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PREPARATION AND CHARACTERISATION OF ANTI-FAT1 POLYCLONAL ANTIBODIES

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ABSTRACT

Fat cadherins comprise the largest of all known members of cadherin superfamily. They are present in all multicellular organisms and retain a high degree of structural conservation. In Drosophila there are two Fat genes: Fat and Fat-like, whilst in vertebrates there are four members called Fat1, Fat2, Fat3 and Fat4. Our lab group focused on the use of various biochemical methods to analyse expression of the FAT1 protein. Because of the high molecular size of FAT1 protein our laboratory has used affinity purification to prepare bespoke rabbit anti-FAT1 antibodies using FAT1 protein prepared as GST fusions. five overlapping segments of the FAT1 cytoplasmic tail designated A, B, C, D and E as GST-fusion proteins contained within pGEX plasmids were assigned in our lab. According to their sequences and hosted rabbit name antibodies were named N34B, N35B and N34E. To test the efficacy of each antibody preparation, two different techniques were undertaken, first Western blotting and second immunofluorescent staining. By this analysis the immunoreactivity of N34B, N35B and N34E compare favourably with the CTD-pAb. N34E produced less than satisfactory results in this test with low signal to noise and these results are omitted here. This staining pattern was largely consistent between N34B and N34E, but the signal to background was a considerably high. According to that the use of the new B and E antibodies was restricted to Western blotting where these reagents fulfilled the functional requirements.

Key words: Fat1, Fat1 antibodies, cadherins, western blot. Immunofluorescence staining.

INTRODUCTION

Adhesion receptors are largely categorized into 4 main groups known as integrins [1], selectins [2], immunoglobulin super family [3] and the cadherins [4]. Within all multicellular organisms these molecules perform a host of functions that extend beyond cell adhesion and migration per se: these include roles in cellular proliferation, apoptosis, intercellular signalling, differentiation and maturation at both the prenatal and postnatal developmental stages [5]. Of these four groups, the cadherins are of particular interest to this project. They comprise a large superfamily of several hundred-membrane proteins and are most well known to be critical for the formation of adherence junctions and desmosomes in epithelia, intercalated disks in cardiac myocytes, and synaptic junctions [6]. All cadherins contains multiple "cadherin" repeat motifs in their extracellular domain although the composition of each protein can be very diverse. They can be divided into several major subfamilies which include the classical cadherins, desmosomal cadherins and protocadherins, together with other smaller groups including the CDH15-23 cadherins and the Daschous and Fat (Ft) cadherins. Fat cadherins comprise the largest of all known members of cadherin superfamily [7]. They are present in all multicellular organisms and retain a high degree of structural conservation. In Drosophila there are two Fat genes, Fat and Fat-like, whilst in vertebrates there are four members called Fat1, Fat2, Fat3 and Fat4. Each Fat cadherin contains 34 cadherin repeats, several EGF-like motifs and one or two Lamin A-G domains in the extracellular domain, a single transmembrane domain and relatively large cytoplasmic domains. Consequently, the Fat cadherin proteins are massive and are comprised of ~4,500 to 5000 amino acids. In general terms, relatively little is known about Fat cadherins and their functions. Most of the important findings to date have come from studies conducted in Drosophil.

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Our previously published manuscripts involved the application of various biochemical methods to analyse expression of the FAT1 protein. Traditionally our laboratory has used affinity purification to prepare bespoke rabbit anti-FAT1 antibodies using FAT1 protein prepared as GST fusions [8]. These are finite resources and an initial task undertaken for that work involved the preparation of new anti-FAT1 antibodies against the cytoplasmic tail of FAT1. This paper details the preparation of these reagents including their characterisation.

METHODS

PREPARATION OF GST-FUSION PROTEINS

Transformed E-coli bacteria containing pGEX plasmids (supplied by Dr Charles De Bock) were grown overnight on LB agar bacterial petri-dishes (5g NaCl, 5g Tryptone, 2.5g Yeast Extract, 7.5g Agar per 500mL dH2O (Amresco)) containing 100□g/ml ampicillin (Amresco). As a starter culture, one colony was then added to 10mL of 2YT media (16g tryptone, 10g yeast extract, 5g NaCl per 1L with 100µg/ml Ampicillin) and this incubated overnight at 37°C in a shaking incubator. The next day, 1 mL of starter culture was added to 500 mL of 2YT ampicillin media for 6 hours before the addition of IPTG to final concentration of 0.1 mM. This culture was further incubated for 16 hours before pelleting the bacteria by centrifugation (3000 rpm, 4°C, 15min) and removing the supernatant. Bacterial pellets (from 250 mL) were then kept on ice or stored at -80°C for later processing. Thereafter the cell pellet was resuspended in PBS containing protease inhibitors (cOmplete protease inhibitor mixture and PhosSTOP, respectively; Roche Applied Science) (3ml PBS/250 mL culture). The resuspended cells were then sonicated with three 10 sec pulses (Soniprep 150, 4 × 10 sec pulses at an amplitude of 12 µ) before adding 1/10 volume of 10% Nonidet™ P 40 (NP-40, Sigma) was added to each to a final concentration of 1% NP-40. This was then incubated for 30 min and the lysate clarified by centrifugation at 4250rpm for 20 min.

PREPARATION OF GST-FUSION AFFINITY COLUMNS

Each crude bacterial lysate containing GST proteins was added to 1000 uL of Glutathione agarose beads (Pharmacia) and the tubes incubated overnight at 4°C on a rotating wheel. The beads were then recovered by centrifugation at 2000 rpm for 3 min before sequential washing with 10 mL 0.1 M borate buffer pH8, 10 mL 0.1 M borate buffer pH9 and then twice with 10ml 0.2 M borate buffer pH9. After the washing steps, the beads were incubated at 4°C in 10ml 0.2M borate buffer (pH9) containing 40mM dimethypimelimidate (Sigma) on a rotating wheel for 1 hour. After incubation, the beads were washed again twice with 10 mL 0.1M borate buffer (pH8) followed by 45 min incubation with 10 mL 40mM ethanolamine in 0.1M borate buffer (pH 8) to quench unreacted crosslinker. Finally the beads were alternatively washed two times with 10 mL cold PBS and 1x with 10 mL 1M K2HPO4, lastly washing again two times with 10 mL PBS. The prepared columns then stored in an equal volume of PBS with 0.1%NaN3 and stored at 4°C. For the antibody purification, the storage buffer was removed from each column by washing with 20 mL PBS containing 0.2% v/v Tween 20 (PBST).

CRYSTORAGE AND REVIVAL OF CELLS

Cells designated for storage in liquid nitrogen were carefully harvested with trypsin-EDTA solution (Lonza) and pelleted by centrifugation at 200x g for 5 min. Cells were resuspended in freezing media (10% DSMO in FBS) and aliquots taken into cryotubes before being gradually frozen in a Styrofoam container at -80°C for 2-4 hours. Thereafter the vials were transferred to liquid nitrogen for long-term storage. For revival, vials were taken from liquid nitrogen and cell suspensions liquefied quickly in a 37°C water bath. The cell suspension was added to 9 ml complete warm media softly mixed and centrifuged at 200 x g. The supernatant was discarded and the cells was resuspended in complete media and cultured as described above.

PREPARATION OF CELL LYSATES

Culture media was removed from cells and the monolayer washed two times with PBS by gentle rocking the dish or flask. The monolayer was treated with suitable amount of ice-cold NDE lysis buffer (1% Nonidet P-40, 0.4% sodium deoxycholate, 66mM Tris-HCl, pH 7.4) supplemented with protease and phosphatase inhibitors (cOmplete protease inhibitor mixture and PhosSTOP, respectively; Roche Applied Science). The homogenate then was transferred to 1.5 ml tube and kept in ice for 15 min. Thereafter, samples were centrifuged at 10,000 x g for 15 min at 4°C to pellet insoluble material. The supernatant was stored at -20°C for later analysis. After thawing, protein concentrations were estimated (see next section) and samples containing equal protein amounts prepared with 4x lithium dodecyl sulphate buffer, 50 mM DTT (1M stock) and water, were heated in 70 °C for 10 min, then samples briefly centrifuged for 10 sec.

IMMUNOPRECIPITATION PROTOCOL

Cells lysates were prepared from T75 culture flasks according to Section 2.4. Cell lysates (500-1000) were precleared twice with 30 µl of Protein A/G Plus-agarose beads (Santa Cruz Biotechnology) for 1–2 h, and lysates then immune-precipitated with 1–2 µg of the indicated antibody along with 20 µl of beads. Samples were rotated overnight at 4°C on a mixing wheel before the washing steps. After briefly centrifuges the sample to pellet the beads, unbound proteins were washed away using subsequent wash steps. The beads were washed alternately SDS RIPA buffer (1% Nonidet P-40, 0.1% SDS, 0.1% sodium deoxycholate, 150 mm NaCl in 50 mm Tris-HCl, pH 8) then high salt RIPA buffer (1%

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Nonidet P-40, 0.5% sodium deoxycholate, 0.5 m NaCl in 50 mm Tris-HCl, pH 7.4), a total of four times. Immune-complexes were eluted using lithium dodecyl sulfate sample buffer as described in Section 2.4. Prepared samples were run in commercial acrylamide gels (NuPAGE® Novex 3–8% Tris acetate mini/midigels, Invitrogen) using Tris acetate running buffer (20x stock solution) (Tricine 89.5g, Tris 60.6g SDS 10g up to 500 ml water) according to company protocol (150V for 1:10h).

WESTERN BLOTTING

Lysates were prepared as previously described in lithium dodecyl sulfate sample buffer (Section 2.4). For high molecular weight proteins NovexTM Tris acetate gels were used as described in Section 2.6. For other protein targets, 4- 12% Bis-Tris 10 wells gels were used in MES SDS Running Buffer (20x stock; MES 97.6 g, Tris Base 60.6 g, SDS 10 g, EDTA 3.0g in 500 ml water) (Novex®, Invitrogen) according to company protocol (165V for 35 min). Transfer to nitrocellulose membranes was performed using a semidry blotting system (iBlot® Transfer Stack and iBlot device, Invitrogen). After transfer in nitrocellulose membranes were then washed with water and blocked with 5% (w/v) skim milk powder dissolved in 1x Tris tween Buffered Saline (TTBS) (10x TTBS: 24.23 g Tris base, 87.66 g NaCl, 10 ml Tween-20 in 1L water) for one hour. Membranes then incubated overnight at 4°C with specific primary antibodies diluted in skim milk (see Table 1 and Appendix 1). After incubation the membrane was continuously washed three times (for 5 min each) with TTBS. After washing membrane incubated with specific rabbit or mouse secondary antibodies (all diluted 1:5000 in 5% skim milk) (Cell Signalling Technology or BioRad) according to the species of primary antibody. Following to secondary antibody incubation, each membrane was washed again three times with TTBS. Protein detection was then performed as described previously using an ECL-based detection system [9], and the results were recorded using a cooled charge-coupled device camera system (Fuji-LAS-4000, Fujifilm Life Science Systems).

IMMUNOFLUORESCENCE STAINING

Cells were prepared for imaging according to previous methods developed in our laboratory [104]. Cells were seeded in 24-well plates containing 13 mm circular glass coverslips in 2ml media mixed containing 1:400 Matrigel (1:400) (BD Biosciences). Prior to staining, media was removed and cells were washed three times with PBS, and then fixed in 2% paraformaldehyde in PBS for 10 min at room temperature. Coverslips were then washed 2 times in PBS to remove residual paraformaldehyde, and samples stored submerged in PBS at 4 ºC until use. For antibodies targeting the intracellular compartment, cells were permeabilized with 0.3% Triton X-100 made in PBS solution for 15 min. Thereafter coverslips were washed 3 times with PBS, followed by blocking with 10% goat serum for 30 min. Primary antibodies were diluted to the desired concentration in 250 µl 10% goat serum in PBS. Antibody solutions were gently dropped onto the coverslip (so the cells are all covered) and incubated for 30 min or longer at 37ºC. Initial antibody optimization steps were accomplished in a range of primary antibody concentrations up to 5µg/ml adjusted to suit optimal reactivity. After primary antibody incubation, coverslips washed again 3 times with PBS. Thereafter coverslips were incubated with fluorochrome-conjugated secondary antibodies (Alexa Fluor conjugated; Invitrogen) diluted 1:1000 in 10% goat serum/PBS). Further washing with PBS was used to remove unbound antibodies. Coverslip then covered with 250µl of the nuclear staining reagent 4',6-diamidino-2-phenylindole (DAPI) for 10 min followed with 3 times washing with PBS. Finally, coverslips were mounted in SlowFade Gold (Invitrogen) and epifluorescent images were then acquired using an Axiocam MRm(v3) and Axioplan2 epifluorescent microscope (CarlZeiss, Thorn-wood, NY). Images presented were prepared using the Axiovision software package (v4.6).

RESULTS AND DISCUSSION

Previously a GST-fusion protein encompassing the entire cytoplasmic domain of the FAT1 had been used to prepare affinity purified antibodies (see Figure A1). In this instance, two selected segments of the FAT1 cytoplasmic tail were implemented. Elham Sadeqzadeh in our laboratory had produced five overlapping segments of the FAT1 cytoplasmic tail designated A, B, C, D and E as GST-fusion proteins contained within pGEX plasmids (Figure 1; [8]). Towards purification of affinity purified antibodies against FAT1, selected cultures of bacteria containing pGEX plasmids were induced to produce GST-fusion proteins according to the Methods described in Section A1.2. GST-, B-FAT1 and E-FAT1 proteins were then bound and cross-linked to glutathione agarose to produce three affinity columns for later use in the purification of the FAT1-specific antibodies.

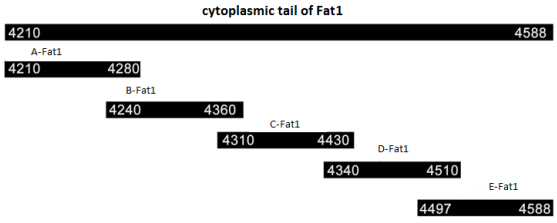

Figure 1: Schematic representation of the FAT1 cadherin cytoplasmic tail divided into five overlapping segments.

Each designated segment (A-E respectively) was cloned into the pGEX-2T plasmid to permit expression of recombinant GST-fusion proteins. The numbering indicates the corresponding amino acids in full length FAT1 (modified from [8]).

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ASSESSING GST-FUSION PROTEIN PURIFICATION

To assess the relative yield and purity of GST-fusion proteins bound to the glutathione affinity matrix, a small fraction of beads from each column was eluted and then subjected to PAGE (Section 2.7). The gels were then stained with Coomassie Blue (R-250; Bio-Rad) with the results shown in Figure 2. The GST protein was observed predominantly as a single band at ~28 kDa consistent with its predicted molecular mass of 26.9 kDa. The two FAT1 GST-fusion proteins (B-FAT1 and E-FAT1) also mainly resolved as single bands around 40 kDa. The E-FAT1 band was slightly smaller than the B-FAT1 band consistent with the predicted masses of 37 and 40, respectively. For both E- and B-FAT1 there were also some smaller products appearing below the main band but this is typical of such purifications and not excessive. By this analysis all of the column matrices appeared suitable for use in purifying the anti-FAT1 antibodies.

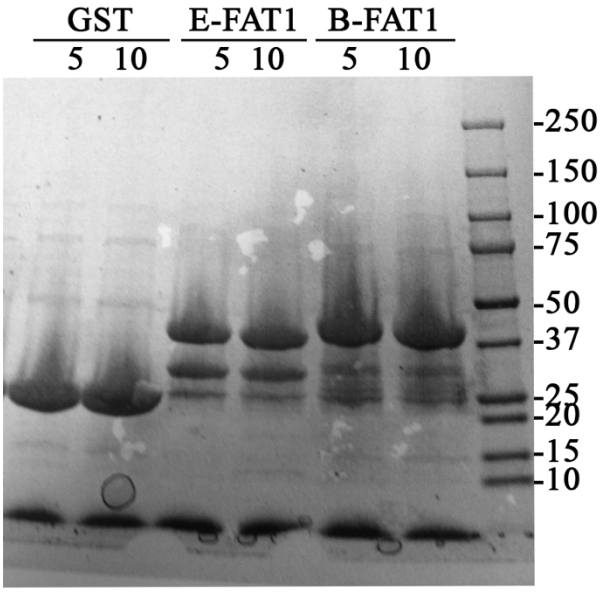

Figure 2: Validation of GST-fusion protein purifications using PAGE.
After preparing the affinity matrices bound with GST, E-FAT1 and B-FAT1, 25µl of the beads was eluted using an equal volume of 1X LDS sample buffer containing DTT (Section 2.7). The eluted samples (5µl or 10µl) were then resolved on Bio-Rad gradient PAGE gels before staining with Coomassie Blue R-250.

AFFINITY PURIFICATION OF THE ANTI-FAT1 ANTIBODIES

Immune serum from two rabbits designated N34 and N35 was available as starting material. The rabbits had previously been immunised with a GST-fusion protein encompassing the entire cytoplasmic tail of FAT1. As the immune response of individuals can be different, the notion was to use both rabbit sera individually against both B-FAT1 and E-FAT1. This would notionally provide four antibody permutations to evaluate. The general scheme for purification is shown in Figure 3. One mL of each serum was diluted 1:10 with PBS and passed through the descending columns and this repeated for a total of five passes. After washing the column with 20 mL PBST, 5 mL of 0.2M glycine/HCl (pH 2.5) was used to elute bound antibodies into tubes containing a small quantity of 2M Tris (pH 8), i.e. sufficient to neutralise the acidic elution buffer. To restore the columns, they were then washed with 20 mL PBST. The procedure was repeated for N34 and N35 sera four times giving 16 batches of affinity purified antibodies (i.e. 2 rabbit sera x 2 affinity matrices x 4 purification batches).

After elution, downstream processing was conducted for antibody batch where the samples were buffer exchanged to PBS and concentrated down to ~1 mL (performed using Amicon Ultra-2 mL Centrifugal Filters (YM-30; Millipore) according to the manufacturer's instructions). A sample of each batch of antibody was then evaluated by PAGE under reducing conditions (Figure 4). The anticipated Mr of antibody heavy chains is around 50 kDa. For all 15 batches evaluated (one being lost after tube breakage) the strongest band detected in each sample was 50kDa in size. Some other possible contaminants were also noted at higher Mr. Irrespective of these, the next step was to determine if these antibodies could adequately detect FAT1 in biochemical assays. Because the content of the samples appeared identical, it was decided to pool the batches to arrive at four reagents for testing (N34B, N35B, N34E, N35E).To sum up, it can be seen the utility of the FTIR, DSC and SEM techniques in identification and characterisation the carbamazepine. In addition, the three instruments were effective techniques to provide whole information about drug which cannot be separately obtained. This information included chemical structure, functional groups such as amine NH2, alkenes, and aromatic rings. Additionally, melting point 191.88 C°, enthalpy 187.96 C° and prismatic morphology with coarse surface also determine amount of carbamazepine in the sample.

Furthermore, it can improve and develop pharmaceutical analysis by defining purpose of analysis, well understanding for analysis process to know all factors which have potential effect on result of process such as instrument setting, process parameters and sample characterizations, ensures selection of the optimal technique and to access accurate results.

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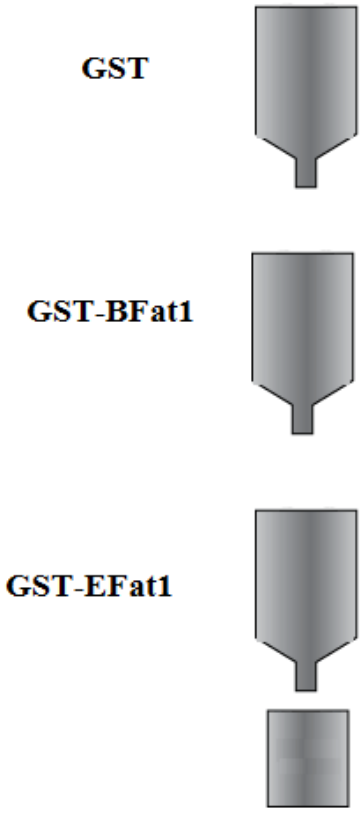

Figure 3: Schematic showing the arrangement of columns used for preparing the affinity purified antibodies (see text for details).

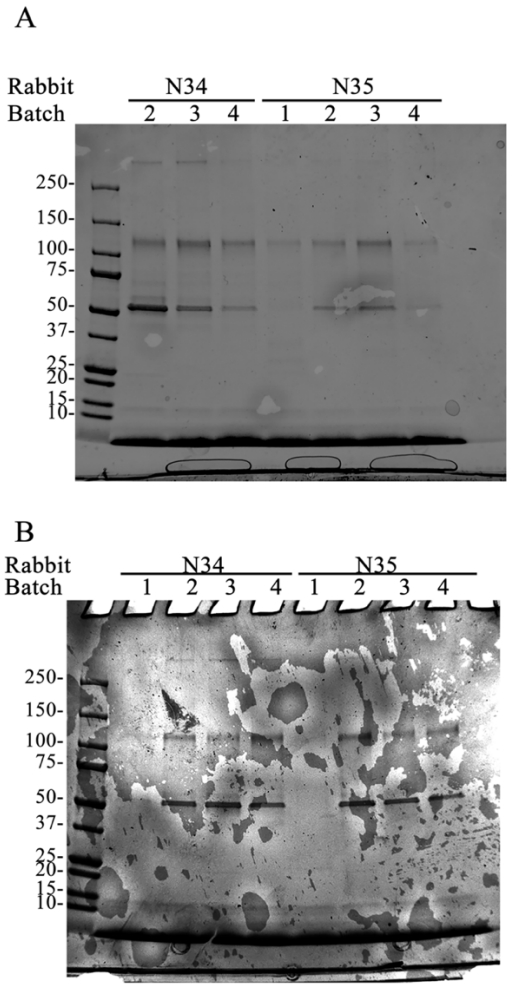

Figure 4: Evaluation of purified anti-FAT1 antibodies using PAGE.
A sample of each batch of affinity purified antibodies was applied to Bio-Rad gradient PAGE gels before staining with Coomassie Blue R-250.
A- Seven batches of antibodies purified against B-FAT.
B- Eight batches of antibodies purified against E-FAT.

FUNCTIONAL EVALUATION OF AFFINITY PURIFIED ANTI-FAT1 ANTIBODIES

To test the efficacy of each antibody preparation, two different techniques were undertaken, first Western blotting and second, immunofluorescent staining. For Western blotting, replicate membranes were prepared with two types of samples containing FAT1 protein. Cell lysates were prepared from human HaCaT keratinocytes and the lysates subjected to immunoprecipitation with anti-FAT1 monoclonal antibodies (CTD and NTD) prepared by our lab [8]. Note that these monoclonal antibodies are not functional in immunoblotting and hence the need for alternative reagents.

Both cell lysates and FAT1-immunoprecipitate samples were used to test the efficacy of the new purified antibodies. The results are shown in Figure 5A comparing these reagents against the CTD-pAb used previously in our laboratory [194]. By this analysis the immunoreactivity of N34B, N35B and N34E compare favourably with the CTD-pAb. N34E produced less than satisfactory results in this test with low signal to noise and these results are omitted here.

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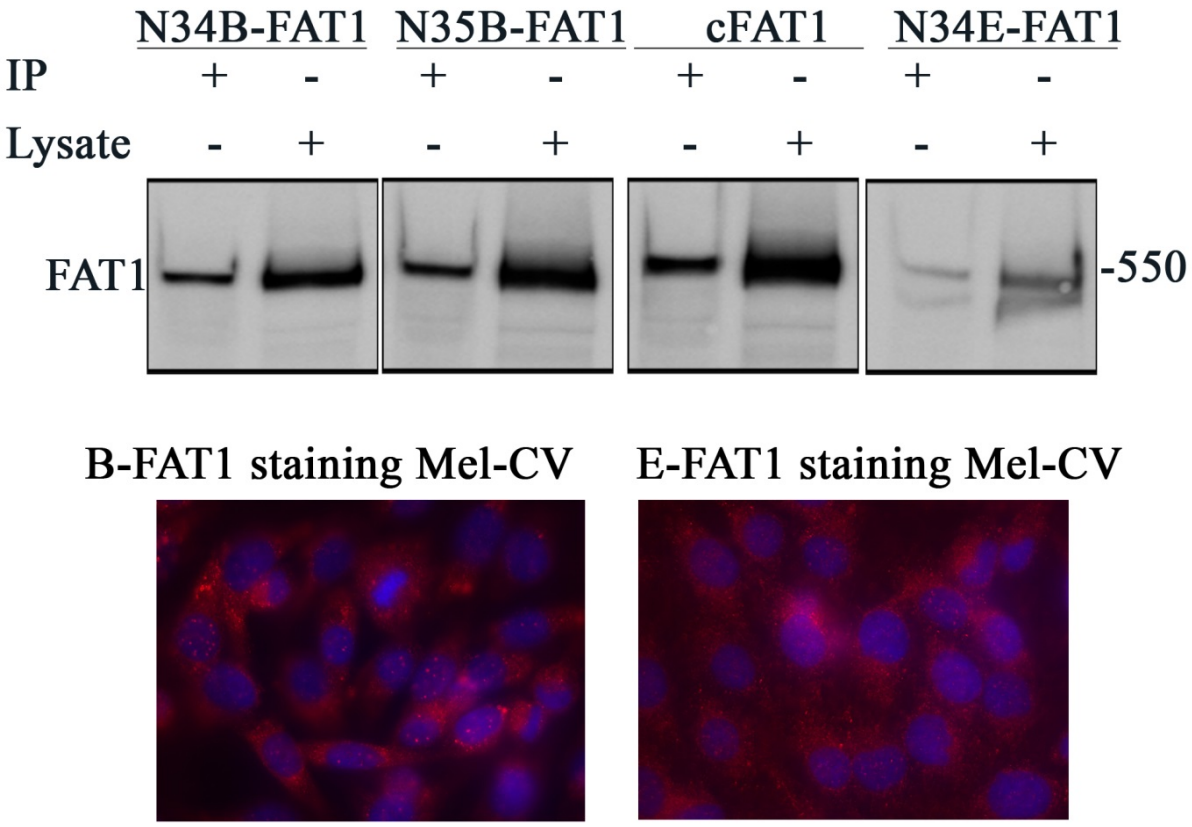

Figure 5: Functional evaluation of the affinity purified anti-FAT1 polyclonal antibodies.
A. Western blotting against FAT1-immunoprecipitates and cell lysate samples prepared from HaCaT keratinocytes. Samples were resolved using Tris-Acetate gels required for high Mr proteins. For this analysis each primary antibody was diluted 1:1000 with subsequent ECL detection performed as described in Section 2.7.
B. and C. Representative microscopic images of cultured MelCV melanoma cells stained by indirect immunofluorescence with the N34B (B) or N34E (C) affinity purified polyclonal antibodies. For this analysis each primary antibody was diluted 1:350 with subsequent detection performed as described in Section 2.8. The secondary antibody used was an anti-rabbit IgG conjugated with Alexa Fluor 594 (red colour). Cell nuclei were counterstained using DAPI (blue colour).

For Western blotting, replicate membranes were prepared with two types of samples containing FAT1 protein. Cell lysates were prepared from human HaCaT keratinocytes and the lysates subjected to immunoprecipitation with anti-FAT1 monoclonal antibodies (CTD and NTD) prepared by our lab [8]. Note that these monoclonal antibodies are not functional in immunoblotting and hence the need for alternative reagents. Both cell lysates and FAT1-immunoprecipitate samples were used to test the efficacy of the new purified antibodies. The results are shown in Figure 5A comparing these reagents against the CTD-pAb used previously in our laboratory [194]. By this analysis the immunoreactivity of N34B, N35B and N34E compare favourably with the CTD-pAb. N34E produced less than satisfactory results in this test with low signal to noise and these results are omitted here.

The next evaluation concerned the ability of the antibodies to decorate FAT1 in situ using the immunofluorescence technique.

The inability to adequately stain FAT1 in whole cells has been particularly problematic with uncertainty over the specific pattern of staining.

In melanoma cells it was demonstrated that FAT1 staining at the periphery of cells could be abolished using siRNA treatment [194]. Therefore the new antibodies were tested against melanoma cells to determine if affinity purification against specific regions (i.e. B and E) might improve the staining characteristics in this technique. Immunofluorescence staining was conducted against MelCV melanoma cells with representative results shown for N34B and N34E (Figure 5B). The antibodies decorated punctate structures on the cell membrane with some focalisation in the cell peripheries. This staining pattern was largely consistent between N34B and N34E although the signal to background was higher for N34B. Based on prior experiments conducted in our laboratory, the performance of these antibodies was not convincingly better than other available reagents such and the polyclonal antibodies directed against the N-terminus of FAT1. Thus the use of the new B and E antibodies was restricted to Western blotting where these reagents fulfilled the functional requirements.

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