a 1:1 mixture of DMEM (GIBCO) and Ham's F12 (GIBCO) medium supplemented with 5 % heat inactivated fetal calf serum in a humidified atmosphere of 5 % (v/v) CO_2/air. Cells used in immunofluorescence experiments were plated on glass coverslips at a low density and allowed to grow to either subconfluence where the cultures contained discrete islands of cells or to confluence for 10-14 days. To halt trafficking at the Golgi, cells were incubated at 15°C for 2 hours and in some experiments 10 μM cycloheximide was added to the media 30 minutes prior to incubation at 15°C to block protein synthesis. Where indicated cells were incubated in 1 μM Brefeldin A from Penicillin brefeldianum (Sigma) for 1 hour to disrupt the Golgi.

Antibodies, Immunoprecipitation and Western analysis

Affinity-purified polyclonal anti-FAM rabbit antibody raised to the peptide sequence TATTRGSPVGGNDNQGQAPC were generated as described previously (Kanai-Azuma et al., 2000). Antibodies to E-cadherin, β-catenin (mouse monoclonal), p120, AF6, GM130, Lamp1 and ZO-1 were purchased from BD Transduction Laboratories. Antibodies to myosin, β-catenin (rabbit polyclonal), β-tubulin and anti-rabbit Cy3 conjugated antibody were purchased from Sigma. Anti-mouse Alexa 488 conjugated antibody was purchased from Molecular Probes. HRP conjugated anti-mouse and anti-rabbit antibodies were purchased from Dako.

For immunoprecipitation cells were lysed in buffer A (50 mM Tris pH 7.5, 1 mM EDTA, 50 mM NaCl, 0.5 % Triton X-100 containing one protease inhibitor cocktail tablet per 10 ml

[Complete, Mini; Roche]), centrifuged to remove cell debris and incubated with protein A agarose for 1 hour to remove non-specifically bound proteins. The resulting supernatant was then incubated with antibody bound to protein A agarose for 1 hour then unbound proteins were washed away using buffer A followed by a final wash in PBS. The Protein A agarose was then boiled in SDS sample buffer to elute the bound proteins.

For Western blot analysis, samples were separated by SDS-PAGE (Laemmli) and proteins were transferred onto nitrocellulose (Towbin et al., 1979). Immunoblotting was performed using the ECL kit (Amersham Pharmacia Biotech) according to the manufacturers instructions.

Plasmids and Transfections

The E-cadherin expression plasmid (PGK-E-cadherin-hygro) was a gift from Rolf Kemler (Max-Planck Institute for Immunobiology, Freiburg). The pDEST-FAM-V5 was derived by the insertion of FAM cDNA into pEFDEST51 (Invitrogen) by an LR Gateway reaction. Transient transfections were performed using DOTAP Liposomal Transfection Reagent (Roche) according to their instructions. Briefly, MCF-7 cells were grown to 60-70 % confluence either in flasks or on glass coverslips and transfected with the amount of DNA indicated using the solutions provided. After 24 hours the media was changed and the cells grown for a further 24 hours then either fixed with ethanol or scraped, lysed in buffer A and the lysate used for subsequent experiments.

Mass spectrometry Peptide mass fingerprinting

Proteins were resolved by SDS-PAGE and stained with Coomassie Blue R-250.

Individual bands were excised, destained, S-amidomethylated and subjected to in-gel tryptic digestion essentially as described (Speicher et al., 2000). Extracted tryptic peptides were

resolved by reversed phase HPLC using a 1 × 30 mm column packed with Zorbax C-18 Extend matrix (Agilent) and introduced into a Q-TOF^2 mass spectrometer (Micromass, Manchester, U.K.) at 10µl/min. The eluted peaks were analysed and the monoisotopic masses of the peptides were determined. Lists of peptide masses were used to interrogate the non-redundant NCBI protein sequence database (version NCBInr.03.26.2002) using the programs MS-Fit (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm) or MASCOT (http://www.matrixscience.com). Hanson Institute Protein Core Facility.

Separation of T84 cell lysates by Gel Filtration Chromatography

T84 cells were washed in ice cold PBS then lysed in 250 µl of buffer A. Cell extracts were centrifuged to remove cell debris and 200 µl of the cell lysate was separated by gel filtration. Gel filtration was carried out using a Superose 6 FPLC column (Pharamcia) equilibrated with buffer B (20 mM Tris pH 7.5, 10 % glycerol, and 100 mM NaCl). Fractions (1 ml) were collected.

Immunofluorescence microscopy

Cells were fixed in 100 % ethanol for 20 minutes at - 20°C and then the ethanol removed. Coverslip were either used immediately or stored at -20°C. Cells were washed in PBS then blocked with blocking solution (1 % BSA in PBS). Cells were incubated at room temperature, with primary antibody then secondary antibody antibodies for 1 hour each.

Images were captured using the BioRad Radiance 2100 confocal microscope (Bio-Rad Microscience Ltd, UK). The dual labelled cells were imaged with two separate channels in a sequential setting. The image data were further analysis using the Confocal Assistant Software (Todd Clark Brelje. USA).

T84 cells are columnar cells 20-30 μm in height. Data used throughout this paper has been selected to most clearly show the results and may not contain the nuclei due to the height of the cells. Where relevant a section through the basolateral region and a section at the subapical/apical point are shown.

Detergent solubilization of MCF-7 epithelial monolayers

The assessment of cytoskeleton associated β-catenin in MCF-7 cells transfected with pDEST-FAM-V5 was performed according to the protocol of Svastova et al., (2003)

Results

FAM localises to points of protein trafficking

In the polarised intestinal cell line T84, FAM localises to puncta of varying sizes throughout the cytoplasm from the nucleus to a point at or near the plasma membrane (Figures 1A and 2). Similar results were also obtained in Caco-2 intestinal epithelial cells (data not shown). The vesicular FAM staining was present throughout the basolateral region of the cell but absent from the apical region stopping at a point just below the localisation of the tight junction protein ZO-1 at points of cell-cell contact. The number of FAM-associated puncta gradually increased towards the apical region of the cell (Figure 1A). To identify with which membrane-bound compartment(s) FAM was associated, cells were co-labelled with antibodies specific for FAM and for proteins highly enriched in specific organelles. FAM partially co-localised with the Golgi apparatus marker GM130, with LAMP1, a marker of multi-vesicular bodies (MVBs) and the lysosome, but little, if any, colocalisation was found with EEA1, an early endosome marker (Figure 1B) or the endoplasmic reticulum marker BiP (data not shown). The majority of FAM puncta did not colocalise with any of the organelle markers, however indicating that FAM localised to sites of protein sorting and trafficking in T84 cells.

Identification of proteins co-localising with vesicular FAM

In light of the fact that two of FAM's substrates, β-catenin and AF-6, are adhesion junction proteins, and hence need to be trafficked to the plasma membrane, we investigated whether these and other adhesion junction proteins co-localised with FAM in T84 cells. In sub-confluent T84 cells FAM and β-catenin extensively colocalised in cytoplasmic vesicles throughout the basolateral region of the cell (Figure 2A). FAM did not however colocalise with β-catenin at the cell surface. This was observed in apical regions of subconfluent T84s as

well as in confluent cells when all β-catenin was present at cell-cell contacts and there was no overlap with FAM which remained in cytoplasmic vesicles (Figure 2B). Vesicles containing FAM and AF-6 were also detected in the basolateral but not apical regions of sub-confluent T84 cells (Figure 2A). Similar results were seen with other cell adhesion molecules including ZO-1, a tight junction protein and $p120^{ctn}$, an adherens junction protein (Figure 2A).

Detection of FAM at a number of intracellular localisations raised the possibility that distinct populations of FAM exist in T84 cells, possibly in differing protein complexes. To begin the identification of FAM-associated proteins we performed immunoprecipitation of whole T84 cell lysates and co-immunoprecipitated greater than 40 bands as visualised by Coomassie Blue staining (data not shown). Eight predominant, high molecular weight bands (>100kDa) were analysed by MALDI-TOF mass spectometry. Seven of these were identified as either components of the cytoskeleton or motor proteins and included, spectrins (non-erythrocyte alpha I and alpha II and beta), alpha 4 actinin, non-muscle myosin II and villin as well as a protein with no significant matches to sequences in the non-redundant NCBI protein sequence database (see Materials and Methods). Given that these proteins are present in both cytoskeletal structures as well as sites of vesicular protein transport we performed immunofluorescence to determine if, and where, these proteins colocalised with FAM. Alpha actinin is an actin binding protein and localises to both the actin cytoskleleton and vesicles (Pol et al., 1997). Myosin II is a motor protein involved in actin cytoskeleton rearrangements at cellular extensions and also in the budding of a subpopulation of vesicles from the trans-Golgi network (TGN) (Allan et al., 2002; Ikonen et al., 1997). In both instances FAM colocalised only with the punctate cytoplasmic staining (Figures 3A and B) suggesting that the interaction of these proteins was also on vesicles.

FAM associates with E-cadherin and β-catenin complexes

To further investigate whether the co-localisation of FAM and β-catenin reflected their presence in common complexes the elution profile of FAM was determined using gel filtration chromatography to separate proteins and protein complexes by size followed by immunoblot analysis (Figures 4A and B). FAM was present in at least two distinct complexes in both sub-confluent and confluent T84 cells. Population I (PI) eluted around fractions 8-9 in the size range of 2 MDa, while population II (PII) peaked around fraction 13 (500 kDa) eluting over several fractions which vary in size from approximately 290 kDa, free FAM, to part of a complex of at least 400-700 kDa. Both populations have the potential to contain several FAM associated complexes. The proportion of FAM located in the two populations did not vary from sub-confluent to confluent cells (compare Figures 4A and 4B). Lysates extracted from Caco-2 cells fractionated with a very similar elution profile (data not shown). β-catenin co-eluted in PI and PII, the majority eluting in PI (Figures 4A and B), however the ratio of β-catenin changed depending on cell confluence such that the amount in PII is reduced in confluent T84 cells. Similar results were also seen for E-cadherin such that little, if any, E-cadherin was present in PII at confluence (Figures 4A and B). In addition, p120 catenin, which associates with the cadherin complex at the plasma membrane (Davis et al., 2003) was also only detected in the large molecular weight complex in confluent T84 cells (Figure 4B).

Although proteins co-elute this does not necessarily mean that they are in a complex together. Therefore to test whether β-catenin and E-cadherin were in a complex with FAM in either PI or PII, immunoprecipitation with anti-FAM antibodies was performed from fractions 8-9 and fractions 12-15 and the FAM associated proteins analysed (Figure 4C). E-cadherin and β-catenin co-immunoprecipitated with FAM from PII but not PI despite the fact that the vast

majority of β-catenin and E-cadherin was present in PI. The predicted size of an E-cadherin, β-catenin and FAM complex (approximately 500 kDa) is consistent with elution around fractions 13, suggesting that the FAM may be in a complex with these proteins alone. p120 catenin did not co-immunprecipitate with FAM, from either sub- or confluent T84 cells under the conditions used (data not shown).

FAM localises to the Golgi independently of β-catenin T84 cells

β-catenin associates with E-cadherin at the ER before the dimer is transported to the Golgi and ultimately the plasma membrane (Chen et al., 1999). Given FAM's interaction with β-catenin in T84, as well as others cells (Taya et al., 1999; Taya et al., 1998) we wished to determine if FAM's localisation to the Golgi was dependent on β-catenin. Firstly, cells were treated with Brefeldin A (BFA), which disrupts Golgi architecture (Lippincott-Schwartz et al., 1989). This treatment resulted in the severe disruption of FAM localisation throughout the entire cell (Figure 5A). To further analyse FAM's association with the Golgi, trafficking was halted at the pre-Golgi stage by incubation of cells at 15°C (Saraste and Kuismanen, 1984). After incubation at 15°C for 2 hours a much greater proportion of FAM was found localised at the Golgi than in cells grown under normal conditions (compare Figures 5B and 1B). However, this localisation at the Golgi was independent of β-catenin which was not present at the Golgi (Figure 5C). The lack of β-catenin at the Golgi was anticipated as the cells were treated with 10 μM cycloheximide, a translation inhibitor, for 30 min prior to incubation of the cells at 15°C for 2 hours. Therefore nascent proteins of the secretory pathway, including E-cadherin and associated β-catenin, would have trafficked beyond the Golgi.

Expression of E-cadherin in cadherin-negative fibroblasts redistributes FAM from the Golgi to cytoplasmic vesicles

Although FAM localised to the Golgi independently of E-cadherin and β-catenin we wished to determine if the E-cadherin - β-catenin complex could nevertheless influence FAM's localisation in the cell. Therefore we investigated the localisation of FAM in L cell fibroblasts, a cadherin-negative cell line, in the presence or absence of E-cadherin. In the absence of E-cadherin, free cytoplasmic β-catenin is rapidly degraded by the ubiquitin-proteasome system (Aberle et al., 1997; Kitagawa et al., 1999) and consequently L-cells have low levels of β-catenin protein. The majority of FAM localised to the Golgi in L-cells but low levels were also detected in cytoplasmic puncta (Figure 6A) confirming FAM's association with the Golgi in the absence of β-catenin.

We generated stably transfected L-cell lines expressing exogenous E-cadherin. This resulted in changes to cell morphology, with the cells becoming more epithelial-like. The localisation of FAM was also clearly altered with the majority of FAM localised to vesicles in the cytoplasm rather than to the Golgi (Figure 6B). In some cells punctate FAM staining was detected predominantly in sub-membranous regions below sites of cell-cell contact (Figure 6B). This alteration in FAM localisation was not due to changes in overall cellular FAM levels (Figure 6C). FAM was found to be associated with E-cadherin in these cells (Figure 6D).

Overexpression of FAM in MCF-7 epithelial cells increases the level of β-catenin at the plasma membrane

Finally, we wished to determine if FAM could influence β-catenin levels and/or intracellular localisation. To this end we over-expressed V5 epitope-tagged full length FAM in polarised MCF-7 epithelial cells which express both β-catenin and E-cadherin (Zhu et al., 2001) and are more amenable to transient transfection than T84 cells. Expression of exogenous FAM

resulted in increased levels of β-catenin, E-cadherin and p120 catenin (Figure 7A). These increases appeared to be correlated with the level of exogenous FAM expressed (Figure 7A compare lanes 2 and 3). To determine where β-catenin was localised in cells expressing exogenous FAM immunofluorescence was performed. There was no colocalisation of β-catenin and FAM in transfected cells as detected by the V5 epitope (Figure 7B) with β-catenin predominantly at the plasma membrane and FAM in cytoplasmic vesicles. β-catenin was not detected in the nucleus in any transfected cells. This suggested that FAM increased β-catenin in stable cadherin-catenin adhesion complexes at sites of cell-cell contact. Similar results were observed in HEK293T cells (data not shown). Functional cadherin-catenin adhesion complexes are bound to the cytoskeleton and hence insoluble in Triton X-100 whereas membrane inserted E-cadherin-β-catenin complex not involved in cell adhesion is Triton X-100 soluble (Svastova et al., 2003). Therefore to directly assess whether the FAM-induced increase in β-catenin, E-cadherin and p120 catenin levels was due to increased adhesion complexes the proportion of β-catenin in Triton X-100 soluble and insoluble fractions was determined. At sub-confluence a higher proportion of β-catenin in the soluble fraction in untransfected cells was observed (Figure 7C). The presence of exogenous FAM however resulted in a higher proportion of β-catenin in the insoluble fraction (Figure 7C) although FAM was found entirely in the soluble fraction indicating that FAM itself not associated with mature adhesion complexes. Together these data indicate that FAM facilitates the transport of the cadherin-catenin complex to the plasma membrane.

DISCUSSION

FAM localises at sites of protein trafficking

Here we demonstrate that the FAM deubiquitylating enzyme localises to sites of protein trafficking and/or sorting in epithelia and fibroblasts. These included the Golgi, late endosomes and/or lysosomes as well as cytoplasmic vesicles but not EEA1-positive early endosomes nor the endoplasmic reticulum. The Golgi apparatus was the predominant site of FAM localisation in L-cell fibroblasts (Figure 6) and HEK293T cells (data not shown). In addition, blocking protein trafficking at the Golgi in T84 cells resulted in most of FAM colocalising with the Golgi marker GM130 (Figure 5B) suggesting that the majority, if not all, FAM associates with the Golgi at some point. Similarly, disruption of endocytosis also resulted in dramatic re-localisation of all FAM throughout the cell (data not shown). This suggests that in T84 cells FAM is not statically resident at particular sub-cellular localisations and raises the possibility that it may remain associated with vesicles as they traverse between different compartments.

That ubiquitin plays a role in the regulation of protein trafficking is now well established although not fully understood (Katzmann et al., 2002; Schnell and Hicke, 2003). The ubiquitylation status of endocytosed proteins determines their fates en route to the lysosome. If the protein remains ubiquitylated it is sequestered into internal vesicles of multi-vesicular bodies and hence is delivered into the lysosome (Katzmann et al., 2002). A number of ubiquitin ligases and ubiquitin interacting proteins regulating these processes have been identified suggesting a networking or scaffolding role for ubiquitin in forming protein trafficking complexes (Schnell and Hicke, 2003). The yeast deubiquitylating enzyme Doa4 functions at the late endosome /prevacuolar compartment to facilitate the recycling of ubiquitin from substrates as they are sorted into internal vesicles. Although FAM and Doa4p

both localise to late stages of the endocytic pathway, they are not homologues. They share little sequence similarity beyond their ubiquitin-specific protease motifs and, Doa4 (Ubp4p) was unable to functionally replace FAM's homologue in Drosophila, Fat Facets (Faf) whereas Fam could (Chen et al., 2000; Wu et al., 1999). The identification of liquid facets, the Drosophila homologue of epsin, as a critical substrate of Faf during photoreceptor development further implicates the Fat Facets deubiquitylating enzymes in the regulation of protein trafficking (Chen et al., 2002). Genetic studies have also established that Faf facilitates endocytosis (Cadavid et al., 2000).

FAM's localisation at the Golgi apparatus raises the possibility that it may also play a role in regulating trafficking in the biosynthetic pathway. Targeting of proteins in the biosynthetic pathway can also be influenced by ubiquitylation especially at the TGN. Monoubiquitylation of the yeast permease Gap1 results in transport to the plasma membrane whereas polyubiquitylation targets it to the vacuole, a process regulated by the ubiquitin ligase Rsp5 and the ubiquitin ligase adaptors BUL1 and BUL2 (Helliwell et al., 2001). The role of deubiquitylating proteins in this process however, is unknown. FAM's colocalisation with, and immunoprecipitation of, vesicular myosin II, suggests it may be involved in the trafficking of a subset of vesicles targeted to basolateral domains as they bud from the TGN (Allan et al., 2002; Musch et al., 1997). FAM is the first DUB to be shown to be localised to the Golgi apparatus and may be involved in regulating protein trafficking by antagonizing the function of ubiquitin ligases such as Rsp5 (Kaminska et al., 2002; Wang et al., 2001). Recently a function for the yeast Ubp3 in mediating COPII vesicle traffic between the endoplasmic reticulum and cis-Golgi has been proposed (Cohen et al., 2003). However, although Ubp3p's substrate Sec23 functions in this region, Ubp3's interaction with Sec23 at this cellular location was not demonstrated (Cohen et al., 2003).

Interestingly, the localisation of FAM in polarised T84 epithelial cells differs from that observed in MDCK cells where it tightly localises to sites of cell-cell contact (Taya et al., 1998). The differences reported may be due to the different targeting mechanisms employed by the two epithelia. MDCK cells directly target cargo to the basolateral membrane from the TGN. However intestinal epithelial cells, such as T84 employ an indirect route involving a series of membrane insertions and recycling between the plasma membrane and sorting endosomes (Mostov et al., 2000). In T84 cells therefore, FAM may have a shorter resident time at sites of cell-cell contact. Alternatively the differences may reflect that the antibodies used were raised to different FAM epitopes. However, FAM has also been detected in a vesicular staining pattern in other epithelia including the polarised trophectoderm cells of preimplantation mouse embryos (Pantaleon et al., 2001) and in both primary and cultured, (HaCaT), keratinocytes (SAW unpublished observations).

Association of FAM with the E-cadherin / β-catenin complex during trafficking

We have shown previously that FAM interacts with and stabilises the cell adhesion-associated and signalling molecules β-catenin and AF-6 (Taya et al., 1999; Taya et al., 1998). In T84 cells FAM colocalised with a number of cell adhesion associated-proteins including β-catenin, AF-6, α-actinin, ZO-1 and p120 catenin. Colocalisation was only seen in the cytoplasmic puncta with all these proteins and not at sites of cell-cell contact although some punctate staining was evident just beneath the plasma membrane. The most extensive colocalisation observed was with the FAM substrate β-catenin in subconfluent cells (Figure 2A).

In epithelia, β-catenin associates with the cytoplasmic domain of E-cadherin at the endoplasmic reticulum (Chen et al., 1999) before the complex is transported through the Golgi apparatus and to the basolateral plasma membrane. Although we have previously speculated that in preimplantation mouse embryos FAM stabilises cytoplasmic β-catenin (Pantaleon et al., 2001), several of the data presented here indicate that FAM can also associate with another pool of β-catenin, namely the E-cadherin- and vesicle-associated form in transit to, or from the plasma membrane. Firstly, as mentioned above, FAM only colocalised with the punctate staining of β-catenin in subconfluent cells (Figure 2A) whereas in confluent cells FAM did not colocalise at sites of cell-cell contact nor did the proteins immunoprecipitate. In subconfluent cells the adhesion junctions are unstable and undergoing constant rearrangement and insertion of new cadherin-catenin complexes (Le et al., 1999) whereas at confluence cadherin complexes are stable and contain many more associated proteins.

Secondly, the co-immunoprecipitation of FAM with β-catenin and E-cadherin from the smaller fractions of PII only and failure to detect FAM at sites of cell-cell contact suggests that FAM does not associate with mature E-cadherin-catenin adhesion complexes. The size of fractions from PII are consistent with a complex containing FAM and β-catenin and E-cadherin alone, although the broad range of molecular weights covered in the complex does not preclude the presence of a small number of other proteins. The majority of E-cadherin and β-catenin was in PI which contains higher molecular weight complexes in the order of 2MDa which are most probably stable cell adhesion complexes at the plasma membrane. The presence of p120catenin in PI only and the failure of FAM to immunoprecipitate with p120catenin supports the argument that FAM does not associate with mature cadherin adhesion complexes. In addition E-cadherin is not present in PII in confluent cells when there

is decreased transport of E-cadherin out to PM and little recycling (Le et al., 1999). Interestingly in no instances did FAM immunoprecipitation pull down β-catenin and not E-cadherin also, even though some β-catenin was detected in PII in confluent cells and E-cadherin was not. This suggests that in T84 cells FAM associates with the E-cadherin / β-catenin complex alone.

In addition, introduction of E-cadherin into L cells resulted in the relocation of a large fraction of FAM to vesicular structures in the cytoplasm (Figure 6B) strongly arguing that FAM is associated with the trafficking of E-cadherin / β-catenin complexes. Reciprocally, increased expression of FAM in MCF-7 cells, which are polarised epithelia expressing both β-catenin and E-cadherin, resulted in an increase in β-catenin, E-cadherin and p120 catenin levels (Figure 7A) The increased β-catenin localised at the plasma membrane and not in the nucleus (Figures 7B and C) as occurs in the presence of Wnt signaling (Hecht and Kemler, 2000). These data indicate that FAM's effect are on the cadherin-catenin adhesion complex and not on stabilisation of free cytoplasmic β-catenin. The fact that FAM was found entirely in the soluble fraction and not in the adhesion complexes suggest that its primary role is to facilitate transport of the complex to the plasma membrane. This raises the interesting possibility that FAM and E-cadherin can simultaneously bind the armadillo repeats of β-catenin (Huber et al., 2001; Shapiro, 2001; Taya et al., 1999).

Two types of vesicles would be expected to contain β-catenin in subconfluent epithelia: those of the biosynthetic pathway as well as endocytic vesicles. The colocalisation of FAM with virtually all basolateral β-catenin vesicles suggest that it is associated, at least in part, with β-catenin as it traffics toward the plasma membrane. This is supported by FAM's colocalisation with myosin II which labels vesicles as they bud form the TGN (Ikonen et al., 1997).

We propose that FAM is a deubiquitylating enzyme associated with intracellular protein trafficking. In epithelial cells it appears that FAM may regulate the trafficking of both tight and adherens junction-associated proteins including AF-6, ZO-1, and the E-cadherin / β-catenin dimer. Both β-catenin and E-cadherin are known to be ubiquitylated. The cytoplasmic tail of E-cadherin is unstructured in the absence of bound β-catenin (Huber et al., 2001). It also contains PEST sequences which overlap with the β-catenin binding sites and so E-cadherin uncomplexed is degraded at the ER in a ubiquitin-dependent manner (Chen et al., 1999). E-cadherin at the plasma membrane can also be ubiquitylated by the ubiquitin ligase, Hakai in response to Src kinase (Fujita et al., 2002). The ubiquitylation of β-catenin has been well characterised but has generally been thought to be restricted to the free cytoplasmic pool. However Hakai activation also results in the ubiquitylation of β-catenin at the plasma membrane (Fujita et al., 2002).

The data here show that the mammalian deubiquitylating enzyme FAM plays a role in the trafficking of β-catenin and E-cadherin in epithelia. The data also suggest that this occurs in part, as the E-cadherin / β-catenin complex moves through the biosynthetic pathway. This may reflect a wider role for FAM in regulating the trafficking of other adhesion junction proteins including another FAM substrate, AF-6. Whether FAM's function as a deubiquitylating enzyme is important in these processes requires further investigation.

Abbreviations used in this paper: DUB, deubiquitylating enzyme; UBP, ubiquitin-specific protease; TGN, trans-Golgi Network; ER, endoplasmic reticulum; MDCK, Madin-Darby canine kidney; MVB, multi-vesicular body

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FIGURE LEGENDS

Figure 1. FAM localises to sites of protein trafficking and sorting in T84 cells.

A. Individual confocal sections from the apical and basolateral regions of subconfluent T84 cells were immunolabelled with anti-FAM antibodies. FAM is found predominantly in vesicles present throughout the cytoplasm and close to or at the plasma membrane in the basolateral region of the cells.

B. Confocal sections from the basolateral region of subconfluent T84 cells were immunolabelled for FAM, GM130, LAMP1 or EEA1. FAM partially colocalises in vesicles with the Golgi marker GM130 and the MVB marker LAMP1 but not with EEA1, an early endosome marker.

Bar represents 10 μm

Figure 2. Co-localisation of FAM with junction proteins in vesicles.

A. Individual confocal sections from basal regions of subconfluent T84 cells were co-immunolabelled for FAM and the cell adhesion proteins β-catenin, p120catenin, AF-6 and ZO-1. FAM extensively colocalised with the apical junction protein β-catenin found in vesicles in the basolateral region of subconfluent T84 cells and also, but to a lesser extent with p120catenin, AF-6 and ZO-1. Inserts show boxed regions at a higher magnification and arrows indicate colocalising puncta. The bar in the top panel is 10 μm.

B. Individual confocal sections from an apical region of confluent T84 cells were immunolabelled for FAM and β-catenin. FAM which remains cytoplasmic does not colocalise with β-catenin which is found at sites of cell-cell contact.

Figure 3. FAM localises with punctate forms of α-actinin and myosin in T84 cells.

A. Individual confocal sections from both the apical and basolateral region of T84 cells were immunolabelled for FAM and α-actinin. FAM partially colocalises with α-actinin in vesicles but not with that found at the plasma membrane.

B. Individual confocal sections from the basolateral region of subconfluent T84 cells were immunolabelled for FAM and myosin. FAM and myosin partially colocalise in basolateral cytoplasmic vesicles.

Figure 4. Two populations of FAM exist in T84 cells, one containing a proportion of cellular E-cadherin and β-catenin.

A. & B. Subconfluent and confluent (respectively) T84 cell lysates were separated by gel filtration chromatography using a Superose 6 column. Fractions were immunoblotted with anti- FAM, E-cadherin, β-catenin and p120catenin antibodies. FAM elutes as at least two distinct populations in both subconfluent and confluent T84 cells both of which elute with β-catenin, although the proportion of β-catenin eluting with PII is reduced in confluent cells.

C. Proteins eluted in PI (fractions 8-9) and PII (fractions 12-15) were immunoblotted, either before or after immunoprecipitaion with anti-FAM antibodies, with antibodies against FAM, β-catenin and E-cadherin. Both β-catenin and E-cadherin associate with FAM in a complex of approximately 500 kDa but not with the larger FAM complex(es).

Figure 5. Disrupting the Golgi alters FAM localisation while halting traffic at the Golgi in T84 cells localises FAM to the Golgi.

A. T84 cells were incubated with 1 μM Brefeldin A for 1 hour at 37°C to disrupt the Golgi. Individual confocal sections were immunostained for FAM and GM130. FAM localisation is altered when the Golgi is disrupted.

B. & C. Individual T84 cells were incubated with 10 μM cycloheximide for 30 minutes at 37°C to deplete protein production prior to incubation at 15°C for 2 hours to halt trafficking at the Golgi. Individual confocal sections from the basolateral region of T84 cells were immunostained for FAM, GM130 and β-catenin. A dramatic increase in the proportion FAM localising with GM130 is seen when trafficking is halted at the Golgi but this is independent of β-catenin which did not colocalise with GM130..

Figure 6. Exogenous E-cadherin in L-cell fibroblasts redistributes FAM from the Golgi to vesicles in the cytoplasm.

A. Individual confocal sections of L-cells, which have no E-cadherin, were immunostained for FAM and GM130. In the absence of E-cadherin FAM localises to the Golgi.

B. Individual confocal sections of L-cells stably transfected with full length E-cadherin were immunostained with anti-FAM antibodies. FAM relocalises from the Golgi to vesicles after the addition of E-cadherin to L-cells.

C. L-cells stably transfected with full length E-cadherin were lysed and immunoblotted with antibodies against FAM, E-cadherin and β-tubulin. Expression of E-cadherin does not increase the level of FAM.

D. Lysates from untransfected (top row) and E-cadherin-expressing clone A (bottom row) were immunprecipitated with antibodies against FAM or E-cadherin before western analysis with FAM antibodies. FAM associates with E-cadherin in L-cells after the addition of exogenous E-cadherin.

Figure 7. Overexpression of FAM in MCF-7 cells increases the level of β-catenin at the plasma membrane.

A. MCF-7 cells were analysed for adhesion protein expression 48 hours following transient transfection with 0, 4 or 8 μg of pDEST-FAM-V5. V5 epitope was detected in both transfected populations (4μg lane clearly visible on longer exposure). When compared to the β-tubulin loading control, the level of all adhesion proteins was increased in the presence of exogenous FAM. β-catenin and p120catenin appeared to show a grade response to the different levels of FAM-V5.

B. Confluent MCF-7 cells were analysed for the localisation of exogenous FAM as detected by the V5 epitope, and β-catenin. FAM-V5 and β-catenin did not colocalise, with FAM predominantly in cytoplasmic vesicles and β-catenin concentrated at the plasma membrane. Only 10% - 20% of the cells appeared to have been transfected.

C. Soluble and insoluble fractions of β-catenin in subconfluent MCF-7 cells transiently transfected with pDEST-FAM-V5. Although exogenous FAM is exclusively localised to the soluble fraction it results in a higher proportion of β-catenin in the insoluble fraction.

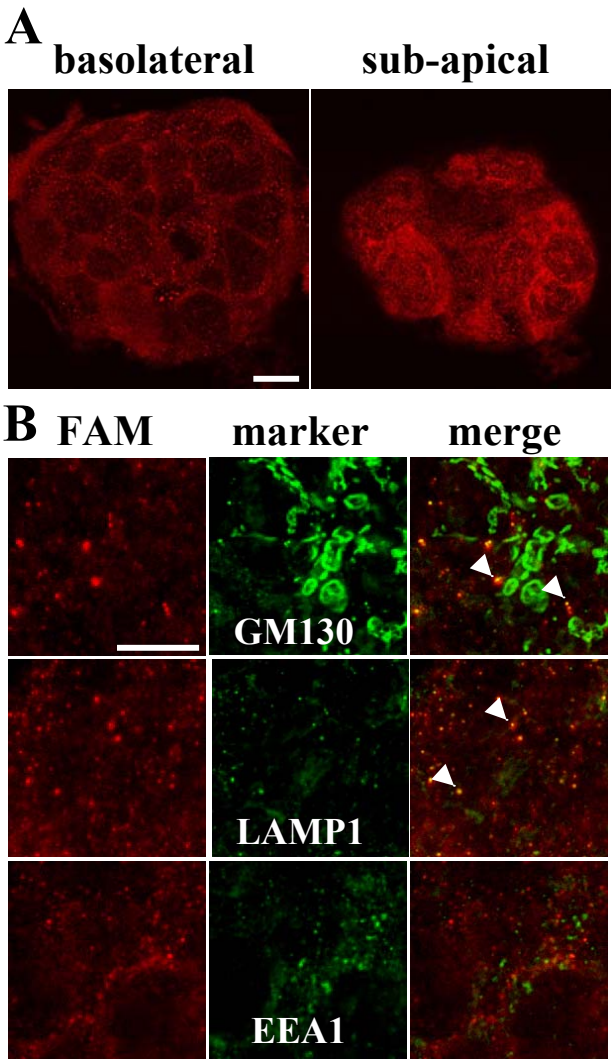

A
basolateral
sub-apical
B
FAM
marker
merge
GM130
LAMP1
EEA1

Figure 1

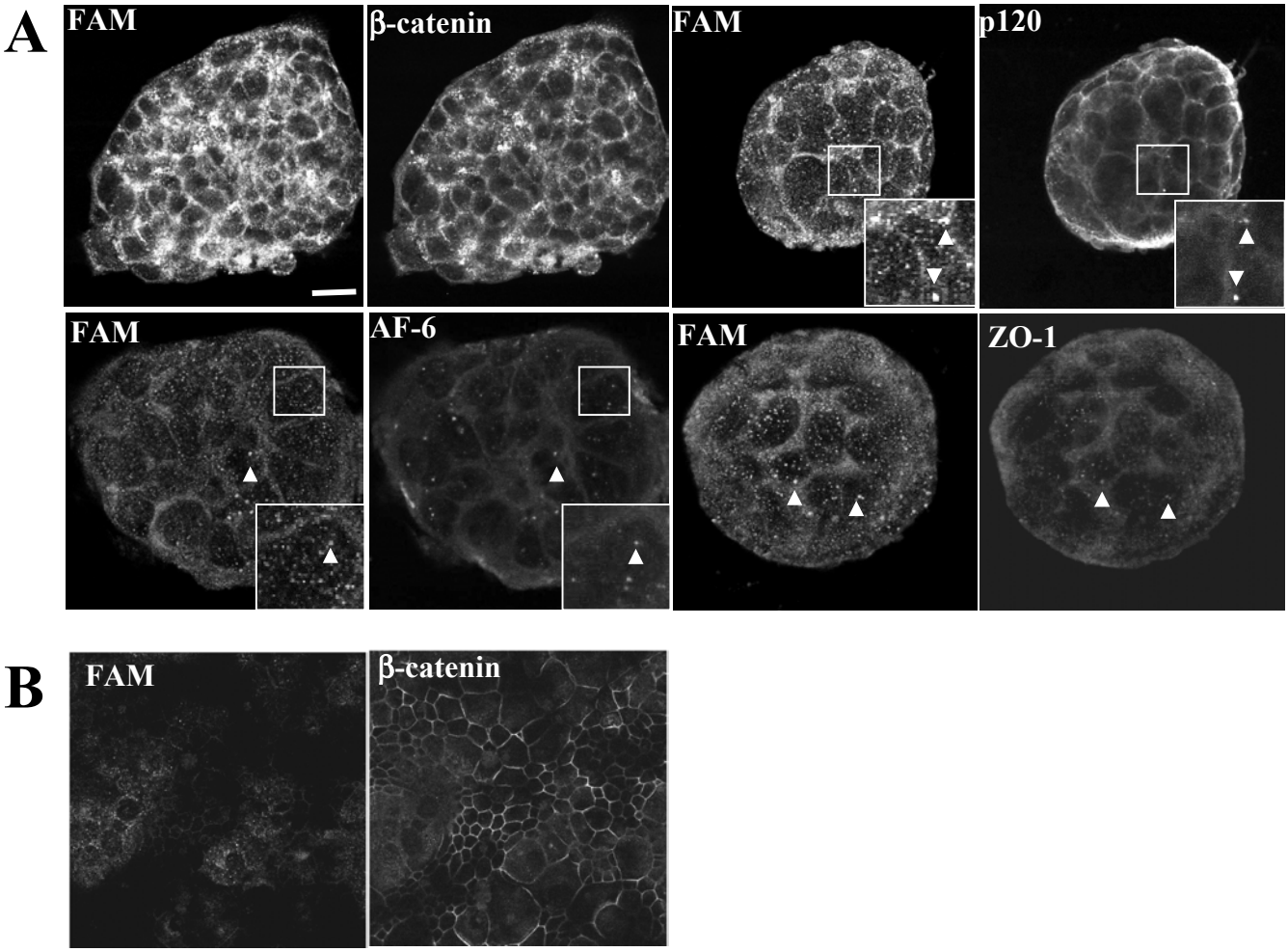


Figure 2

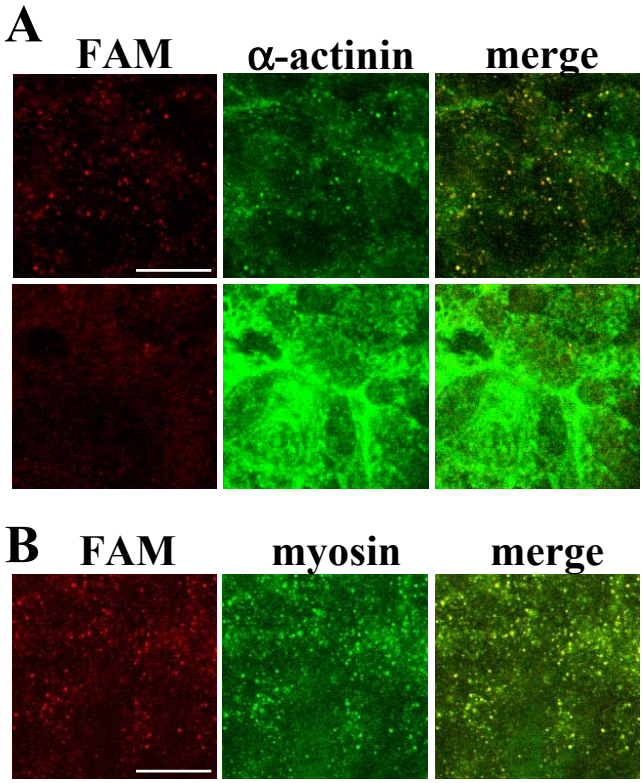
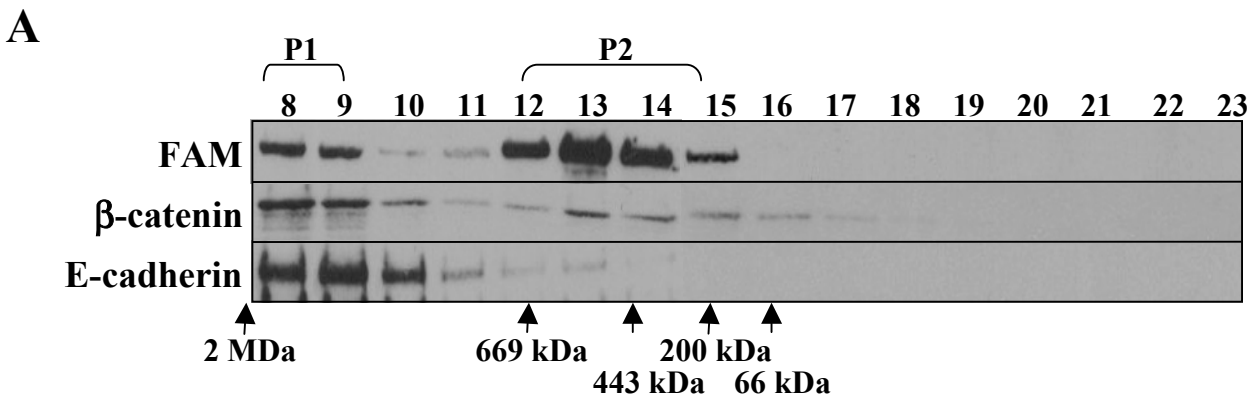
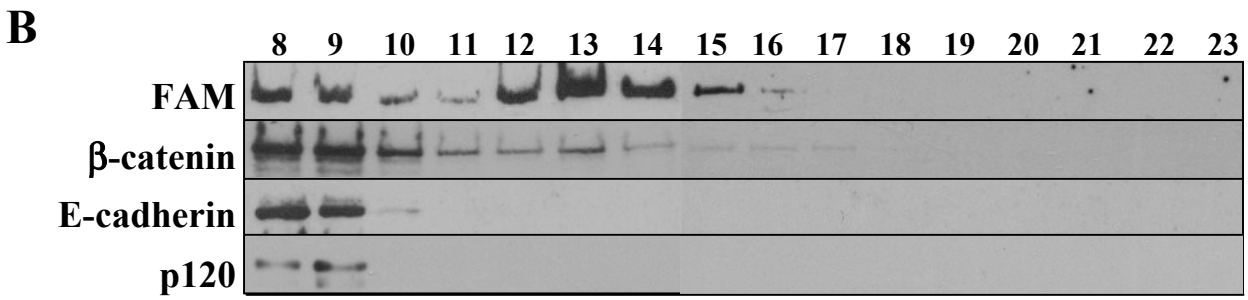
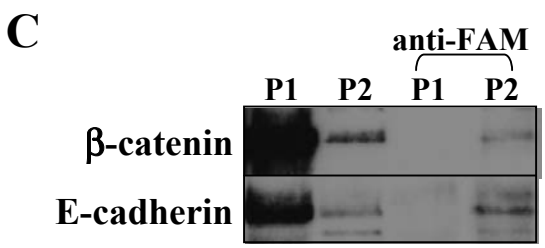


Figure 3


A
P1
P2
8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
FAM
β-catenin
E-cadherin
2 MDa
669 kDa
443 kDa
200 kDa
66 kDa


B
8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
FAM
β-catenin
E-cadherin
p120


C
anti-FAM
P1 P2 P1 P2
β-catenin
E-cadherin

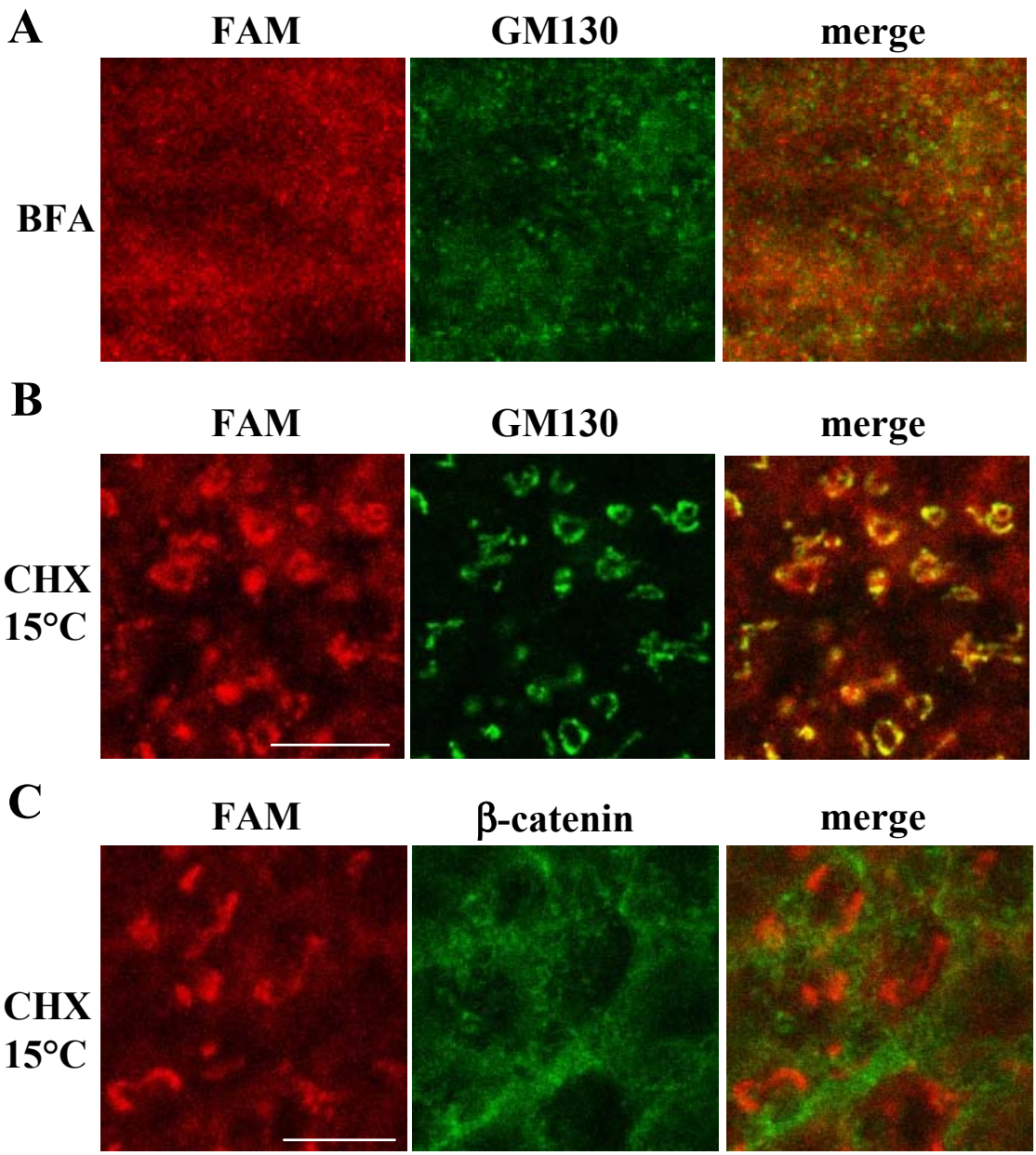

A
FAM
GM130
merge
BFA
B
FAM
GM130
merge
CHX
15°C
C
FAM
β-catenin
merge
CHX
15°C

Figure 5

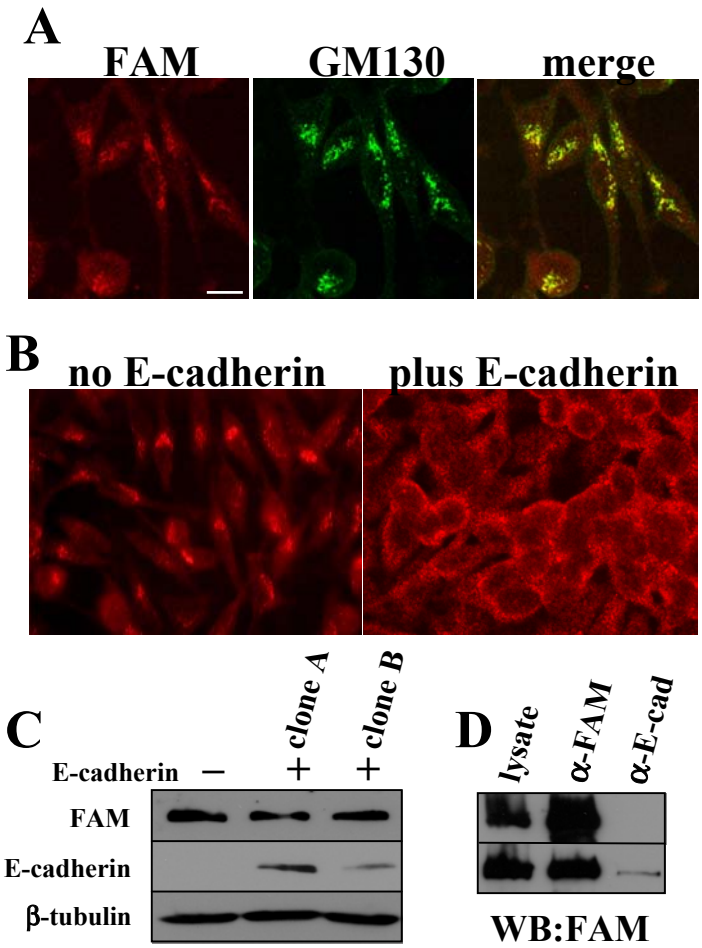
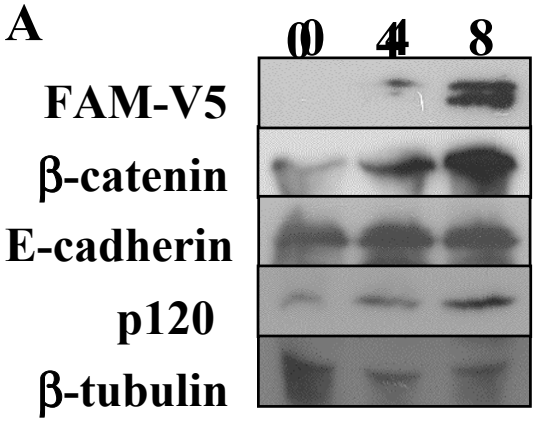
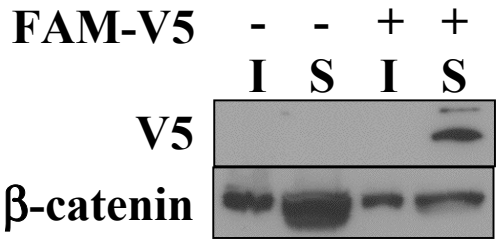

A
FAM
GM130
merge
B
no E-cadherin
plus E-cadherin
C
E-cadherin
–
+ clone A
+ clone B
FAM
E-cadherin
β-tubulin
D
lysate
α-FAM
α-E-cad
WB:FAM

Figure 6

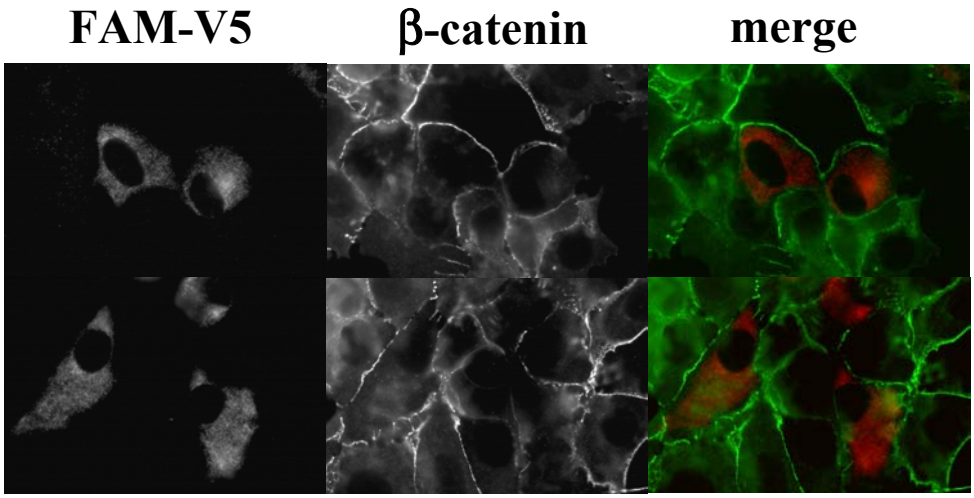
A


0
4
8
FAM-V5
β-catenin
E-cadherin
p120
β-tubulin

C


FAM-V5
- - + +
I S I S
V5
β-catenin

B


FAM-V5
β-catenin
merge