Chapter 2

Materials and Methods
2.0 - Materials and Methods:

2.1 - Materials

Go 6976 (ab141413), phorbol 12-myristate 13-acetate (PMA) (ab120297) and staurosporine (ab120056) were purchased from Abcam (Cambridge, UK). QuikChange Lightning Site-Directed Mutagenesis Kit, Isopropylthio-β-galactoside (IPTG) and X-gal were purchased from Agilent Technologies (Berkshire, UK). IMR-32 CCL-127™ neuroblastoma cell line was from ATCC™ (Manassas, VA). Illford PQ universal developer and Illford rapid fixer were purchased from Avon film productions (Bath, UK). DNA HyperLadder 1 was purchased from Bioline (London, UK). Advanced DMEM (11520436), Agar purified powder (Oxoid), Amersham hyperfilm ECL nitrocellulose membrane, Amersham hyperfilm ECL, cacodylic acid sodium crystals, calcium chloride, dimethyl pimelimidatedihydrochloride, disodium hydrogen phosphate, ethylenediaminetetraacetic acid (EDTA), glacial acetic acid, glycine, hydrogen chloride, magnesium chloride, MES free acid, methanol, poly-l-lysine, potassium chloride, PVDF membrane (IPFL00010), Restriction enzyme FastDigest Ascl, Restriction enzyme FastDigest PacI, sodium azide, sodium bicarbonate, sodium chloride, sodium dodecyl sulphate (SDS), sodium hydroxide, Spectra ladder (Fermentas), triethanolamine, Tris, Tween® 20 (Acros), triptone powder (Oxoid), urea and yeast extract powder (Oxoid) were purchased from Fisher Scientific (Loughborough, UK). His Mag Sepharose Ni, HiTrap™ Q HP column, PD10 column, and Protein G Mag Sepharose beads were purchased from GE Healthcare (Buckinghamshire, UK). Ampicillin, Bluo-gal, Gateway® Entry Vector pENTR221 with the hBCATc gene, Gateway® Entry Vector pENTR221 with the hBCATm gene, Gateway® LR Clonase® II enzyme mix, Gateway® pcDNA™DEST47, Gateway® pDEST™26 (GeneArt), GlutaMAX supplement, kanamycin sulphate, Library Efficiency® DH5α™ Cells, NuPAGE 4-12% Bis-Tris gel, NuPAGE LDS sample buffer 4 X, NuPAGE MES SDS running buffer, NuPAGE
MOPS SDS running buffer, PureLink™ HiPure Plasmid Miniprep Kit, trypsin, Ultrapure™ Agarose, Ultrapure™ 10 mg/mL ethidium bromide, Ultrapure™ Tris 1 (Gibco®), (Invitrogen™) were purchased from Life Technologies (Carlsbad, CA). L-Glutamine and RPMI 1640 media were from Lonza (Basel, Switzerland). Phosphate buffered saline (PBS) was from Oxoid (Hampshire, UK). jetPRIME® DNA and siRNA buffer was purchased from Peqlab (Sarisbury Green, UK). BM chemiluminescence blotting substrate, Complete mini EDTA-free protease inhibitor cocktail tablets and Pefabloc SC were purchased from Roche (West Sussex, UK). Bovine Serum Albumin (BSA) was purchased from Sera Laboratories (West Sussex, UK). 30% Acrylamide, adenosine triphosphate (ATP), ammonium persulphate, β-mercaptoethanol, bromophenol blue dye, Coomassie blue G dye, dithiothreitol (DTT), ethanolamine, ethidium bromide, foetal bovine serum (FBS), glucose, glycerol, imidazole, magnesium chloride, NZ amine, RPMI 1640 non-essential amino acids solution, sodium fluoride, sodium glycerol-phosphate, TEMED, thrombin and Triton™ X-100 were purchased from Sigma-Aldrich (Dorset, UK). Vectashield hardset mounting medium with DAPI and Vectastain elite ABC kit were purchased from Vector Labs (Peterborough, UK). Ethanol and phosphoric acid (BDH) were purchased from VWR (Leicestershire, UK). Ni-NTA Agarose was purchased from Qiagen Ltd. (West Sussex, UK). Marvel was purchased locally.

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Primers were synthesised by Eurofins MWG Operon (London, UK). The sequences of the hBCATm with no stop codon primers are as follows:

**hBCATm - no stop**

**Sense**

5’ – cacgagtggagttcccccgtggccttaattaacccagctttcct – 3’

**Anti-Sense**

5’ – aagaaagctggatttaattagccacgggaacatccactctg – 3’
2.2 - IMR-32 and SH-SY5Y neuroblastoma cell culture.

IMR-32 cells were propagated in RPMI 1640 media containing 10% foetal bovine serum (FBS), 1 X non-essential amino acids and 1 X L-glutamine. SH-SY5Y cells were grown in Advanced DMEM media containing 10% FBS and 1 X GlutaMax. Both cell lines were maintained at 50-80% confluence and incubated at 37°C, 5% CO$_2$. Cell treatments were either in the respective cell media or Earle’s Balanced Salt Solution (EBSS).

2.3 - Bradford assay.

A stock solution of Coomassie blue G dye (330 mg Coomassie blue G dye dissolved in 100 mL phosphoric acid/ethanol 2:1) was prepared fresh. The working solution was prepared from 3% stock dye, 8% phosphoric acid and 3.8% ethanol in distilled water. Protein standards were prepared using BSA at a concentration of 1 µg/µL. The samples were diluted by 1/5 and 1/10, and 5 µL of each was added in triplicate to a 96 well plate. Working dye solution (200 µL) was added to each well and the absorbance monitored at λ620 nm. The protein concentration of each sample was calculated using the standard curve produced by the protein standards.

2.4 - Wet transfer western blot.

Protein samples were added to loading buffer (Bromophenol blue dye, 8% SDS, 40% Glycerol, 0.7 M β-mercaptoethanol, in 200 mM Tris, pH 6.8), heated at 73 °C for 10 minutes and separated on either an 8% SDS PAGE gel (1% APS, 1% SDS, 0.06% TEMED, 8% acrylamide and 1.5 M Tris, pH 8.8) at 100 V for 20 minutes followed by 185 V for 75 minutes in running buffer (25 mM Tris, 190 mM Glycine, 1% SDS, pH 8.3), an 8% Native PAGE gel (1% APS, 0.06% TEMED, 8% Acrylamide and 1.5 M Tris, pH 8.8) at 185 V for 95 minutes in running buffer (without SDS), or a NuPAGE Novex 4-12% Bis-Tris gel for 185 V for 75 minutes in 1 X NuPAGE® MOPS SDS Running Buffer. The proteins were then transferred from
the gel to either a nitrocellulose or a PVDF membrane for 18 hours at 35 V in transfer buffer (20% Methanol, 25 mM Tris, 190 mM Glycine, pH 8.3). The membrane was washed three times with Tris buffered saline with Tween (TBST: 0.1% Tween, 200 mM NaCl, 2 mM Tris, pH 7.5) for 10 minutes each and blocked with 5% Marvel in TBST (10 mL) for 1 hour. Primary antibody in 5% Marvel was added to the membrane and incubated for 14 hours at 4°C. After three 10 minute washes with TBST and an additional block with 5% Marvel for 30 minutes, secondary antibody (HRP linked) in 5% Marvel was added for 1 hour. Following three 10 minute washes with TBST, chemiluminescent HRP substrate was added for 1 minute and exposed to film for various time periods.

2.5 - Immunoprecipitation.

IMR-32 cells were scraped and removed on ice in IP buffer (20 mM Tris, 150 mM NaCl, 1% Triton™ X-100, 2 mM MgCl₂, 1 μM CaCl₂, 0.5 mM ATP, 1 mM sodium glycero-phosphate, 50 mM NaF, 0.5 mg/mL Pefabloc SC and 1 X EDTA-free Protease inhibitor), extracted through a G21 needle twenty times and centrifuged at 15,000 X G for 10 minutes at 4°C. Protein concentrations of the cell lysates were obtained via a Bradford assay, and the extracted proteins diluted to 1 μg/μL in IP buffer. Protein G Mag Sepharose beads were vortexed for 30 seconds and aliquoted out into 50 μL samples and re-suspended in 100 μL IP buffer. As a pre-clearing step the extracted proteins (800 μg) were incubated with 50 μL of Protein G Mag Sepharose beads for 1 hour on a rotary wheel at 4°C. Antibodies (anti-BCATm and IgG) were diluted to 0.004 μg/μL in 250 μL IP buffer and incubated with 50 μL of Protein G Mag Sepharose beads for 1 hour on a rotary wheel at 4°C. The supernatant was then removed from the antibody-bead samples and the pre-cleared lysate added and incubated overnight on a rotary wheel at 4°C. The Protein G Mag Sepharose beads were then washed three times with IP buffer, two times with IP buffer (0.1% Triton X-100™), and once with IP buffer (without
Triton X-100™), incubating for 5 minutes on a rotary wheel at 4°C for each wash. The samples were re-suspended in 50 mM glycine pH 2.7 and loading buffer, and the proteins were separated by SDS-PAGE and analysed by western blotting.

2.6 - Heterologous expression of hBCATm-His.

The heterologous expression and purification of the hBCAT proteins in *E. coli* has been previously described by Davoodi *et al.*, (1998). Briefly, 100 mL of 2-YT growth medium (1.6% tryptone, 1% yeast, 0.5% NaCl) containing 30 µg/mL kanamycin sulphate was inoculated with *E. coli* BL21.DE3 containing a pET-28a plasmid with hBCATm-cDNA and incubated at 37°C overnight at 180 rpm. The *E. coli* were then expanded into 1 L of 2-YT containing 30 µg/mL kanamycin sulphate and incubated at 30°C for 24 hours at 155 rpm. Expression of hBCATm was induced with the addition of 1 mM IPTG at 37°C for 4 hours after which the *E. coli* were pelleted by centrifugation at 8000 rpm for 10 minutes at 4°C and the pellets stored at −80°C.

2.7 - Extraction and purification of hBCATm-His.

*E. coli* BL21.DE3 pellets were re-suspended in 25 mL of buffer AU (0.5 M Na₂HPO₄ pH 8.0, containing 25 mM Tris, 5 M urea and 0.035% β-mercaptoethanol) and sonicated for 10 minutes with 15 second intervals every 15 seconds. The cell suspension was then centrifuged at 18,500 rpm for 10 minutes at 4°C and the supernatant transferred to a conical flask and stored on ice. The pellet was then re-suspended in 25 mL of buffer AU and once more sonicated and centrifuged as described. The supernatants were pooled and 8 mL of Ni-NTA agarose, equilibrated in buffer AU, was added and mixed gently for 1 hour at 4°C. The Ni-NTA agarose supernatant mix was transferred to a 20 mL glass column, the flow through collected and the column sequentially washed with three column volumes of buffer B (0.5 M Na₂HPO₄ pH 7.4, containing 25 mM Tris, 0.5 M NaCl, 20% glycerol
and 0.035% β-mercaptoethanol), buffer C (0.5 M Na₂HPO₄ pH 6.0, containing 25 mM Tris, 1.5 M NaCl, 20% glycerol and 0.035% β-mercaptoethanol) and buffer C50 (0.5 M Na₂HPO₄ pH 6.0, containing 25 mM Tris, 75 mM imidazole, 10% glycerol and 0.035% β-mercaptoethanol).

The hBCAT was then eluted from the column in 15 mL of buffer D (0.5 M Na₂HPO₄ pH 6.0, containing 25 mM Tris, 0.75 M NaCl, 0.75 M imidazole, 10% glycerol and 0.035% β-mercaptoethanol). The hBCATm-His was exchanged into Mono Q buffer A (0.1 M HK₂PO₄, pH 8.0) and 4 mM Ketoisocaproate (KIC) was added before being loaded onto a HiTrap™ Q HP column for ion-exchange chromatography. The protein was eluted from the column via a concentration gradient of 0 to 0.5 M NaCl in Mono Q buffer A at a flow rate of 1 mL/min for 20 minutes. The eluted hBCATm-His protein was dialysed in Stable buffer (50 mM Tris-HCl pH 7.5, containing 0.15 M NaCl, 5 mM glucose, 1 mM EDTA, 1 mM KIC and 5 mM DTT) overnight at 4 °C. A final concentration of 30% glycerol was added to the dialysed protein before being aliquoted and stored at –20°C.

2.8 - IMR-32 cell lysate pull-down using Ni Sepharose™.

hBCATm-His pure protein was diluted to 50 µg, 250 µg and 1 mg in 1 mL of buffer AU and added to 175 µL samples of Ni Sepharose™ beads for 2 hours on a rotary wheel at 4°C. IMR-32 cells were scraped and removed on ice in IP buffer containing 5 mM imidazole, extracted through a G21 needle twenty times and centrifuged at 15,000 X G for 10 minutes at 4°C. Protein concentrations of the cell lysates were obtained via a Bradford assay, and the extracted proteins diluted to 1 µg/µL in IP buffer. Ni Sepharose™ beads bound to hBCATm-His were washed three times with 600 µL IP buffer containing 5 mM imidazole and re-suspended in 760 µL. Cell lysates (1 mg) were added to the beads bound to hBCATm-His and incubated overnight on a rotary wheel at 4°C. The Ni Sepharose™ beads were then washed three times with IP buffer, two times with IP buffer (0.1% Triton X-
100™), and once with IP buffer (without Triton X-100™), incubating for 5 minutes on a rotary wheel at 4°C for each wash. The samples were re-suspended in IP buffer (without Triton X-100™) containing 767 mM imidazole and loading buffer. The proteins were separated by SDS-PAGE and analysed by Western blotting.

2.9 - Preparation of Lysogeny Broth (LB) agar plates.

The LB broth was prepared using 5 g of Bacto-Tryptone, 2.5 g of Bacto-Yeast and 5 g of NaCl dissolved in 500 mL of distilled deionised water. The pH was adjusted to pH 7.0 before adding 7.5 g of purified Agar. The media was sterilised through autoclaving at 121°C for 15 minutes. Once the media had cooled to 50°C, antibiotics were added and 25 mL was added per plate. After the media had set the plates were stored upside down at 4°C.

2.10 - LR recombination reaction.

Samples were prepared containing 150 ng of specified Gateway® destination vector with either 50 ng, 100 ng or 150 ng of purified hBCATm/hBCATc pENTR221. A positive control containing 100 ng pENTR™-gus with 150 ng destination vector and a negative control containing 150 ng of purified hBCATm/hBCATc pENTR221 with 150 ng destination vector were also prepared. To each sample TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA) was added to make a total of 8 µL reaction samples to which 2 µL of LR Clonase® II enzyme mix was added, except for the negative control where 2 µL of TE buffer was added instead. The samples were gently mixed and incubated in a hot-block at 25°C for 3 hours. To stop the LR reaction, 1 µL of proteinase K solution was added to each sample and incubated at 37°C in a water bath for 10 minutes.

2.11 - Transformation of Library Efficiency® DH5α™ Cells.

Library Efficiency® DH5α™ Cells were thawed on ice alongside the required number of 50 mL Falcon tubes. To each tube 50 µL of cells was aliquoted, followed
by the addition of 1.5 µL of the LR recombination reaction. In order to assess the transformation efficiency, 25 pg of pUC19 plasmid was added to 50 µL of cells. All of the samples were incubated on ice for 30 minutes and then heat-shocked for 30 seconds at 42°C in a water bath. The samples were placed back on ice for 2 minutes before the addition of 450 µL of Super Optimal broth with Catabolite repression (S.O.C.) and 1 hour incubation at 37°C with shaking at 225 rpm. From the gus, 50, 100 and 150 ng samples, 50, 100 and 200 µL was spread on an LB ampicillin (100 µg/mL) plate. From the transformation with pUC19, 50 µL was spread on LB ampicillin (100 µg/mL), Bluo-gal (100 µg/mL) and IPTG (40 µg/mL). All plates were incubated at 37°C for 24 to 48 hours until blue/white colonies were visible to select.

2.12 - Plasmid isolation from E.coli using the PureLink™ Hi-Pure Plasmid Mini Prep Purification Kit.

A single white colony was selected from each plate with a sterile P200 tip and placed into 5 mL of 2-YT growth medium containing the same antibiotic selection (100 µg/mL ampicillin or 50 µg/mL kanamycin sulphate) and incubated overnight at 37°C with 180 rpm shaking. From the overnight cultures, 3 mL was centrifuged at 10,000 rpm for 10 minutes at room temperature in sterile microcentrifuge tubes. The supernatant was discarded and the bacterial pellet re-suspended in 400 µL of re-suspension buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.0) containing RNase A, followed by the addition of 400 µL of lysis buffer (0.2 M NaOH, 1% SDS). After a 5 minute incubation at room temperature, 400 µL of precipitation buffer (3.1 M potassium acetate, pH 5.5) was added and the sample mixed by inversion. The mixture was centrifuged at 15,000 rpm for 10 minutes at room temperature, during which the columns supplied with the kit were equilibrated with 2 mL of equilibration buffer (0.1 M sodium acetate, 0.6 M NaCl, 0.15% Triton™ X-100, pH 5.0).
The supernatant was applied to the column, followed by two washes with 2.5 mL of wash buffer (0.1 M sodium acetate, 0.825 M NaCl, pH 5.0) and then eluted with 900 µL of elution buffer (100 mM Tris-HCl, 1.25 M NaCl, pH 8.5). The eluted plasmid DNA was then precipitated by the addition of 630 µL of isopropanol, a 15 minutes incubation on ice and then centrifugation at 15,000 rpm for 30 minutes at 4°C. The supernatant was carefully removed, the pellet re-suspended in 1 mL of 70% ethanol and centrifuged at 15,000 rpm for 5 minutes at 4°C. The supernatant was removed and the pellet left to air dry for 10 minutes before re-suspending in 40 µL of TE buffer (10 mM Tris-HCl, 0.1 M EDTA, pH 8.0). The DNA yield was calculated from absorbance measurements at 260 nm using the nano-drop (Thermo Scientific). The quality of the DNA was examined by separating the fragments on a 0.8% agarose gel (see 2.13) and imaging under UV trans-illumination.

2.13 - Agarose gel electrophoresis.

Ultrapure agarose (0.8%) was dissolved in 1 X TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and heated in a microwave until the agarose had melted. Ethidium bromide was added to produce a final concentration of 0.5 µg/mL and poured into sealed agarose plates and allowed to set at room temperature. The tape seal and the comb were removed and the gel mounted in an electrophoresis tank containing 1 X TAE buffer. The DNA samples were made up in FastDigest green buffer and DNA/RNA free water, and were loaded into the wells alongside DNA HyperLadder I (Bioline). The samples were separated at 150 V for 45 minutes and the bands visualised under UV trans-illumination on a gel dock.

2.14 - Transfection of IMR-32/SH-SY5Y cells with jetPRIME® transfection reagent.

IMR-32/SH-SY5Y cells were grown in 6-well plates at an initial density of 1.9 X 10^5 cells/well in 2 mL of media (RPMI-1640 containing 10% FBS, 1 X non-
essential amino acids and 1 X L-glutamine/Advanced DMEM media containing 10% 
FBS and 1 X GlutaMax) and incubated for 24 hours. For each well, stated amounts 
of hBCAT expression vector DNA was added to 200 µL of jetPRIME® buffer and 
gently vortexed before adding a 2:1 volume (µL of jetPRIME®:µg plasmid DNA) of 
jetPRIME® transfection reagent. This solution was vortexed for 10 seconds, 
centrifuged briefly and incubated at room temperature for 15 minutes. The cell 
media was changed and the transfection mix added to each well drop-wise and in 
an even distribution. The 6-well plates were rocked back and forth and side to side 
and then incubated for 48 hours. The transfected cells were washed twice with 1 
µL of PBS and removed using 500 µL/well of Trypsin.

2.15 - Flow cytometry.

Eppendorf tubes containing extracted cells were centrifuged for 2 minutes at 
5000 rpm, the supernatant removed, and the pellet re-suspended in 500 µL of PBS. 
This was repeated twice before re-suspending the cells in 250 µL of PBS and 
running the samples on an Accuri C6 flow cytometer. For each sample 10,000 cells 
were counted and gated (R1) to remove cell debris. The cells were excited (488 
nm) and their emission recorded using the FL-1 channel (533/30 nm) to examine 
the GFP positive population. The results were processed using the CFlow program 
version 1.0.227.4.

2.16 - IMR-32 cell lysate pull-down using His Mag Sepharose Ni beads.

IMR-32 cells were transfected with hBCATm-His, removed using Trypsin 
and extracted in IP buffer (without Triton X-100™) through a G21 needle twenty 
times and centrifuged at 15,000 g for 10 minutes at 4°C. Protein concentrations of 
the cell lysates were obtained via a Bradford assay, and the extracted proteins 
diluted to 1 µg/µL in IP buffer (without Triton X-100™). Specified amounts of cell 
lysate were then added to 60-75 µL of His Mag Sepharose Ni beads for 30 minutes
on a rotary wheel at room temperature. The bound proteins were eluted in 50 µL of 500 mM imidazole and separated by SDS-PAGE for analysis by Western blotting.

2.17 - QuikChange Site-directed mutagenesis.

The hBCATm entry vector was purified by mini-prep and used as the template DNA for the stop codon mutants. All reagents were provided with the kit and samples were prepared on ice. A control reaction was prepared containing 5 µL of 10 X reaction buffer, 5 µL (25 ng) of pWhitescript 4.5 kb control plasmid, 1.25 µL (125 ng) of oligonucleotide control primer 1, 1.25 µL (125 ng) of oligonucleotide control primer 2, 1 µL of dNTP mix, 1.5 µL of QuikSolution reagent and 35 µL of DNA/RNA free water. The hBCATm entry vector stop codon mutants were prepared as described for the control using a total of 50 ng and 100 ng of template DNA, and 125 ng of both sense and anti-sense primers for the stop codon mutation. The reaction was started by the addition of 1 µL of QuikChange Lightning Enzyme to each of the 50 µL reaction mixtures. The tubes were placed into a thermal cycler with a hot-top assembly and the following cycling parameters used: 1 cycle of 2 minutes at 95°C, 18 cycles of 20 seconds at 95°C, 10 seconds at 60°C and 2 minutes at 68°C, 1 cycle of 5 minutes at 68°C. The reaction was then held at 4°C at the end of the PCR reaction.

For post-mutant strand synthesis, 2 µL of Dpn I restriction enzyme was added to each reaction and incubated at 37°C for 5 minutes. After digestion, XL10-Gold Ultracompetent cells were thawed on ice and 45 µL was aliquoted into a pre-chilled 14 mL BD Falcon round bottom tube. To each aliquot of cells 2 µL of β-mercaptoethanol was added and incubated on ice for 2 minutes before adding 2 µL of the Dpn I-treated DNA and incubating for 30 minutes on ice. A transformation control was prepared using 1 µL of 0.01 ng/µL pUC18 control instead of the Dpn I-treated DNA. The cells were heat-shocked for 30 seconds at 42°C and then
incubated on ice for 2 minutes. Preheated NZY broth was added (500 µL) to the cells and incubated at 37°C for 1 hour with shaking at 250 rpm.

The pWhitescript mutagenesis control (10 µL) and pUC18 transformation control (2.5 µL) were spread on LB plates containing 100 µg/mL ampicillin, 100 µg/mL Bluo-gal and 40 µg/mL IPTG. The BCATm stop codon mutants were spread on LB plates containing 50 µg/mL of kanamycin sulphate and incubated for 24 hours at 37°C. Six colonies in total were selected from the 50 ng and the 100 ng BCATm stop codon mutant plates for overnight growth in 3 mL of 2YT media containing 50 µg/mL of kanamycin sulphate and purification by mini-prep.

Samples were prepared for sequencing as instructed by Eurofins MWG Operon. Purified DNA samples with greater than 1.85 260:280 nm ratios were diluted to a final concentration of 100 ng/µL in sterile water, from which 15 µL was sent for sequence analysis.

2.18 - Cross-linked co-immunoprecipitation.

Before beginning extraction, IMR-32/SH-SY5Y cells were incubated in a phospho buffer containing 5.6 mM KCl, 0.2 mM KH₂PO₄, 137.6 mM NaCl, 2.4 mM Na₂HCO₃, 5.6 mM glucose, 0.4 mM MgSO₄, 0.5 mM MgCl₂, 20 mM HEPES (pH 7.4), 1.26 mM CaCl₂ and 10 mM ATP for 2 hours at 37°C at 5% CO₂. The cells were then scraped and removed on ice in IP buffer (20 mM Tris, 150 mM NaCl, 1% Triton™ X-100, 2 mM MgCl₂, 1 µM CaCl₂, 0.5 mM ATP, 1 mM sodium glycerophosphate, 50 mM NaF, 0.5 mg/mL Pefabloc SC and 1 X EDTA-free Protease inhibitor), extracted through a G21 needle twenty times and centrifuged at 15,000 X G for 10 minutes at 4°C.

Protein concentrations of the cell lysates were obtained via a Bradford assay, and the extracted proteins diluted to 1 µg/µL in IP buffer. Protein G Mag Sepharose beads were vortexed for 30 seconds and aliquoted out into 30 µL
samples and re-suspended in 400 μL binding buffer (50 mM Tris, 150 mM NaCl, pH 7.5). A total of 1 μg of antibody (hBCATc, hBCATm or IgG isotype control) was added to the beads in 500 μL binding buffer and incubated on a rotary wheel for 30 minutes at room temperature. The beads were then washed once with 500 μL binding buffer, once with 500 μL crosslink solution A (200 mM triethanolamine, pH 8.9) and then incubated with 500 μL crosslink solution B (50 mM dimethyl pimelimidate dihydrochloride, 200 mM triethanolamine, pH 8.9) for 1 hour on a rotary wheel at room temperature. A 500 μL wash with crosslink solution A then preceded a 15 minute incubation with crosslink solution C (100 mM ethanolamine, pH 8.9). Any unbound antibody was then washed off using 500 μL of elution buffer (0.1 M glycine-HCl, 2 M urea, pH 2.9) and the beads washed twice with 500 μL binding buffer. Cell lysates (800 μL) were added to the beads and incubated on a rotary wheel at room temperature for 1 hour. The beads were washed three times with 500 μL of wash buffer (50 mM Tris, 150 mM NaCl, 2 M urea, pH 7.5) and incubated with 50 μL elution buffer for 5 minutes. The eluted samples were separated by SDS-PAGE and analysed by Western blotting.

2.19 - Subcellular fractionation.

After appropriate treatment, IMR-32/SH-SY5Y cells were washed twice in ice cold PBS before being removed by scraping in PBS. The cells were centrifuged for 5 minutes at 900 X G and re-suspended in Tris buffer (50 mM Tris, 150 mM NaCl, 1 X EDTA-free Protease inhibitor, pH 7.5), extracted through a G25 needle twenty times and incubated on ice for 30 minutes. To remove the nuclear fraction, the cell lysates were centrifuged at 600 X G for 5 minutes at 4°C. The supernatant was removed and centrifuged at 20,000 X G to isolate the membrane fraction from the cytosolic, which was then re-suspended in MES-buffered saline (25 mM MES, 150 mM NaCl, 1% Triton X-100™, 1 X EDTA-free Protease Inhibitor, pH 6.5) and incubated on ice for 60 minutes. After centrifugation at 20,000 X G for 30 minutes
at 4°C, the non-raft membrane fraction was collected as supernatant and the raft-membrane pellet was re-suspended in Tris buffer containing 60 mM β-octylglucoside and 1% Triton X-100™ and incubated on ice for 30 minutes. The raft-membrane fraction was then collected as supernatant after centrifugation at 15,000 X G for 20 minutes at 4°C. Protein concentrations were calculated using the Bradford assay, the samples were separated by SDS-PAGE and analysed by Western blotting.

2.20 - Immunocytochemistry.

Sterile glass coverslips were treated with 150 µL poly-l-lysine for 20 minutes, washed three times with distilled water, and 5 X 10⁴ SH-SY5Y cells were plated and incubated for 48 hours. After specified treatments, the cells were washed twice with sodium cacodylate solution (0.1 M sodium cacodylate in 0.1 M sucrose pH 7.4) and fixed with glutaraldehyde (0.25% in sodium cacodylate solution) for 20 minutes. Once the fixing solution was removed the cells were subject to separate 5 minute incubations with sodium cacodylate solution, glycine (100 mM in sodium cacodylate solution) and fetal bovine serum (FBS) (10% in sodium cacodylate solution). To permeabilize the cells, after washing twice with sodium cacodylate solution, they were incubated for 20 minutes with Triton™ X-100 (0.2% in sodium cacodylate solution). After two washes with sodium cacodylate solution, cells were incubated with NaBH₄ (1 mg/mL in sodium cacodylate solution) for four 10 minute incubations. After two washes with sodium cacodylate solution the cells were left in blocking buffer (3% bovine serum albumin (BSA) in sodium cacodylate solution) overnight at 4°C. Primary antibodies were used at final concentrations in blocking buffer as stated, of which 70 µL was applied for 1 hour. After two washes with sodium cacodylate solution the secondary antibodies were applied at concentrations of 1:500 for anti-rabbit Alexa Fluor 488 and 1:250 for anti-mouse Alexa Fluor 568 in blocking buffer, using 70 µL per coverslip for a 1 hour incubation in the dark. Following two washes with sodium cacodylate solution the coverslips were mounted
on sterile slides using Vectashield hard set mounting medium with DAPI. The slides were then stored at 4°C in the dark until viewed. Images of the labelled cells were acquired using a Zeiss Axiovert 200 confocal microscope.

2.21 - Statistical analysis.

Data were statistically analysed using GraphPad Prism 5.0 software. Values are expressed as mean ± SEM, and statistical comparisons were made by one-way ANOVA with a Dunnett’s post hoc test, where statistical significance was (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$. A one-way ANOVA test was used to compare the means of two or more independent groups to determine whether there is a significant difference between the associated population means. A post hoc Dunnett’s test was then used to test for significant differences between the mean of each factor level compared to the control group.