

DUAL PROCESSING OF FAT1 CADHERIN BY HUMAN MELANOMA CELLS GENERATES DISTINCT PROTEIN PRODUCTS*

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The giant cadherin, Fat1, is one of four vertebrate orthologues of the *Drosophila* tumor-suppressor, Fat. It engages in several functions, including cell polarity and migration, and in Hippo signaling during development. Homozygous deletions in oral cancer suggest Fat1 may play a tumor suppressor role although over-expression of Fat1 has been reported in some other cancers. Here we show using Northern blotting that human melanoma lines variably but universally express Fat1 and less commonly Fat2, Fat3 and Fat4. Both normal melanocytes and keratinocytes also express comparable Fat1 mRNA relative to melanoma cells. Analysis of the protein processing of Fat1 in keratinocytes revealed that, like *Drosophila* Fat, human Fat1 is cleaved into a non-covalent heterodimer before achieving cell surface expression. The use of inhibitors also established such cleavage requires the proprotein convertase furin. However, in melanoma cells the non-cleaved pro-form of Fat1 is also expressed at the cell surface together with the furin-cleaved heterodimer. Moreover furin-independent processing generates a potentially functional proteolytic product in melanoma cells: a persistent 65kDa membrane-bound cytoplasmic fragment no longer in association with the extracellular

fragment. *In vitro* localization studies of Fat1 showed melanoma cells display high levels of cytosolic Fat1 protein whereas keratinocytes, despite comparable Fat1 expression levels, exhibited mainly cell-cell junctional staining. Such differences in protein distribution appear to reconcile with the different protein products generated by dual Fat1 processing. We suggest that the uncleaved Fat1 could promote altered signaling, and the novel products of alternate processing provide a dominant negative function in melanoma.

Fat was first identified in *Drosophila* as a giant member of the cadherin superfamily that functioned as a tumor-suppressor gene (1). The first vertebrate Fat to be cloned (subsequently re-named Fat1) showed considerable homology to *Drosophila* Fat in encoding a type 1 transmembrane protein containing 34 cadherin repeats, 5 EGF-like repeats and a laminin A-G domain in the extracellular region and a cytoplasmic tail that was quite distinct from classical cadherins (2). Limited studies on human tissues indicated that Fat1 expression is developmentally regulated and largely confined to embryonic tissues, findings confirmed in zebrafish, rats and mice (3-5). Four Fat genes have now been identified in vertebrates, and McNeill and

colleagues (6) have shown that Fat4 is the true structural orthologue of *Drosophila* Fat in mammals.

Despite this, several pieces of experimental data support the notion proposed by Skouloudaki *et al* (7) that the functions of *Drosophila* Fat signaling are shared between Fat1 and Fat4 in vertebrates. Thus, as with *Drosophila* Fat which co-operatively regulates planar cell polarity through binding to Atrophin (8), human Fat1 also physically binds Atrophins 1 and 2 to regulate cell orientation in smooth muscle cells (9). In *Drosophila*, Fat also participates in the Hippo pathway that regulates cell growth and organ size (10,11), a function likely relating to its role as a tumor suppressor. Fat1 also shares this functional role, at least in zebrafish where it binds and co-ordinates with Scribble to regulate Yes-associated protein 1 (YAP1), a key downstream regulator of the Hippo pathway during pronephros development (7). There are also data to suggest that this shared functionality between *Drosophila* Fat and Fat1 may extend to a suppressor function for human Fat1. In a study designed to identify the location of candidate tumor suppressor genes in oral cancer, homozygous deletions (HD) of Fat1 were identified in a genome-wide screening of a primary oral cancer (12). Further analysis by genomic PCR revealed that 80% of 20 primary oral cancers exhibited exonic HD of Fat1. Also, in an immunohistochemical study of 31 cases of intrahepatic cholangiocarcinoma, Settakorn *et al* (13) found that Fat1 expression showed a significant inverse association with the Ki67 index, and that loss of membrane localization for Fat1 correlated with more aggressive tumors.

Paradoxically, in their original cloning paper, Dunne *et al* (2) recorded that human Fat1 mRNA expression was high in epithelial cells from some breast and colorectal cancers, and in immunochemical studies of *ex vivo* breast (14) also shown high levels of

cytoplasmic Fat1 expression in the tumor cells. In this report an analysis of the distribution of Fat1 in cell lines found contrasting expression patterns comparing normal keratinocytes with melanoma cells. In keratinocytes, Fat1 was expressed mainly at cell-cell junctions whereas melanoma cells displayed abundant intracytoplasmic Fat1 staining. Northern blotting analysis did not show greatly increased levels of transcription or obvious splice variants in the melanoma cells compared to keratinocytes, therefore we considered the post-translational processing of Fat1 in these cells as a possible explanation.

Fat1 processing has not been studied, but the processing of *Drosophila* Fat, and murine Fat4 has been examined in two recent studies (6,15). It was shown that Fat was intrinsically cleaved in the early secretory pathway before being expressed on the cell surface as a non-covalently associated heterodimer. Further processing to generate an intracellular fragment able to traverse to the nucleus was dependent upon ligand binding resulting in casein kinase-dependent phosphorylation followed by enzymic cleavage likely involving an ADAM type metalloprotease sequentially followed by further intracytoplasmic cleavage by the γ -secretase complex.

We show here that in human keratinocyte and melanoma cell lines, Fat1 is processed by a similar intrinsic cleavage pathway and further demonstrate that the enzyme involved is furin. However, in the melanoma cells we have identified an alternative intrinsic pathway of Fat1 processing that is furin independent and results in the generation of a membrane-bound fragment (p65) that could account for the cytoplasmic staining for Fat1 seen in these cells. Our interpretation of these findings is that such alternative processing of Fat1 could enable the tumor cells to utilize the advantageous role of Fat1 in cell migration (16-18) while simultaneously negating the

suppressive-suppressive role of Fat1 in the Hippo pathway.

Experimental Procedures

Cell lines and culture- Neonatal human foreskin keratinocytes (a kind gift of Dr. Sean Geary and Prof. Leonie Ashman (University of Newcastle) were cultured in Keratinocyte-SFM (Invitrogen). Normal human melanocytes were kindly provided by Dr P Parsons (Queensland Institute of Medical Research, Qld, Australia) and cultured in medium purchased from by Clonetics (Edward Kellar, Vic, Australia). The immortalised adult human keratinocytes (HaCaT) cell line was obtained under an MTA from Deutsches Krebsforschungszentrum (German Cancer Research Center) and cultured in DMEM (Lonza) supplemented with 5-10% fetal bovine serum (Sigma-Aldrich). The panel of human melanoma cell lines used were obtained from various sources as previously described (19-21). Where indicated, cells were treated with 25 μ M Decanoyl-RVKR-CMK (a cell-permeable compound known to irreversibly inhibit furin at a K_i ~1nM ((22); BIOMOL) for 16 h prior to analysis. A 20mM stock solution of the inhibitor was made in DMSO with the equivalent volume of DMSO used as a control.

Polyclonal (pAb) and monoclonal (mAb) anti-Fat1 antibody production- Serum was collected and stored from two rabbits immunized with a GST-fusion protein incorporating the entire cytoplasmic tail of human Fat1 cadherin (GST-Fat1-aa4203-4588). Antibodies were affinity purified from the serum using a GST-fusion protein incorporating the last ~1/3 of the cytoplasmic tail (GST-Fat1-aa4454-4588). Affinity purified polyclonal antibodies recognizing the extracellular domain of Fat1 were similarly prepared from antiserum obtained

commercially (AbSea, Beijing, PRC) from rabbits immunized with a GST-fusion protein incorporating amino acids 1162-1326 (GST-Fat1-aa1162-1326). Murine monoclonal antibodies against the extracellular domain or cytoplasmic domain of Fat1 were produced as described previously (23) from mice immunized with either the GST-Fat1-aa1162-1326 or GST-Fat1-aa4203-4588, respectively. Monoclonal antibodies were purified using Protein G HP SpinTrap columns according to the manufacturer's instructions (GE Healthcare). An isotype-matched mAb (11H5 anti-CD36 mAb, murine IgG1 (23)) was used throughout as a negative control.

Northern blotting- Analyses were performed as described previously (24) with minor modifications. Total RNA was isolated from melanoma cells using Illustra RNAspin Mini Isolation Kit (GE Healthcare), and 20 μ g per sample was electrophoresed on 1.2 % denaturing agarose gels, transferred to Hybond-N+ membranes (GE Healthcare) and subjected to baking and U.V. crosslinking. Hybridizations were performed in ExpressHyb (Clontech) using ³²P-dCTP cDNA probes (1 x 10⁶ CPM/mL) prepared by random-priming (Prime-a-gene kit; Promega) and purified using ProbeQuant G-50 micro columns (Amersham Biosciences). The Fat1-4 cDNA probes used for hybridization corresponded to the following sequences: Fat1 (nt13091-14751; NM_005245.3), Fat2 (nt12541-14450; NM_001447.2), Fat3 (nt13141 - 14870; NM_001008781.2) and Fat4 (nt13081 - 14580; NM_024582.4).

Preparation of cell lysates and immunoprecipitation- Unless indicated otherwise, soluble cell lysates were prepared using NDE lysis buffer supplemented with protease and phosphatase inhibitors (Complete protease inhibitor cocktail and PhosSTOP, respectively; Roche) as previously described (25). Cell lysates were precleared twice with 30 μ L of Protein A/G-PLUS agarose beads (Santa Cruz Biotechnology) for 1-2 h, lysates

were immunoprecipitated with 1-2 ug of the indicated mAb pre-coupled to 20 uL of beads. The beads were then washed alternately a total of four times with either SDS-RIPA or high salt-RIPA buffers (1% NP40, 0.1% SDS, 0.1% sodium deoxycholate, 150 mM NaCl in 50 mM Tris-HCl, pH 8 and 1% NP40, 0.5% sodium deoxycholate, 0.5 M NaCl in 50 mM Tris-HCl pH 7.4, respectively) before elution of immunocomplexes using LDS sample buffer. In some experiments, samples were treated with 400 U of Lambda Protein Phosphatase (Lambda PP) (New England BioLabs) for 2 h at 30°C prior to analysis.

Tris Acetate-gel electrophoresis and Western blotting- To optimally resolve high Mr proteins, samples prepared in LDS sample buffer were applied to commercial acrylamide gels (NuPAGE® Novex 3-8% Tris-Acetate mini/midi gels; Invitrogen). After electrophoresis, proteins were transferred to nitrocellulose membranes using a semi-dry blotting system (iBlot® Transfer Stack and iBlot® device, Invitrogen). Western blotting was then performed as described previously using an ECL-based detection system (23) with the results recorded using a cooled CCD camera system (Fuji-LAS-4000, Fujifilm Life Science).

In vivo cell labeling- Pulse-chase labeling experiments with ³⁵S-methionine/cysteine were conducted as previously described (26). Gel images were captured using phosphor storage as described above. Densitometric analysis was performed using Multiguage image analysis software (Fujifilm Lifesciences).

Cell surface biotinylation- Where indicated cell surface proteins were labeled with biotinamidocaproate N-hydroxysuccinimide ester (Sigma-Aldrich) as described previously (20) before preparation of cell lysates and immunoprecipitation as described above. After electrophoresis and transfer, biotinylated proteins were detected using the ECL-based detection system described above after

probing with NeutrAvidin-HRP Conjugate (ThermoScientific). To perform subsequent Western blotting analysis, HRP-activity was first quenched by treatment of membranes with 1mM NaN₃ solution for 1h at RT.

siRNA mediated knockdown and qRT-PCR analysis- siRNAs targeting Fat1 were purchased from GenePharma (Shanghai, PRC). The sense and anti-sense strands of the siRNAs used were as follows: Fat1-1223-1241 (5'-CCAGUUCUCUUCUGUAAAUU-3' and 5'-UUUAAACAGAAGAGAACUGGUU-3'), Fat1-2473-2491 (5'-AUAGUUGCUUCAUGAUUGAUU-3' and 5'-UCAAUCAUGAAGCAACUAUUU-3'), Fat1-13890-13910 (5'-GACGACGGCCACUUCGAAGAGUU-3' and 5'-CUCUUCGAAGUGGCCGUCGUCUU-3').

Cells were transfected with a pool of three siRNA using RNAimax (Invitrogen) according to the manufacturer's protocol with a final concentration of 50nM of siRNA. The ability of each siRNA to mediate knockdown of Fat1 mRNA was confirmed using qRT-PCR. Total RNA was extracted as described above and reverse transcription performed with random hexamers using BioScript™ (Bioline, Australia) according to the manufacturer's instructions. Quantitative RT-PCR was performed using SensiMix™ SYBR Kit (Bioline) and with the specific primers for the Fat1 gene: (forward, 5'-GTGTGATTTCGGGTTTTAGGG-3' and reverse, 5'-CTGTACTCGTGGCTGCAGTT-3'). The reaction was carried out in the Applied BioSystem RT-PCR 7500 series for 40 cycles as follows: 95°C for 15 seconds followed by 1 minute of 60°C.

Microsome isolation- Cell monolayers were swelled *in situ* using HB buffer (250 mM sucrose, 1 mM EDTA, 20 mM HEPES-NaOH, pH 7.4 containing a protease inhibitor cocktail (Complete, Roche)) after which the cells were collected using a cell scraper. Pooled cell suspensions were then subjected to nitrogen

cavitation in a Parr Model 4639 device charged at 550 psi for 15min. Disrupted cells were then sequentially centrifuged at 1,000 x g and 10,000 x g for 10 minutes each to remove nuclei and mitochondria respectively. The supernatant was divided and mock treated for 30 minutes with HB buffer or buffer containing 200 mM sodium carbonate, pH 10. Samples were ultracentrifuged at 100,000 x g for 30 minutes (TLA 110, Beckman-Coulter) and the resulting membrane pellets were washed once in HB buffer and centrifuged again for 30min. Pellets were suspended in HB before undertaking Western blotting analysis against either Fat1, CD36 (Long6; (23)) or Annexin II (sc-1924; Santa Cruz).

Indirect immunofluorescent staining and confocal microscopy- Immunostaining and confocal microscopy was performed using the rabbit polyclonal anti-Fat1 cytoplasmic antibodies on formaldehyde fixed, Triton-X-100 permeated cells attached to glass coverslips essentially as described previously (19). Controls were stained in parallel with diluted normal rabbit serum.

RESULTS

Fat1 is the major Fat cadherin expressed by human melanoma cells. To examine the expression of all four vertebrate Fat cadherins in melanoma cells at the mRNA level, Northern blotting using specific probes against Fat1, Fat2, Fat3 and Fat4 was undertaken against a large panel of melanoma cell lines (Fig. 1). This analysis showed that all 16 lines tested express a single major transcript of Fat1 at around 15kb in size (1A). No prominent smaller Fat1 transcripts were identified in any of these lines (Fig. S1). A repeat blot of 15 of the melanoma cell lines showed the same result for Fat1 (not shown) and re-probing for Fat2, Fat3 and Fat4 (1B-D) revealed that Fat1 is the major Fat cadherin expressed by human

melanoma cell lines. Northern blotting of cultured, normal melanocytes, and the immortalized keratinocyte cell line, HaCaT, indicated that these cells expressed the Fat1 transcript at levels that were comparable to many of the melanoma cell lines (Fig. 1).

Fat1 is proteolytically processed before achieving cell surface expression as a non-covalent heterodimer in HaCaT cells. Nothing is known about the processing of Fat1, but both Drosophila Fat and murine Fat4 initially undergo a proteolytic cleavage step in the secretory pathway before cell surface expression (6,15). This process produces a non-covalent heterodimer comprised of a large subunit containing most of the extracellular domain and a smaller subunit that contains the cytoplasmic and transmembrane domains. Apparently similar proteolytic processing of Fat1 was indicated in a series of immunoblotting experiments designed to examine the levels of Fat1 expressed by HaCaT keratinocytes with increasing confluence (Fig. 2A) and time in culture (Fig. 2B). Upon probing the blots with a polyclonal rabbit antibody directed against the cytoplasmic domain of Fat1, a high molecular weight band (~500kDa) consistent with a protein encoded by the Fat1 mRNA was observed and found to show increased intensity at higher confluency and with time in culture. A second band at around 85kDa (p85) showed the same properties, indicating that it may be a cleaved product containing the cytoplasmic domain of Fat1. Because the HaCaT cells progressively form a contiguous epithelial sheet with increasing cell density, the implication of this observation is that Fat1 processing in these cells appears similar regardless of confluence or time of culture.

To provide further evidence that Fat1 undergoes proteolytic processing we carried out pulse-chase experiments in HaCaT cells using pooled mAb against both the extracellular domain and the cytoplasmic domain of Fat1 (see Fig. S2 for mAb details)

to precipitate the labeled proteins before fluorographic and densitometric analysis. The results shown in Fig. 2C clearly reveal such proteolytic processing. After 10 min of chase faint bands are first detected at around 430kDa (p430) and 85 kDa (p85) and the progressive appearance of these bands reciprocates the relative loss of the 500kDa (p500) Fat1 band as a proportion of the total, indicating a precursor, product relationship. An unknown protein of 210kDa is also co-precipitated in the early period of the chase appears to complex with Fat1, likely prior to cleavage, since it appears to disassociate from Fat1 after cleavage begins to occur (see Results below). The nature of this associated protein remains unknown at this time but it does not blot for Fat1.

Next we sought to establish whether p430 and p85 formed a heterodimer at the cell surface of HaCaT cells. Cells were surface labeled with biotin before lysis and immunoprecipitation with mAb directed against an epitope on the extracellular domain (NTD) or the cytoplasmic domain (CTD). Probing the blot with NeutrAvidin to reveal surface proteins identified a single prominent band at around 430 kDa (p430) and this band was precipitated with both the NTD and CTD mAb (Fig. 3A). Re-probing the blot with a rabbit antibody against the C-terminal region of Fat1 illuminated the p500 Fat1 and also a prominent p85 band, both bands also being precipitated with both the NTD and CTD mAb (Fig. 3A). Note that if p430 on the cell surface represents full-length Fat1 (p500) after cleavage of the cytoplasmic domain, this band would not be illuminated with the rabbit antibody against the C-terminus (CTD pAb). Similarly if the p85 band comprised the cleaved cytoplasmic fragment, it would not be expected to display significant labeling with biotin. Indeed, both such predictions are supported by the data (Fig. 3A).

These concepts were further explored when similar immunoprecipitates from HaCaT

cell lysates were subjected to Western blotting using rabbit antibodies against extracellular epitopes (NTD pAb; refer Fig. S2) and the results compared to those using the CTD pAb (Fig. 3B). These experiments showed that both the NTD and CTD pAb illuminated p500 representing full-length Fat1. However, only the NTD pAb revealed p430 (left panel) in contrast to the CTD pAb that specifically decorated the p85 band (right panel). Collectively these data establish that, like *Drosophila* Fat and murine Fat4, human Fat1 in HaCaT cells is cleaved to form a heterodimer before being expressed on the cell surface.

The experiments shown in Figure 3A were carried out under reducing conditions, thus the precipitation of both p430 and p85 by the NTD and CTD mAb indicates a non-covalent association between the subunits. Experiments in which calcium ions were added or chelated before immunoprecipitation did not affect the co-precipitation of the dimeric subunits (Figure 3A) nor did lysis in RIPA buffer containing 0.1% SDS (w/v) and warming (Fig. S3 and data not shown), conditions that have been reported to be sufficient to disrupt the Notch heterodimer (27). However, increasing the concentration of SDS in the cell lysis to 1% showed that the p430 and p85 bands could be individually precipitated by the NTD and CTD mAb, respectively indicating the dissociation of the Fat1 heterodimer (Fig. S3). Although the biophysical mechanism of the association between the chains of the Fat1 heterodimer is yet to be established, this experiment substantiates the occurrence of a non-covalent heterodimer formed between the NTD and CTD domains of Fat1 after proteolytic cleavage.

An additional Fat1 cleavage product, p65, identified in melanoma cells. We then analyzed Fat1 protein in melanoma cells by Western blotting cell lysates with the rabbit antibody against the cytoplasmic domain. Of 7

cell lines tested, all displayed p500 and p85 but, in addition, a prominent band at around 65kDa (p65) not seen in the keratinocytes (data not shown). To ensure that this additional band was Fat1 derived, siRNA was used successfully to knockdown Fat1 expression in 4 of these cell lines before again blotting with the rabbit anti-Fat1 antibody when it was seen that this treatment greatly reduced the expression of all 3 bands (Figure 4A). We have raised a panel of mAb against different epitopes that encompass the entire cytoplasmic tail of human Fat1 (Fig. S2). These were used in immunoprecipitation experiments to substantiate the Fat1 origin of this product and to determine whether p65 contains the entire cytoplasmic region of Fat1. In addition, we examined the cell surface Fat1 precipitated by these mAb with prior biotin labeling and NeutrAvidin blotting. Figure 4B reveals that all 9 mAb precipitated p65 as well as p500, p430 and p85, suggesting that either p65 encompasses the entire cytoplasmic domain of Fat1 or that it co-precipitates with p500 or p85. In addition, blotting with NeutrAvidin indicates that in these cells, in addition to the cleaved heterodimeric subunit (p430), full-length Fat1 (p500) also achieves cell-surface expression.

Next, we assessed whether p65, like p85 in keratinocytes, forms a heterodimer with p430/p85 or p500 by immunoprecipitation with mAb specific for an epitope in the extracellular domain (NTD-7) or the cytoplasmic domain (CTD-7). Again these blots were sequentially developed for cell surface expression and for the Fat1 cytoplasmic domain. The results (Fig. 4C) revealed that both mAb precipitated both p500 and p430 from the labeled cell surface, and both also precipitated p85 as anticipated; however, only the CTD mAb precipitated p65. Hence, these data, together with the results in Fig. 4B, indicate that p65 contains the entire cytoplasmic region of Fat1 but no longer forms a stable association with the

extracellular domain. As with the keratinocytes, neither the addition of CaCl_2 (not shown) or βME , nor calcium chelation with EDTA at room temperature apparently influenced association between p430 and p85, and none influenced the relative amount of p65 precipitated by the anti-CTD mAb (Fig. 4D).

In *Drosophila*, after the initial processing step that occurs as part of its normal maturation, Fat can be subjected to further cleavage to yield a smaller product that contains the cytoplasmic domain but no longer associates with the extracellular domain fragment (15). However, prior to this process, the cytoplasmic tail of the heterodimer is phosphorylated by the *Drosophila* homologue of casein kinase 1 δ and ϵ , an event that is stimulated by ligand binding (6,15). By analogy with the processing of the Notch receptor, Feng and Irving describe the initial processing step as S1 cleavage. Therefore, it seems reasonable to propose that, again analogous to the processing of the Notch receptor, an S2 cleavage of the p85 subunit of Fat1 could result in the generation of the p65 fragment seen in the human melanoma cells. However, the ligand for Fat1 has not been identified therefore it cannot be established whether such postulated S2 cleavage is ligand induced or spontaneous in these cells. However, the broad bands seen for both p85 and p65 can occasionally be seen to run as a doublet in Western blots that are slightly underexposed (see Fig. 4D), suggesting the possibility that a proportion of these cleavage products is phosphorylated. This was confirmed by treatment of the immunoprecipitates with lambda protein phosphatase when the diffuse p85 and p65 bands collapsed into single sharp bands that shared the mobility of the lower band of the doublet (Fig. 5).

The p65 Cleavage Product is Derived from Full-length Fat1. An in silico analysis of the Fat1 protein showed that it contained a

number of predicted furin and general proprotein convertase cleavage motifs (Table S1). In order to investigate whether the proteolytic processing of Fat1 is furin-mediated, as is the case for other cadherins, we utilized the colon carcinoma cell line, LoVo, which expresses only a non-functional furin mutation. In these experiments, as before, cells were labeled on the surface with biotin before precipitation of lysates with mAb against an extracellular (NTD) or cytoplasmic (CTD) epitope of Fat1 (together with an irrelevant control mAb, 11H5) and blotting for surface proteins with NeutrAvidin and for Fat1 with a rabbit antibody against the cytoplasmic tail of Fat1. As illustrated in Fig. 6A, blotting with the rabbit antibody showed the absence of a specific p85 band, showing that p500 Fat1 had not been processed to form the heterodimer found in keratinocytes and thereby implicating furin in the cleavage process. The NeutrAvidin blot from the same experiment showed clearly that in these cells uncleaved Fat1 achieved surface expression as p500. However, quite unexpectedly, in the rabbit anti-Fat1 blot, in addition to the p500 band, there was a prominent band at about 65kDa that was precipitated by the CTD mAb but not by the NTD mAb. The specific nature of this p65 band was confirmed by knocking down Fat1 with siRNA in the LoVo cells (Figure 6B). In these experiments Fat1 knockdown was less than complete, but the reduction seen in the p500 band was mimicked by the reduction in the p65 band. This result suggests that if the p65 band identified in the LoVo cells is the same as p65 in melanoma cells then the origin of this band derives from uncleaved rather than processed Fat1.

We addressed this possibility by treating melanoma cells with the peptide Dec-RVKR that is an efficient inhibitor of furin function (Fig. 6C). In these experiments Dec-RVKR abrogated almost completely the appearance of p430 at the cell surface but the cell surface expression of full-length p500 was

not affected. In the rabbit anti-Fat1 blots p500 was relatively unaffected by this treatment but the appearance of p85 was almost totally abrogated, confirming a role for furin in the cleavage process. Significantly, however, the p65 band precipitated only with the CTD mAb was unaffected by treatment with this peptide.

Further to this, in the same experiment we also tested the effect of growth factor stimulation by increasing the concentration of serum since the expression of Fat1 can be stimulated by serum in vascular smooth muscle cells (16). Figure 6C additionally shows that melanoma cells similarly respond with increased Fat1 expression but this does not alter the ratio of p500:p430 (uncleaved:cleaved) Fat1 on the cell surface of melanoma cells nor the relative amount of the p65 product. Collectively these data establish that there exists an alternative, furin-independent, pathway of Fat1 processing in the melanoma (and LoVo) cells that results in the generation of an intracellular p65 fragment that contains the entire cytoplasmic region of Fat1 but no longer associates with the extracellular domains.

The p65 CTD Fragment of Fat1 is Membrane Associated. We next sought to determine the fate of the fragments of Fat1 generated by dual processing in the melanoma cells. Immunofluorescent staining of permeabilized cells with rabbit antibodies against the ECD of Fat1 showed a messy picture with apparent staining of the extracellular matrix being observed in addition to cell-specific staining (data not shown). This alerted us to the possibility that the extracellular product may have been cleaved at the cell surface and released, or secreted after cleavage within the secretory pathway. Western blotting experiments undertaken with the Fat1 NTD pAb in MelCV cells showed both Fat1 NTD and CTD mAbs immunoprecipitated the expected p500 and p430 bands from cell lysates. In contrast, immunoprecipitations from the cell supernatant identified 2 weak

bands of higher motility that were specifically precipitated by only the NTD mAb (Fig. S4). Therefore, this result suggests the release of the cleaved extracellular Fat1 fragment, which may possibly be subjected to progressive cleavage at its C-terminal end. Since no similar fragments could be detected in HaCaT cell supernatants (data not shown), these data suggest that alternative Fat1 cleavage may be responsible for the appearance of these Fat1 fragments. However given this process is catalysed by an unknown enzyme(s), it will be necessary to establish the identity of the cleavage enzyme(s) before further work can be pursued.

To determine whether cleavage also releases the p65 product from membranes, we prepared microsomes from C32 melanoma cells and extracted these with high pH carbonate buffer, a treatment that releases the contents of microsomes and removes non-integral membrane proteins including those that peripherally associate with membranes. As anticipated, Western blotting with the rabbit anti-CTD antibody showed both full-length p500 Fat1 and the p85 fragment pelleted with the membranes, and so also did p65. Upon carbonate extraction none of these bands was significantly reduced (Fig. 7, left panel). The extraction conditions were verified using blotting against CD36, an integral membrane protein (25), and Annexin II, a protein known to peripherally associate with membranes (28). As shown in Fig. 7 (right panel), CD36 was not removed by carbonate treatment whereas Annexin II was effectively depleted by carbonate extraction. Therefore these data indicate that p65 is predominantly integrated into the membrane, a supposition in keeping with its observed size. A summary comparing the dual mechanisms of Fat1 processing is presented in Fig. 8.

The cellular distribution of Fat1 cadherin may reconcile with the products of dual proteolytic processing. Neonatal human keratinocytes, HaCaT keratinocytes and MV3 melanoma

cells were stained *in situ* with a rabbit polyclonal antibody against the cytoplasmic tail of Fat1 to examine its localization (Fig. 9). This analysis showed both the *ex vivo* keratinocytes and those from the HaCaT cell line displayed almost exclusively cell-cell junctional staining. The melanoma cells also exhibited a degree of cell junctional staining, but most staining was distributed throughout the cytoplasm, with occasional nuclear localization. Similar patterns were found in a number of different melanoma cell lines (data not shown).

Given the similarities between the distribution of Fat1 in melanoma cells and the major differences in comparison to keratinocytes, these data suggest that the intracellular accumulation of Fat1 by melanoma cells may represent the novel Fat1 proteolytic product (p65) identified in our preceding biochemical experiments.

DISCUSSION

There have been relatively few studies conducted on Fat cadherins in mammals despite their undoubted importance in development and their relevance to cancer progression (29). In part this likely reflects the difficulties encountered in working with such large molecules. Magg *et al* (30) circumvented this problem by examining Fat1 processing in transfection studies with a chimeric fusion protein between the extracellular domain of E-cadherin fused to the transmembrane domain and cytoplasmic tail of Fat1. These authors demonstrated a two step cleavage process resulting in the release of the E-cadherin extracellular domain and then, following cleavage by γ -secretase, the release of the intracellular domain which travelled to the nucleus. Based upon the more recent work on endogenous *Drosophila* Fat and murine Fat4 (6,15) it can be suggested that Magg *et al* (30)

were observing S2/3 cleavage, an event that does not normally occur spontaneously but is driven by ligand binding (6,15). Extrapolating from studies of spontaneously cleaved Notch mutations (31,32) it can be suggested that the S2/3 cleavage seen with the Fat1 chimera was the result of the aberrant exposure of a proteolytic cleavage site that is normally cryptic until ligand engagement induces a conformational change. In the present study, we found no evidence of spontaneous S2/3 cleavage of endogenous Fat1 in the two cell types studied. Whether or not such an event can be similarly induced awaits the discovery of the ligand for Fat1.

Our work has shown that the initial intrinsic processing of Fat1 involving S1 cleavage appears to be the same as that shown previously for *Drosophila* Fat and murine Fat4 (6,15), and we have extrapolated upon these reports by demonstrating that the enzyme involved is furin. This result was not unexpected given the structural homology seen in the different Fat molecules. Quite unexpected was our discovery of an alternative spontaneous pathway of cleavage in the melanoma cells that resulted in the generation of entirely distinct protein products from those of the "classical" pathway. This finding raises a number of questions requiring discussion: for example, where does cleavage occur; what is the enzyme involved; is such alternative processing specific to tumor cells, and if so how does it benefit tumor metastasis; can this finding be exploited towards a novel therapy?

Whether the products of alternative processing (released extracellular domain; intact cell-surface Fat1; p65) have any specific functions that differ from "classically" processed Fat1 is not known. However, it is interesting to note that Bush *et al* (33) have recorded that Notch1, which like the Fats generally undergoes intrinsic cleavage by a furin-like convertase, can also be expressed on the cell surface as an intact molecule. Further,

the uncleaved Notch1 at the surface was able to participate in signal transduction that differed from cleaved Notch1, and the authors proposed a novel paradigm in signal transduction, one in which two isoforms of the same cell-surface receptor could mediate two distinct signaling pathways in response to ligand. It is conceivable, therefore, that the full-length surface Fat1 identified on the surface of melanoma cells can engage in an alternative signal pathway.

Notably, the experimental protocols that enabled Bush *et al* (33) to readily identify uncleaved Notch included the use of a furin inhibitor and also the introduction by mutation of furin target sequences within Notch1 itself. Relating to these findings is our result that cells from the LoVo colon carcinoma cell line - which contain an inactive furin mutation - also expressed full-length, uncleaved Fat1 at the cell surface. However, there are no data to suggest that melanoma cells contain defective furin, illustrated in our results showing "classical" Fat1 processing in the same cells. This might suggest that the melanoma cells may be expressing an isoform of Fat1 that resists furin cleavage, whether by splicing or mutation. On the former possibility there are several predicted furin and general proproteinase convertase cleavage sites (refer Table S1) that could relate to the cleavage that produces p85. However there were no obvious multiple transcripts in our Northern analysis (Fig. 1) to indicate that regions of Fat1 may have been spliced out, but the absence of a small exon in the large Fat1 ~15kb mRNA would likely evade detection. Three distinct splice isoforms of Fat1 have been reported as distributing to different locations in migrating cells (34), but whether any of these resists furin cleavage is not known.

Part of our evidence establishing that the p65 product was derived from uncleaved Fat1 and not from the further proteolytic processing of the cleaved Fat1 heterodimer was by the use of the furin-defective LoVo

cells. These results also illustrate that alternative Fat1 processing is not restricted to melanoma cells but is also seen in this colon cancer cell line. We have also examined Fat1 processing in two breast cancer cell lines since most *in vivo* breast cancers also exhibit strong cytoplasmic staining for Fat1 (14), and immunoprecipitation and blotting experiments with these cells have shown them to be indistinguishable from the melanoma cells in prominently displaying both forms of processing (unpublished data). Thus, dual Fat1 processing appears to be a feature of some cancer cells and furthermore suggests this process also occurs *in vivo*. No immunohistochemical analyses of Fat1 expression in melanoma tissues have currently been reported so it will therefore be important to determine both the expression and distribution of Fat1 for *in situ* melanomas as well as defining the proteolytic processing patterns of Fat1 in this setting.

Whether normal cells also can process Fat1 by the alternative pathway has been difficult to evaluate because of the very low levels of Fat1 expressed by most cell types; such studies are also hampered by the very large size of Fat1 making transfection studies a technical challenge, and the fact that our antibody reagents are human-specific. Our work with the keratinocyte lines in this report shows that alternative processing of Fat1 is not a prominent feature in these cells. However, careful examination of overexposed gels sometimes reveals the presence of low amounts of both full-length Fat1 at the cell surface and of p65. This is an inconsistent finding that we cannot always replicate under different conditions of cell culture, quite reminiscent of a similar inconsistently observed p70 band that Sopko *et al* (6) recorded in their study of Fat processing in *Drosophila*. These data together suggest that there are perhaps isoforms of both Fat1 and *Drosophila* Fat that can undergo alternative

processing in normal cells but these are generally expressed only at very low levels relative to classically cleaved Fat1.

Finally, the question arises as to how alternative processing might explain the paradox of the high expression of Fat1 - a putative suppressor-suppressor gene - by some tumor cells, particularly melanoma. Fat1 directly binds the Ena/VASP proteins that activate the actin polymerization complex (17,18). Loss of Fat1 by knockdown (9,17,18) causes slowed cell migration *in vitro*, and added endogenous Fat1 promotes cell migration (34) in a number of different cell types. Therefore, this function of Fat1 could promote tumor metastasis. The Ena/VASP interaction with Fat1 occurs at the plasma membrane, at the leading edge of lamellipodia, filopodia, and microspike tips (17). Hence, we would suggest, intracellularly localized p65 generated by alternative processing, although having the capacity to bind Ena/VASP, would not interfere with this interaction because it is at the wrong site. Conversely, the role of Fat1 in the Hippo pathway and likely in growth control and tumor suppression, involves direct binding to the PDZ-containing protein, Scribble, at an unknown location (7). We would hypothesize that intracellularly located p65 is able to interfere with this process by sequestering Scribble and thereby acting as a dominant negative. We are in the process of testing this hypothesis in zebrafish and have already shown that an approximate p65 construct indeed acts as a dominant-negative to Fat1 in this model of development, mimicking the effects of Fat1 knockdown (manuscript in preparation). Confirmation of these hypotheses would suggest that the unknown enzyme that catalyses Fat1 cleavage in the alternative pathway will provide an attractive therapeutic target for novel drugs in the treatment of melanoma.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Expression of the Fat cadherin family in human melanoma cells, melanocytes and keratinocytes. Northern blot analysis of all four Fat family cadherins in a panel of human melanoma cell lines compared with cultured melanocytes and the HaCaT keratinocyte cell line. RNA loading and integrity was confirmed by reprobing each membrane with an 18S probe. MeWo RNA was not analyzed for Fat2, 3 or 4 as indicated.

Fig. 2. Proteolytic processing of Fat1 cadherin in keratinocytes. Cell lysates of HaCaT keratinocytes were analyzed for Fat1 expression by Western blotting using the Fat1 CTD pAb after (A) seeding equal numbers of cells into different sized culture dishes and allowed to grow for 2 days or (B) seeding equal numbers of cells into the same size culture dish and grown for 1-4 days. All cultures were lysed in an equal volume and an equal proportion of the lysate applied to the gels. Membranes were then reprobbed with anti-GAPDH as a loading control. The high Mr band observed at the expected ~500kDa size of Fat1 is arrowed together with a prominent band at ~85kDa. (C) HaCaT keratinocytes were pulse labeled with 35S-cysteine-methionine and subjected to chase in unlabelled medium for 0 to 360 min. At each indicated time point cells

were collected, lysed and immunoprecipitated using a mixture of anti-Fat1 mAb (NTD-7 and CTD-7). (i) The samples were then resolved on 3-8% TA gels that were subsequently dried and subjected to storage phosphor imaging. The asterisk indicates a non-specific band also observed in control immunoprecipitates throughout the chase (not shown).” (ii) Densitometric analysis demonstrating the relative expression of p500 to the p430 and p85 bands that reflects the proteolytic processing events occurring in Fat1 (refer to text for further details).

Fig. 3. Fat1 cadherin is cleaved and expressed as a heterodimer on the cell surface of keratinocytes. (A) Cell surface proteins of HaCaT keratinocytes were labeled *in situ* with biotin before being subjected to lysis and immunoprecipitation using mAb directed against epitopes in the extracellular and cytoplasmic tail of Fat1, NTD-7 and CTD-7, respectively. CaCl₂ and EGTA indicate the presence (2 mM CaCl₂) or absence (5 mM EGTA) of calcium ions in the immunoprecipitation buffers used. Membranes were first probed with NeutrAvidin-HRP to reveal cell surface proteins (left panels), followed by quenching of HRP activity with azide and subsequent re-probing membranes with Fat1 CTD pAb using Western blot (right panels). Both mAb precipitate a band at ~430kDa (p430) on the cell surface whereas reblotting using the CTD pAb shows a ~500kDa reactive band (p500) and a strong ~85Kda band (p85). (B) Cell lysates of HaCaT cells were immunoprecipitated with NTD-7, CTD-7 and a control mAb (11H5) were subjected to Western blotting with the either Fat1 NTD pAb or CTD pAb. The NTD pAb recognizes both p430 and p500 bands in both NTD-7 and CTD-7 immunoprecipitates in contrast to the CTD pAb that recognizes only p500 and p85.

Fig. 4. Fat1 cadherin occurs as both unprocessed and heterodimeric forms on the cell surface of melanoma cells. (A) Four different human melanoma cell lines expressing Fat1 were treated with siRNA duplexes against either Fat1 or a GFP control. After 72 hrs, the cells were lysed and together with similarly prepared untreated control cells (nil), the cell lysates were subjected to Western blot analysis using the Fat1 CTD pAb. In addition to the p500 band, two smaller Fat1 related bands were observed at 85 and 65kDa. (B) MelCV melanoma cells were biotinylated on the cell surface, lysed and subjected to immunoprecipitation analysis using the panel of anti-Fat1 carboxylterminal mAbs (CTD-1 – CTD-9) together with a control mAb (11H5). Biotinylated proteins were then detected using NeutrAvidin-HRP followed by quenching with azide and re-probing the same membrane by Western blot using the Fat1 CTD pAb. The immunoprecipitation analysis in (B) was repeated except now directly comparing the results obtained using the aminoterminal anti-Fat1 mAb (NTD-7) with (C) a representative carboxylterminal mAb (CTD-7) or (D) a pool of all nine CTD mAb (CTD-1 – CTD-9). As indicated, duplicate samples were subjected to treatment with either 5 mM EDTA or 0.25 mM 2-mercaptoethanol contained in the immunoprecipitation buffers. Non-specific (ns) bands are denoted by an asterisk. The results show that both the processed p430/p85 heterodimer along with the uncleaved p500 form of Fat1 occur on the cell surface of melanoma cells. Additionally, the p65 Fat1 band was selectively captured with the Fat1 CTD but not NTD mAb.

Fig. 5. Proteolytic cleavage and phosphorylation of Fat1 cadherin in melanoma cells. MelCV cells were lysed and subjected to immunoprecipitation with either the Fat1 CTD-7 or the NTD-7 mAb together with a control. Samples were then subjected to digestion with lamda phosphatase or mock digested without enzyme prior to Western blotting using the Fat1 CTD pAb. This analysis shows that both the p85 band (black arrowhead) and p65 band (open arrowhead) are

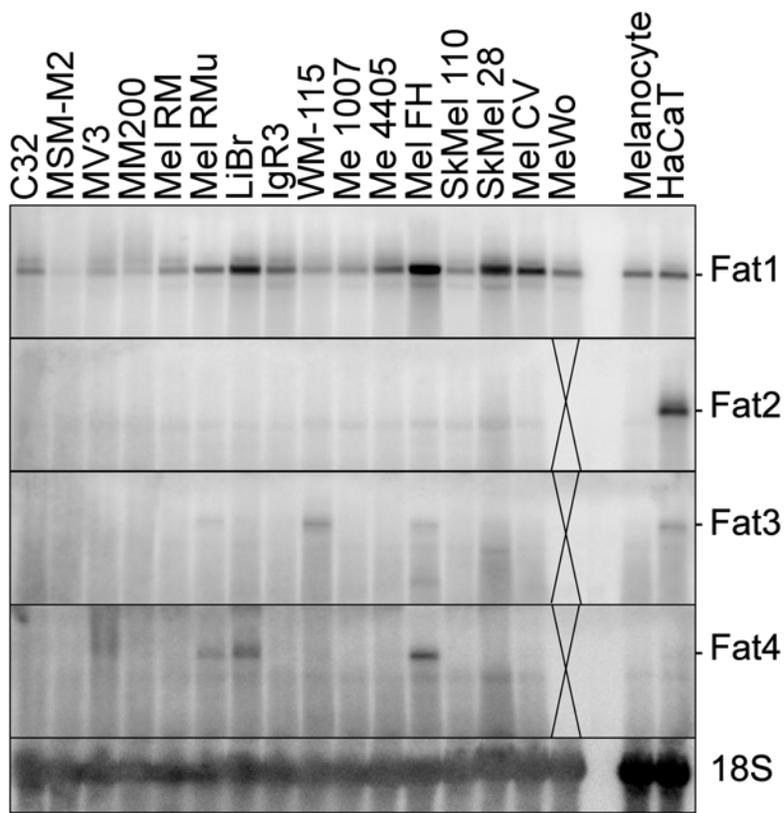
phosphorylated as indicated by the increased mobility after phosphatase digestion. A non-specific band is denoted by an asterisk.

Fig. 6. Furin is necessary for production of the p430/p85 Fat1 heterodimer whereas p65 is a cleavage product derived from the unprocessed p500 molecule. (A) Furin deficient LoVo cells were biotinylated on the cell surface and subjected to immunoprecipitation using the anti-Fat1 CTD-7 and NTD-7 mAb together with a control mAb (11H5). Detection of cell surface proteins with NeutrAvidin-HRP and subsequent re-probing of the same membrane with the Fat1 CTD pAb decorated a specific high Mr protein band (p500). No p85 band was observed as in previous experiments with HaCaT cells but a p65 band was precipitated but only using the CTD-7 mAb. (B) Western blot analysis of immunoprecipitates from LoVo cells treated with siRNA duplexes against either Fat1 or a control sequence (NC). Both the p500 and p65 reactive bands revealed using the Fat1 CTD pAb are diminished after Fat1 knockdown indicating both are Fat1 related products. (C) MelCV melanoma cells were subjected to the same immunoprecipitation analysis used in (A) except cells were first pretreated with either the proprotein convertase inhibitor (Dec-RVKR) or DMSO that was used as a carrier substance for the inhibitor. As indicated, some cells were grown in 10% FBS before harvesting the cell lysates. Treatment with the inhibitor resulted in the greatly diminished expression of both p430 on the cell surface (left panel) together with the p85 band detected by the Western blot using the Fat1 CTD pAb. In contrast, inhibition using Dec-RVKR did not reduce the amount of p65 immunoprecipitated using the CTD-7 mAb.

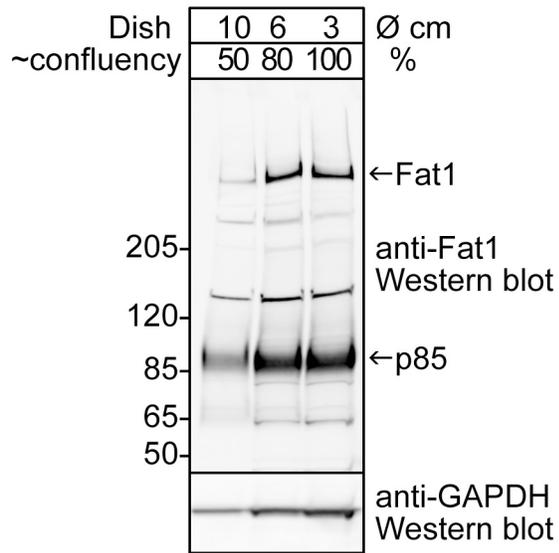
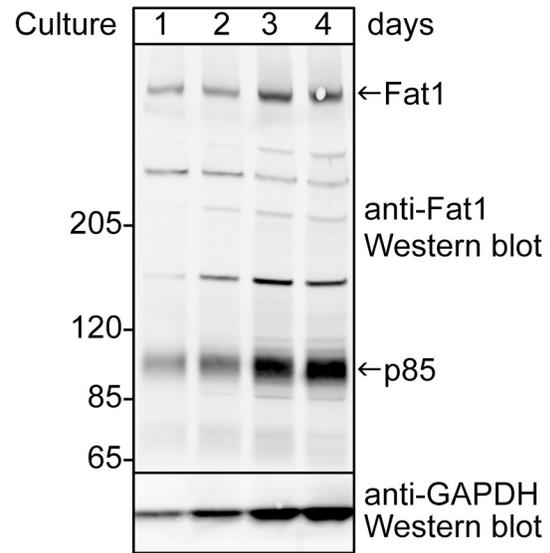
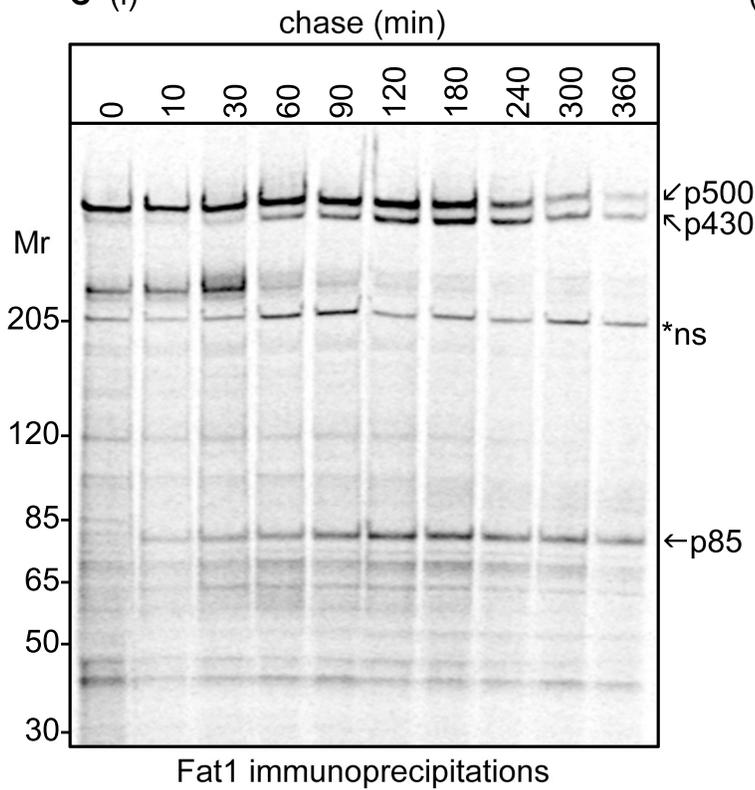
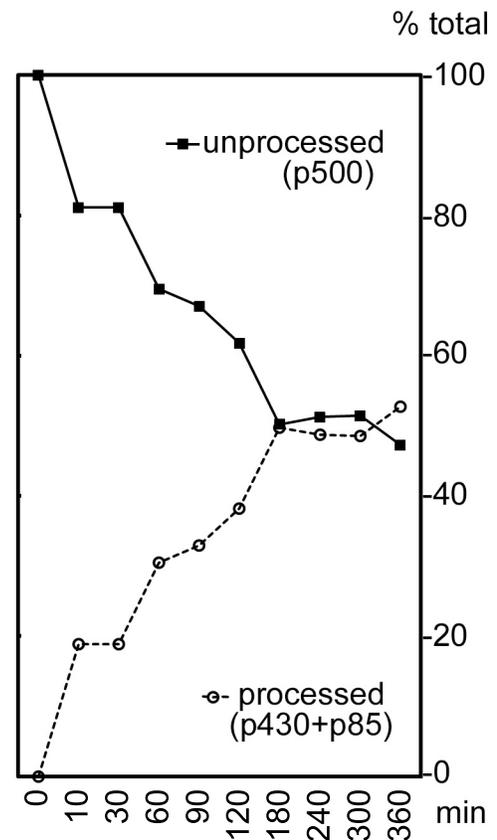
Fig. 7. The p65 cleavage product of Fat1 associated with the alternative processing pathway is membrane associated. Microsomal membranes prepared from C32 melanoma cells were subjected to either mock extraction or extraction with high pH carbonate buffer (carb.) as described in the Experimental Procedures. Samples were subjected to Western blotting either using the Fat1 CTD pAb (left panel) or with antibodies against the integral membrane protein CD36 (25) or the membrane associated protein Annexin II (28) (right panel). Full length Fat1 (p500) and the heterodimer associated p85 chain were detected in microsomes and resisted carbonate extraction. The p65 band was also detected in microsomes and was resistant to carbonate extraction. This suggests that p65 is also an integral membrane protein but consistent our depiction in Figure 8 below the cleavage that generates p65 may be very close to the juxtamembrane transmembrane region.

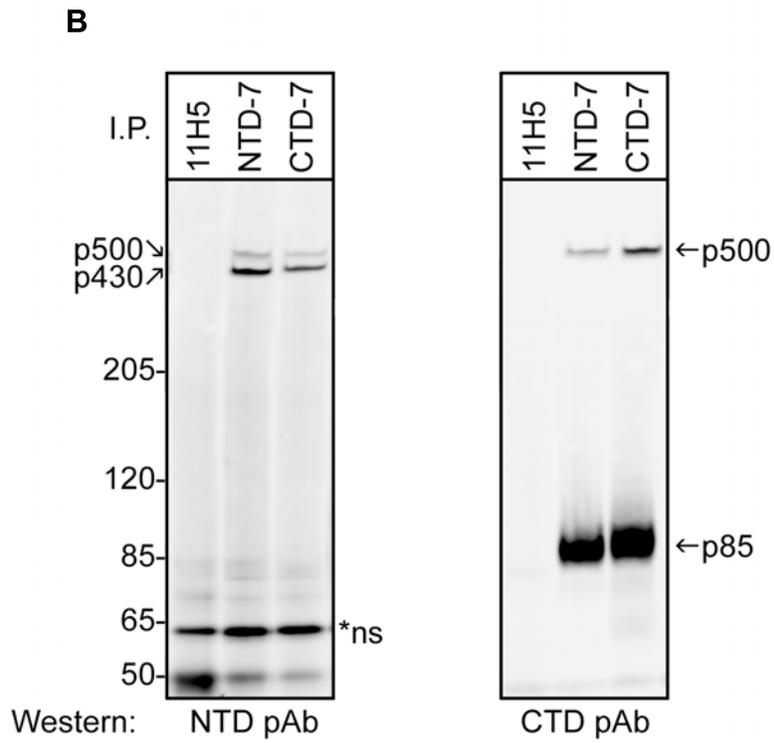
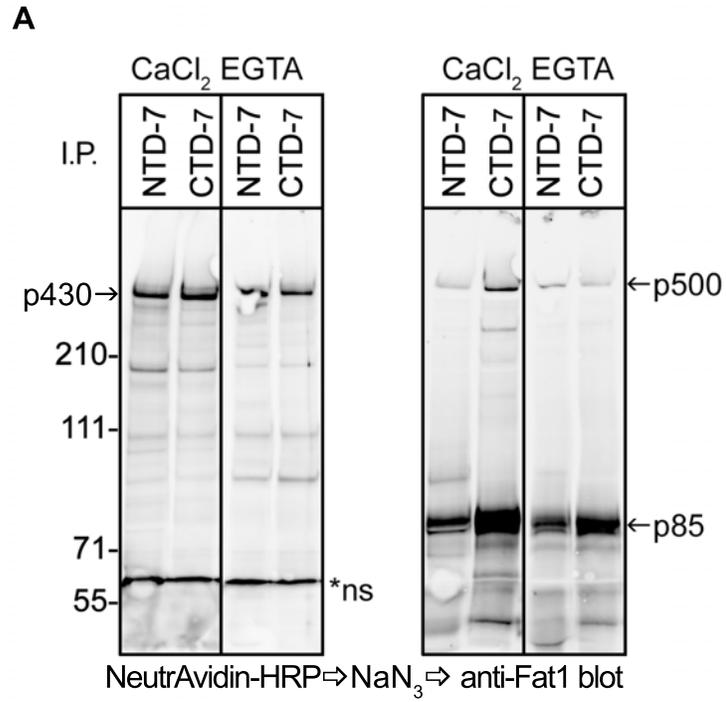
Fig. 8. Schematic illustrating the dual processing of mammalian Fat1 cadherin deduced from the preceding experimental data. “Classical” processing of Fat1 occurs as for *Drosophila* Fat whereas “Alternative” processing has been shown to occur in melanoma cells (further details in text).

Fig. 9. Distribution of Fat1 cadherin *in vitro*. Immunofluorescence microscopy showing the distribution of Fat1 in cultured neonatal keratinocytes, HaCaT keratinocytes and the MV3 melanoma cell line. The analysis shows optical sections of cells stained with the Fat1 CTD pAb in combination with an Alexa-594 secondary conjugate. Cell nuclei were counterstained using DAPI.

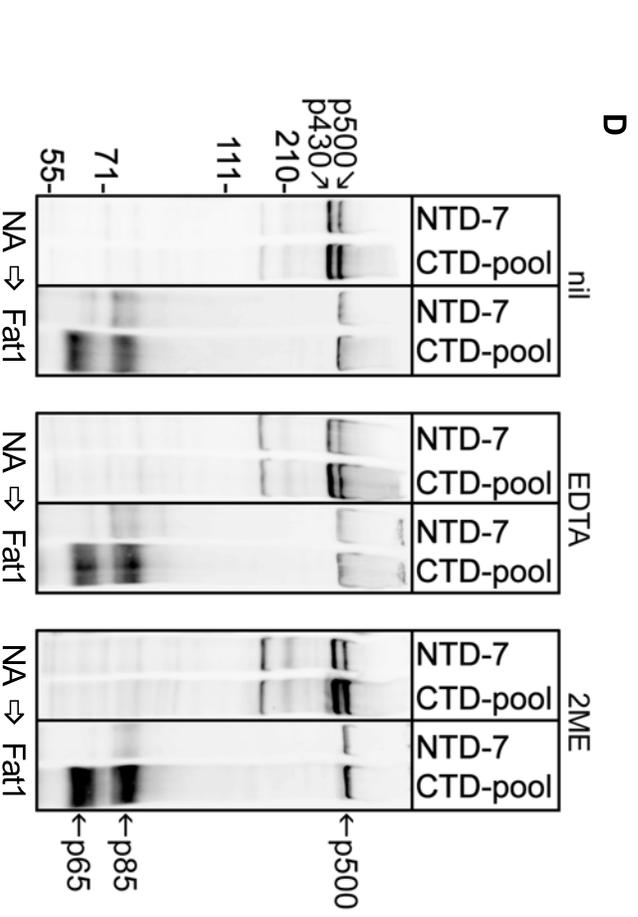
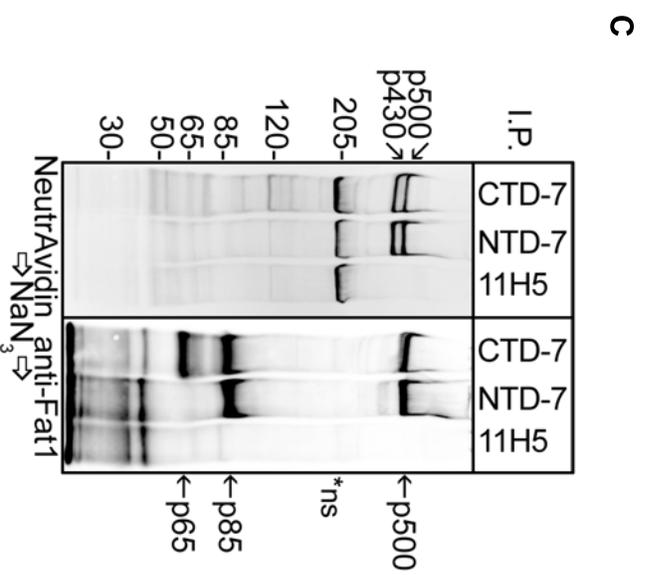
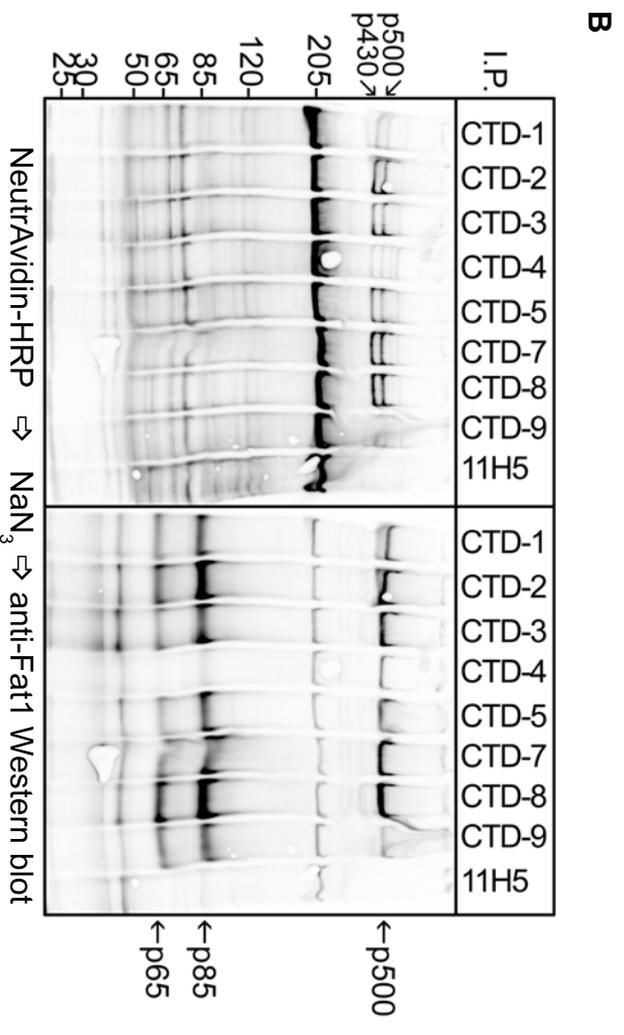
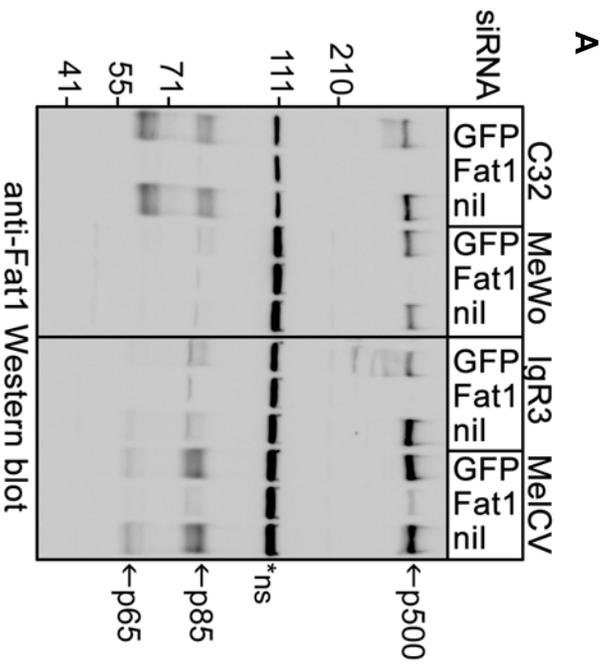


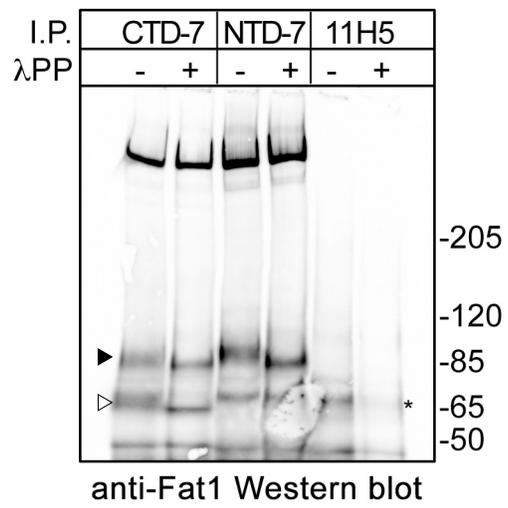
Sadeqzadeh et al, Figure 1

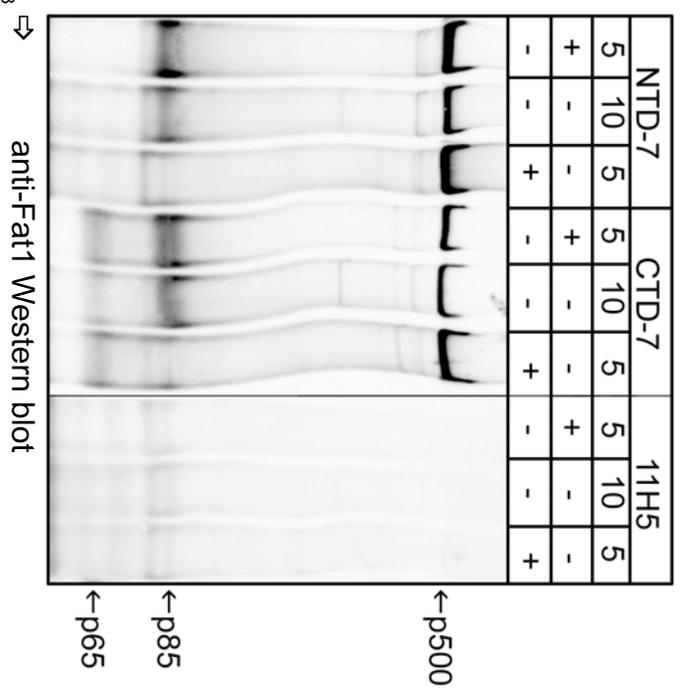
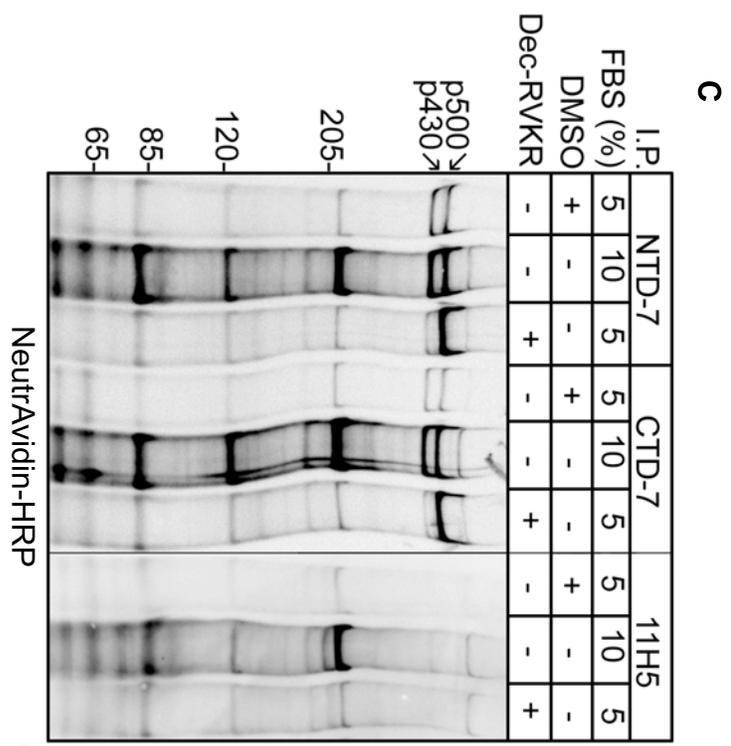
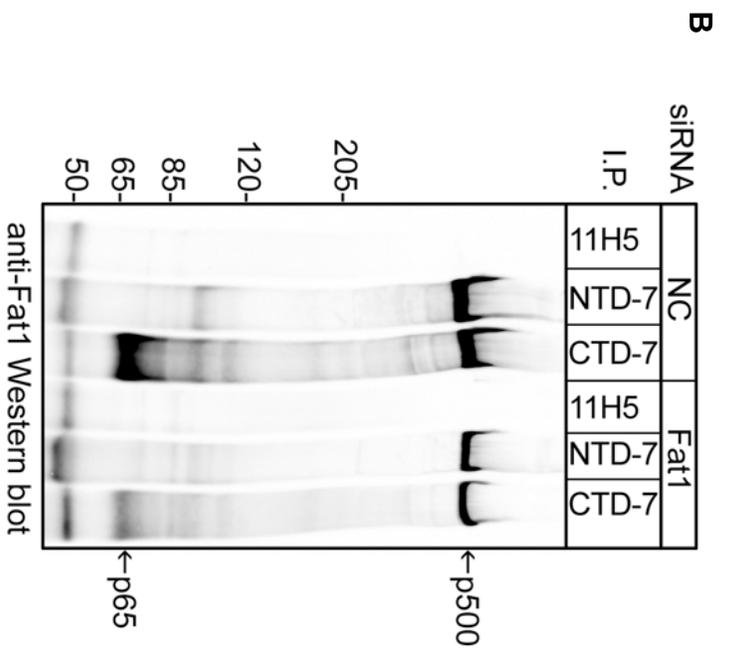
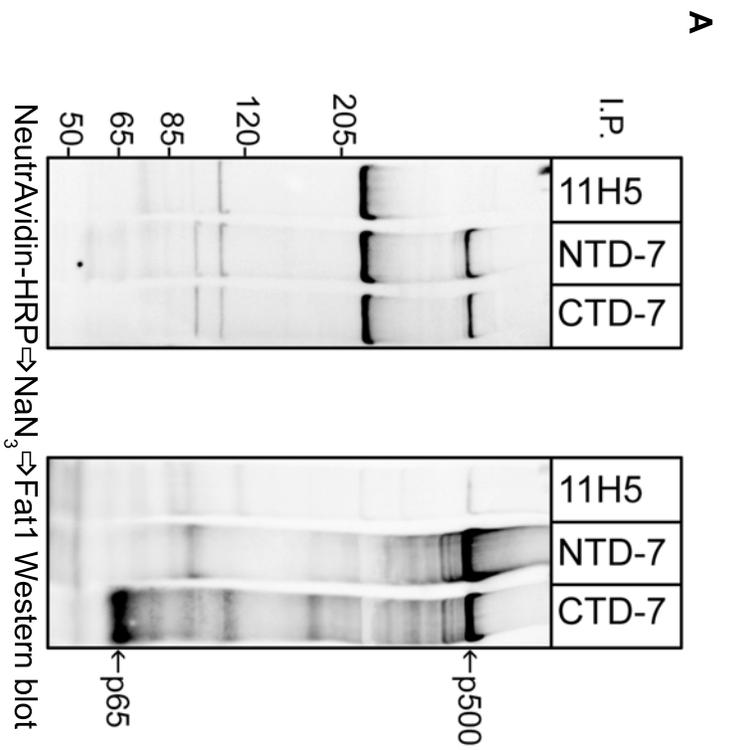
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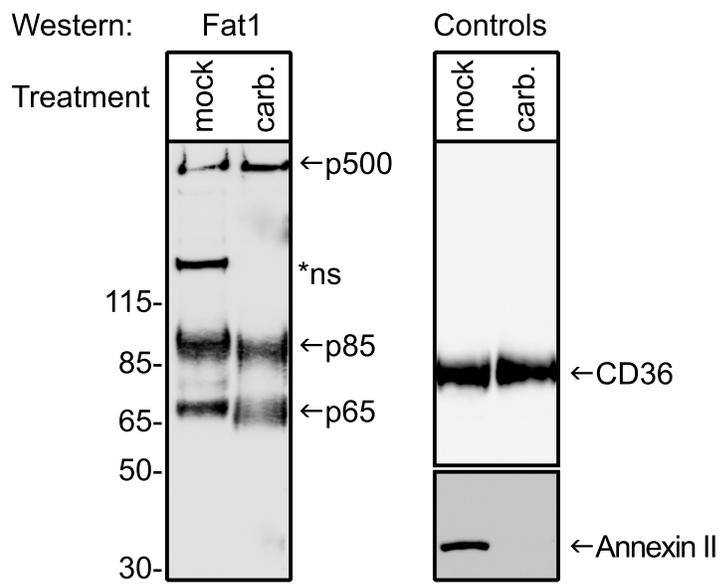


Sadeqzadeh et al, Figure 3



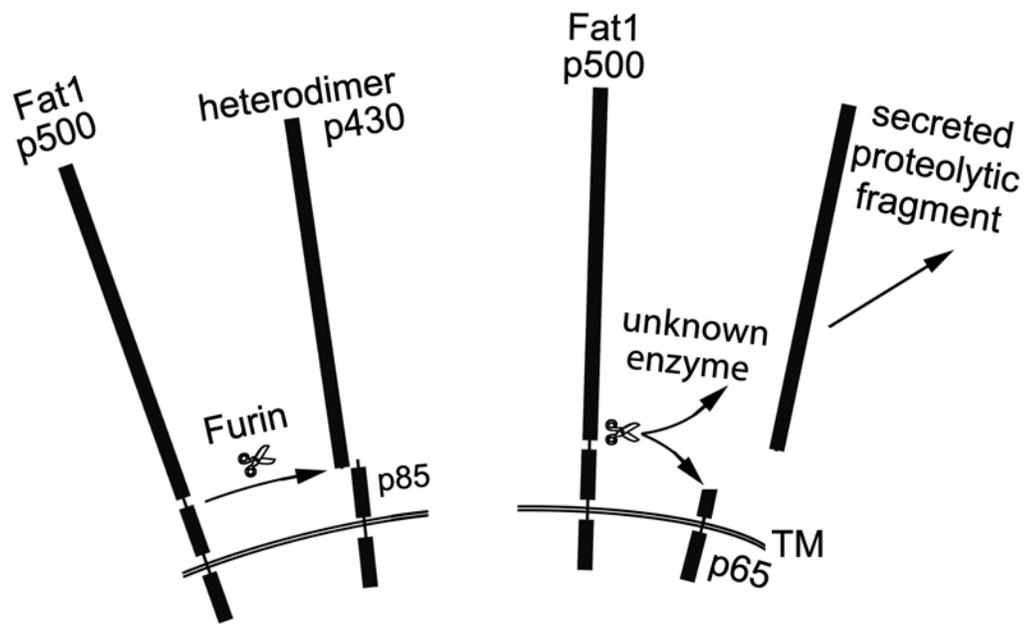




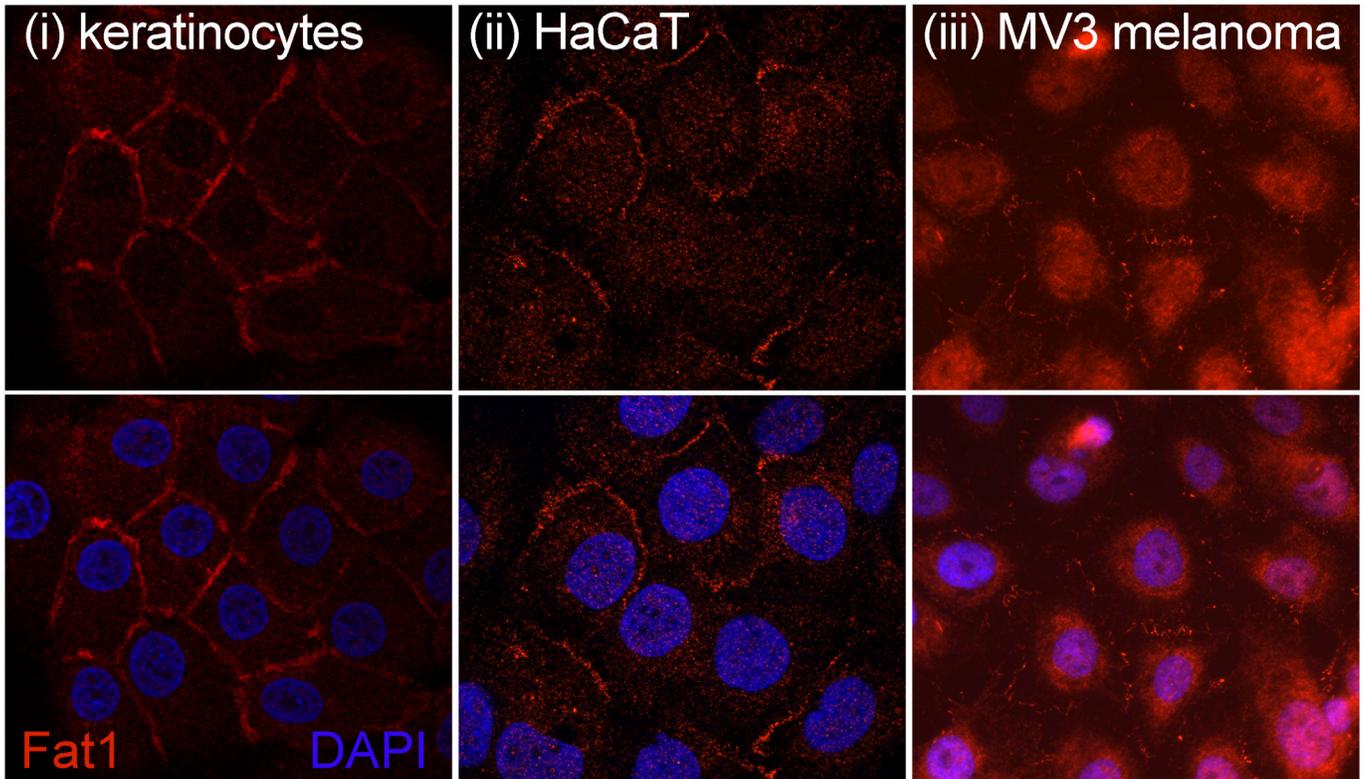


Sadeqzadeh et al, Figure 7

Classical processing vs Alternative processing



Sadeqzadeh et al, Figure 8



Sadeqzadeh et al, Figure 9