The role of hBCATc in breast cancer progression through IGF-1 and insulin signalling cascades

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Abstract

Introduction and Aims: Breast cancer remains the leading cause of cancer mortality in women globally, despite advances in the development of targeted therapies. Driven by oncogenic signals, cancer cells undergo metabolic reprogramming, upregulating metabolite transporters and shuttling nutrients to promote proliferation and migration. The expression of key metabolic enzymes involved in BCAA metabolism (hBCATc and hBCATm), isocitrate dehydrogenase (IDH1 and IDH2) and serine metabolism (PHGDH) have been demonstrated to be upregulated in breast cancer. However, the expression patterns and the role of these metabolic proteins in breast cancer tumourigenesis remains to be elucidated. This research hypothesises that these metabolic pathways converge in tumour cells, whereby particular metabolic pathways are favoured differentially between breast cancer subtypes, which could lead to the identification of novel therapeutic targets. In breast cancer insulin/IGF-1 signalling has been demonstrated to contribute to tumourigenesis through the activation of growth signalling effectors which is mediated by two chief pathways: Akt/mTOR and RAS/MAPK. As nutrient levels of BCAAs particularly leucine, substrates of hBCAT, regulate the mTOR pathway hBCAT was hypothesised to play a role in the regulation of proliferation and migration of triple-negative breast cancer (TNBC) cells through the IGF-1 and insulin signalling pathways.

Methods: Using immunohistochemistry, the expression profile of the metabolic proteins was assessed between breast cancer subtypes. The impact of hBCATc expression on proliferation, migration, invasion, apoptosis and modulation of PI3K/Akt and RAS/MAPK signalling was assessed using molecular biological investigations, Western blot and confocal analysis.

Results and discussion: Using serial sections, hBCATm was found to be significantly associated with IDH1 expression, indicating that these two metabolic pathways are activated concomitantly. Expression of hBCATm and IDH1 correlated with luminal A breast cancer and smaller breast tumours, indicating better prognosis. Differentially, hBCATc expression was found to be significantly associated with the more aggressive HER2+ and TNBC subtypes. For the first time, knockdown of hBCATc was demonstrated to significantly reduce insulin and IGF-1-mediated proliferation, migration and invasion in TNBC cells. An analysis of this pathway showed that when overexpressed hBCATc regulates proliferation through the PI3K/Akt/mTOR axis, whilst simultaneously attenuating the Ras/MAPK pathway, indicating that hBCATc acts as a conduit between these two pathways. Overexpression of hBCATc ultimately led to the increase in FOXO3a, Nrf2 and GRP78, which play fundamental roles in cell proliferation, cell survival and protein folding. Therefore, hBCATc has been demonstrated to provide TNBC cells with metabolic plasticity to alter dependence on the RAS/MAPK and PI3K/Akt/mTOR signalling cascades, in response to IGF-1/insulin, to promote tumour survival and progression.

Poster presentations and Publications

Poster presentations:

- Characterisation of metabolic proteins in breast cancer cell lines, Postgraduate Research Conference, UWE (23/06/2016).

- Characterisation of metabolic proteins in breast cancer cell lines and human breast tissue, CRIB Annual Meeting, UWE (13/01/2017).

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Publications:

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- **Shafei, M.A.**, Harris, M. and Conway, M.E. (2017). Divergent Metabolic Regulation of Autophagy and mTORC1—Early Events in Alzheimer's Disease?. *Frontiers in Aging Neuroscience* (9) pp. 1-9.

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Abbreviations

2HG	2-Hydroxyglutarate
4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
α-KG	α-ketoglutarate
Akt	RAC-alpha serine/threonine-protein kinase
AMPK	5' adenosine monophosphate-activated protein kinase
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BCAA	Branched-chain amino acids
BCAT	Branched-chain aminotransferase gene
BCAT1	Branched-chain aminotransferase gene (cytosolic isoform)
BCAT2	Branched-chain aminotransferase gene (mitochondrial isoform)
BCKA	Branched-chain α-keto acid
BCKD	Branched-chain α-keto acid dehydrogenase
BCKDHA	Branched-chain α-keto acid dehydrogenase, E1α subunit
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
DAB	3,3'-Diaminobenzidine
DAPI	4',6-Diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor

EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ER	Oestrogen Receptor
FASN	Fatty Acid Synthase
FBS	Foetal Bovine Serum
FOXO3a	Forkhead Box O3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDH	Glutamate dehydrogenase
GLUT1	Glucose transporter 1
Grb2	Growth factor receptor-bound protein 2
Grx	Glutaredoxin
GSH	Glutathione (reduced)
GSK3β	Glycogen synthase kinase 3 β
eGFP	Enhanced green fluorescent protein
hBCAT	Human branched chain aminotransferase
hBCATc	Cytosolic human branched chain aminotransferase
hBCATm	Mitochondrial human branched chain aminotransferase
HER2	Human Epidermal Growth Factor Receptor 2
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IDH	Isocitrate dehydrogenase
IGF	Insulin-like growth factor

IGFBP	Insulin-like growth factor-binding protein
IR	Insulin receptor
IRS1	Insulin receptor substrate 1
LC3	Microtubule-associated light chain 3 phosphatidylethanolamine
LDS	Lithium dodecyl sulphate
MAPK	Mitogen-activated protein kinase
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of Rapamycin complex 1
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H- tetrazolium
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
P70S6K	Ribosomal protein S6 kinase beta-1
PBS	Phosphate buffered Saline
PHGDH	Phosphoglycerate Dehydrogenase
PI3K	Phosphoinositide 3-kinase
PR	Progesterone Receptor
RIPA	Radioimmunoprecipitation assay buffer
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
Shc	Src Homologous and Collagen
shRNA	Short hairpin RNA
siRNA	Small interfering RNA

SOS	Son of Sevenless
TBS	Tris-buffered Saline
TBST	Tris-buffered Saline Tween
TEMED	Tetramethylethylenediamine
TCA	Trichloroacetic acid
TNBC	Triple Negative Breast Cancer
Tris-HCL	Tris(hydroxymethyl)aminomethane hydrochloride
Trx	Thioredoxin
TSC1/2	Tuberin sclerosis complex 1/2
TTI	Tritiated thymidine incorporation
WB	Western blotting

Chapter 1

General Introduction

Mai Ahmed Shafei

1.0 Introduction

1.1 Breast cancer

1.1.1 Breast cancer epidemiology

Breast cancer is the most common malignancy worldwide in women accounting for almost one in four cancer cases (Bray *et al.*, 2018). The incidence rate is increasing with a low survival rate of breast cancer globally (Coleman *et al.*, 2008). Incidence rates of breast cancer varies worldwide with higher incidence in high-income regions (92 per 100,000 in North America) compared with lower-income regions (27 per 100,000 in middle Africa and eastern Asia) (Torre *et al.*, 2016). These patterns reflect the availability of screening programmes and therefore the number of detected breast cancer cases (Harbeck *et al.*, 2019). Moreover, several lifestyle factors such as obesity, smoking, alcohol consumption and diet also contribute to higher incidence of breast cancer in higher income countries (Momenimovahed and Salehiniya, 2019). For example, obesity is correlated with breast cancer due to higher rates of conversion of androgenic precursors to oestrogen through increased aromatase activity in adipose tissue (Matthews and Thompson, 2016). Moreover, high levels of insulin and insulin-like growth factors in response to obesity can stimulate the growth of cancer cells (Picon-Ruiz *et al.*, 2017).

Globally, breast cancer is responsible for approximately 627,000 deaths each year (Bray *et al.*, 2018). Despite lower incidence in low- and middle-income countries breast cancer mortality is often higher due to delayed detection, late stage at diagnosis and limited access to treatment (Leong *et al.*, 2010; Bray *et al.*, 2018). In the UK, breast cancer remains the fourth leading cause of cancer death (CRUK, 2019). After being on a plateau for decades, in the 1990s, breast cancer

mortality began to decline in high-income countries such as the UK (Burton and Bell, 2013). Over the last decade, breast cancer mortality rates have reduced by 22% and the 10-year survival rate has doubled to 78% for women in the UK (CRUK, 2019).

Survival rates for breast cancer significantly decrease by stage at diagnosis with a 5-year survival rate of 26.2% for stage 4 tumours compared with 97.9% for stage 1 early breast cancer (CRUK, 2019). Fortunately, the incidence of late stage diagnosis has significantly decreased to an incidence of 13% of breast cancer cases diagnosed at stage 3-4 (CRUK, 2019) from approximately 31% between 1990-1992 (Sant *et al.*, 2003), which is largely due to increased multiple campaigns to promote awareness and encourage self-examination to enhance the chance of early detection and improved methods of detection (Anastasi and Lusher, 2019; Wang, 2017). Breast cancer mortality rates have demonstrated continuous, significant improvement in breast cancer survival rates and mortality rates are projected to fall further by 26% to 31 per 100,000 in the UK by 2035 (Smittenaar *et al.*, 2016; CRUK, 2019).

Additionally, there has been a significant improvement in the management of breast cancer in terms of prognostication and the identification of appropriate treatment strategies (Prat *et al.*, 2015; Ehinger *et al.*, 2017). Understanding the underlying biological mechanisms of carcinogenesis has led to the identification of novel molecular targets and major advances in the development of targeted therapies (Tong *et al.*, 2018). Targeted-therapies have been demonstrated to improve both recurrence-free interval and survival with metastatic disease in the same patients as well as longer survival after recurrence (Perez *et al.*, 2011). This highlights the importance of understanding the pathophysiology of the different

subtypes of breast cancer to further advance the development of novel targeted therapies and improve prognosis (Masoud and Pagès, 2017).

1.1.2 Normal breast development and the progression to breast cancer

The human mammary gland is an apocrine gland consisting of secretory lobules connected by a system of branching ducts embedded in a fat pad formed by adipocytes and infiltrated by vascular endothelial cells, fibroblasts and immune cells (Figure 1.1A) (Macias and Hinck, 2012; Deugnier *et al.*, 2002). The mammary epithelium is composed of a bilayer of inner luminal cells and the basal outer layer of myoepithelial cells which are surrounded by a basement membrane (Figure 1.1B). Luminal cells are responsible for milk production whilst the basal outer layer of myoepithelial cells contract to guide the milk out of the breast ducts (Daniel and Smith, 1999). The mammary glands are one of the few tissues that are subjected to major morphological changes during developmental phases such as puberty and pregnancy (Macias and Hinck, 2012). During breast development, hormonal cues, notably oestrogen and progesterone, orchestrate a series of paracrine interactions between epithelial and stromal cell types to elicit phenotypic changes in the mammary gland (Sreekumar, Roarty and Rosen, 2015). The breast tissue microenvironment has been demonstrated to play an important role to support normal breast development whilst restricting tumour formation; the breast stroma releases regulatory signals initiated by extracellular matrix components such as laminin to promote cell differentiation and the formation of the normal breast architecture (Silberstein, 2001). Breast cancer cells transplanted with adult mouse mammary epithelial cells into epithelium-free fat pads of nude mice were shown to contribute to normal mammary gland development (Bussard and Smith, 2012), indicating the dominance of the tissue microenvironment over cancer cell fate.

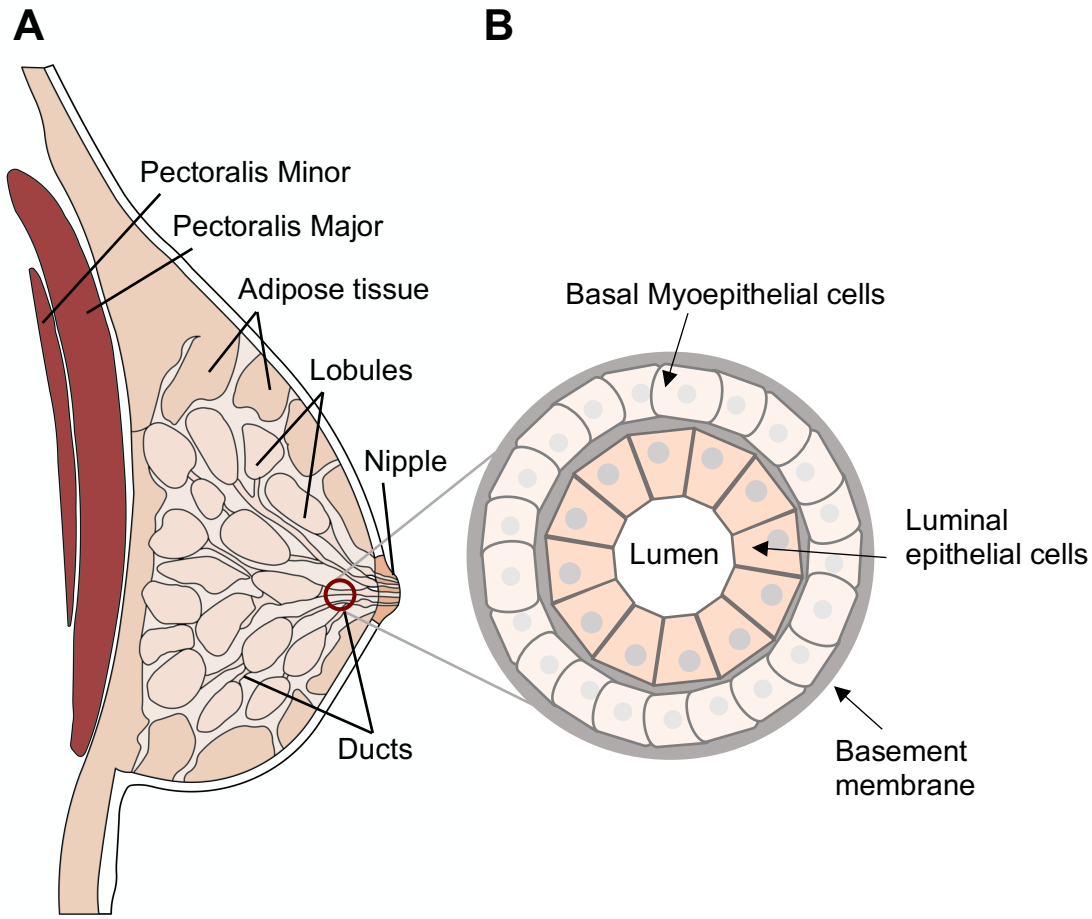

Figure 1.1. Structure of the mammary gland. (**A**) The human breast is composed of lobules connected by a ductal network to the nipple, which are embedded in the stroma. Most breast cancers arise from the lobules or the ducts of the breast. In more advanced breast cancer, the tumour infiltrates the skin or components of the chest wall such as the pectoralis muscles (Macias and Hinck, 2012). (**B**) The ducts are surrounded by an outer basement membrane which is lined with a bilayer of inner luminal cells required for milk production and an outer layer of basal myoepithelial cells required for milk ejection (Daniel and Smith, 1999).

Tumour progression is a multi-step process involving genetic instability leading to an over-proliferation of cells, which become transformed and initiate the development of solid tumours (Hanahan and Weinberg, 2011). Mutations of tumour suppressor genes and abnormal amplification of oncogenes as well as the tumour microenvironment play key roles in breast cancer initiation and progression (Hanahan and Weinberg, 2011; Bussard and Smith, 2012). Breast tumours usually start from ductal hyperproliferation which can develop into benign tumours or even metastatic carcinomas following constitutive stimulation by various carcinogenic factors (Sun *et al.*, 2017).

The initiating event in the development of breast cancer can be caused by inherited gene mutations, such as mutations in the tumour suppressor phosphatase and tensin homolog deleted on chromosome ten (*PTEN),* which can lead to familial breast cancer (Tung *et al.*, 2016). There is an 80% increased lifetime risk for carriers of *PTEN* mutations of developing breast cancer (Ngeow, Sesock and Eng, 2017). *PTEN* is an important tumour suppressor which inhibits phosphatidylinositol 3-kinase (PI3K) signalling by inhibiting phosphatidylinositol (3,4,5) trisphosphate (PIP_3), a critical lipid second messenger in tumourigenesis that activates RAC-alpha serine/threonine-protein kinase (Akt) and other signalling molecules involved in cell survival, proliferation and invasion (Lien, Dibble and Toker, 2017). *PTEN* reduces the pool of PIP_3 hence inhibiting growth and survival signals (Hopkins *et al.*, 2014), therefore loss of *PTEN* allows constitutive activation of PI3K/Akt signalling leading to tumour development (Chalhoub and Baker, 2009). Breast cancer risk is also increased for carriers of other gene mutations such as *breast cancer susceptibility gene1/2* (*BRCA1/BRCA2)* whereby the risk of developing breast cancer doubles by having a first-degree relative with breast cancer (Feng *et al.*, 2018; Ibrahim, Abdelmalek

and Elfiky, 2019). The risk of breast cancer also increases with age as random mutations can gradually accumulate in any breast cell, which can lead to their transformation into tumour cells if/when adequate mutations accumulate (Sun *et al.*, 2017). Other risk factors for breast cancer include obesity (Picon-Ruiz *et al.*, 2017), increased alcohol consumption (Jung *et al.*, 2016), smoking (Kispert and McHowat, 2017), prolonged use of hormone replacement therapy (Shah, Borenstein and Dubois, 2005) and late menopause (Sun *et al.*, 2017).

1.1.3 Diagnosis and screening of breast cancer

Early detection of breast cancer is critical in improving the chance of successful treatment as prognosis significantly decreases at the later stages of breast cancer, particularly for high-risk women that are genetically predisposed (Saadatmand *et al.*, 2015). Screening programmes are employed worldwide for breast cancer with the primary aim of reducing the number of women presenting with late stage cancer thereby reducing mortality rates (Bleyer and Welch, 2012). Meta-analysis of 11 randomised control trials with a 13 year follow up has estimated a 20% reduction of breast cancer mortality rates for women invited for screening (Marmot *et al.*, 2013). The UK has a national screening programme in place which invites women between the ages of 50-71 every 3 years for screening mammography, which involve the use of low-energy X-rays to detect breast cancer through the detection of characteristic masses and microcalcifications (Marmot *et al.*, 2013). Alternatively, ultrasonography, which utilises an ultrasound transducer to measure the acoustic waves reflected from the breast, can be used for subjects where mammography is not suitable especially for women with dense breasts such as those under the age of 40 (Wang, 2017). Upon the detection of an abnormality, a biopsy or fine needle aspiration is taken to collect cells for

microscopical examination which are assessed by a pathologist (Gnant and Harbeck, 2011).

1.1.4 Breast cancer grading and staging

Following diagnosis, breast cancer is initially classified according to stage and grade which aids prognosis. Histological grading provides an insight into how abnormal the cancer cells are whilst staging assesses the extent a tumour has infiltrated other areas of the body (Connolly *et al.*, 2003). Histological grade is based on the microscopic appearance of tumour cells, which assesses the percentage of tubule formation, the degree of nuclear pleomorphism and numbers of mitotic figures (Elston and Ellis, 1991; Bloom and Richardson, 1957). A score of 1-3 is applied depending on the extent of how abnormal the cancer cells are for each criteria; these scores are then combined leading to a grade of 1-3 (table 1.1) (Elston and Ellis, 1991; Bloom and Richardson, 1957). Histological grading provides an insight into the degree of differentiation and growth rate of a tumour, whereby grade 3 represents the least differentiated and fastest growing tumours (Elston and Ellis, 1991; Bloom and Richardson, 1957). Tumour grade reflects the potential aggressiveness of the breast cancer and is a strong prognostic indicator as increasing histologic grade is significantly associated with a progressive decrease in 10-year survival rates of breast cancer patients (Ehinger *et al.*, 2017; Schwartz *et al.*, 2014).

Breast cancer is staged following the guidelines of the American Joint Committee on Cancer (AJCC) which is based on TNM evaluation of primary tumour size (T), degree of lymph node involvement (N) and extent of metastasis (M) (Ravaioli and Tassinari, 2000) as outlined in table 1.2. In the case of multifocal tumours in one

Table 1.1 Breast cancer grading

Feature graded	Clinical feature	Score
Tubule formation	> 75%	1
	10-75%	2
	< 10%	3
Nuclear polymorphism	Small, regular uniform cells	1
	Moderate increase and variability	2
	Marked variation	3
Mitotic figures (per 10 higher power fields)	0-5	1
	6-10	2
	>11	3

Grade	Total Score
1	3-5
2	6-7
3	8-9

Breast cancer is graded according to histological features; the score for each of these is then combined to give the final grade (Elston and Ellis, 1991; Bloom and Richardson, 1957).

Table 1.2 Staging criteria for breast cancer tumours

TNM class	Clinical feature
Tumour (T)	
Tx	Primary tumour cannot be measured
T0	Primary tumour cannot be found
T1	Tumour ≤ 2 cm
T2	Tumour > 2 cm but ≤ 5 cm
T3	Tumour > 5.0 cm
T4	Tumour of any size with direct extension to the chest wall or skin
Lymph Node (N)	
NX	Cancer in nearby lymph node cannot be measured
N0	No regional lymph node metastasis
N1	Metastasis in movable ipsilateral axillary lymph node(s)
N2	Metastasis to ipsilateral axillary lymph node(s) fixed or matted
N3	Metastasis in ipsilateral infraclavicular lymph nodes with or without axillary lymph node involvement
Metastasis (M)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis present

Stage grouping	
Stage 1	T1 N0 M0 T0 N1 M0
Stage 2A	T1 N1 M0 T2 N0 M0
Stage 2B	T2 N1 M0 T3 N0 M0
Stage 3A	T0 N2 M0 T1 N2 M0 T2 N2 M0 T3 N1-2 M0
Stage 3B	T4 N0 M0 T4 N1 M0 T4 N2 M0
Stage 3C	Any T N3 M0
Stage 4	Any T Any N M1

Breast cancer is staged according to the tumour size (T), amount of invasion to lymph nodes (N) and surrounding tissues (M). Following TNM classification, these scores are categorised into stages 1-4 (Egner, 2010; Koh and Kim, 2019a).

breast, the tumour with the highest T category is used for classification (Koh and Kim, 2019b). Breast cancer is then categorised into four stages (1-4) depending on the TNM criteria (Egner, 2010; Koh and Kim, 2019b). Breast cancers with lower tumour stage are strongly associated with better overall survival as the risk of mortality significantly increases with metastatic breast cancer (Saadatmand *et al.*, 2015; Waks and Winer, 2019). Tumour staging provides valuable clinical guidance for control of the local tumour as well as to determine the benefit of systemic therapy (Harbeck *et al.*, 2019). Recently, there has been a significant change in the TNM staging system with the incorporation of biomarkers into the anatomic staging to create prognostic stages (Koh and Kim, 2019b; Amin *et al.*, 2017). The clinical prognostic stage combines the tumour grade, stage with the molecular subtype of breast cancer, whereby different prognostic stages are assigned to tumours of different molecular subtypes with the same anatomic stage as outlined in the 8th edition of the AJCC staging manual (Koh and Kim, 2019b; Amin *et al.*, 2017). As breast cancer is considered a heterogeneous disease with different molecular characteristics that indicate different prognoses, patterns of recurrence and sensitivities to available therapies, combining these parameters provides a more informed indication of prognosis and appropriate treatment strategies (Sims *et al.*, 2007; Amin *et al.*, 2017).

1.1.5 Subclassification of breast cancer

Breast cancer is a heterogeneous disease that has several distinctive histological and biological subtypes (Malhotra *et al.*, 2010; Sørlie *et al.*, 2003). The ability to distinguish between the various subtypes is vital as they each have different prognoses and treatment implications. Histologically, breast cancer is categorised into ductal and lobular types which are further divided into *in situ*, invasive and metastatic carcinomas (Malhotra *et al.*, 2010; Feng *et al.*, 2018). Breast cancer

can also be sub-classified according to a wide range of criteria, including cell type (e.g. apocrine carcinoma), amount, type and location of secretion (e.g. mucinous carcinoma), architectural features (e.g. papillary, tubular, and micropapillary carcinoma), and immunohistochemical profile (e.g. neuroendocrine carcinoma) (Makki, 2015). Rare forms of breast cancer include inflammatory, male and Phyllodes tumours which account for only 5% of all breast cancer subtypes (Feng *et al.*, 2018).

Ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS) are considered pre-invasive tumours, which develop inside normal ducts and lobules in the breast. Both DCIS and LCIS demonstrate a high potential to become invasive without treatment (Feng *et al.*, 2018), hence early treatment is important to prevent the development of tumour progression. Invasive carcinomas are characterised by invasion of the breast cancer cells to the surrounding stromal tissue outside of the normal breast lobules and ducts (Feng *et al.*, 2018). Invasive ductal carcinoma (IDC) is the most common type of breast cancer, which accounts for 80% of breast cancer types (Makki, 2015). Generally, IDCs reflect a wide range of morphological variation, such as tumour size, grade, relative proportion of tumour cell and stroma with variable amount of ductal differentiation (Makki, 2015; Weigelt, Geyer and Reis-Filho, 2010). Invasive lobular carcinoma (ILC) is the second most common type of breast cancer which constitutes 10-15% of all diagnosed breast tumours, which predominantly affects older women in their early 60s (Feng *et al.*, 2018; Weigelt, Geyer and Reis-Filho, 2010). ILC is characterised by typically round, small, relatively uniform, and non-cohesive tumour cells which have a characteristic growth pattern with single-file infiltration of the stroma (Makki, 2015; Weigelt, Geyer and Reis-Filho, 2010). These invasive tumours, IDC and ILC, have the potential to spread to other sites of the body,

such as the lymph nodes or other organs thus entering the classification of metastatic breast cancer (Feng *et al.*, 2018).

Metastatic breast cancers are late stage breast cancers, which can be found in lymph nodes in the armpit, and/or in distant sites such as the lung, liver, bone and brain (Friberg and Nystrom, 2015). Following primary tumour excision, microscopic tumour cells or micro-metastases may remain in the body, which allows the cancer to return and disseminate (Friberg and Nystrom, 2015). Unfortunately, 6-7% of breast cancer are only detected at stage 4, whilst approximately 30% of women diagnosed with early-stage breast cancer will develop a metastatic form of the disease (Saadatmand *et al.*, 2015; Feng *et al.*, 2018). Breast cancer metastasis is the leading cause of breast cancer mortality (Caswell-Jin *et al.*, 2018). Therefore, early detection of breast cancer as well as understanding the mechanisms by which primary tumours undergo metastasis is paramount in reducing metastatic breast cancer morbidities.

Molecular classification of breast cancer, which reflects the biological diversity of tumour cells, allows clinicians to identify appropriate treatment plans (Normanno *et al.*, 2009). These molecular breast cancer subtypes are defined by the status of oestrogen receptor (ER), progesterone receptor (PR) and overexpression/amplification of human epidermal growth factor receptor 2 (HER2) (Kondov *et al.*, 2018). Additionally, the cell proliferation marker (Ki67) is used to determine the prognosis of breast cancer patients (Sims *et al.*, 2007). Molecular profiling has led to the identification of four intrinsic molecular subtypes; luminal A, luminal B, HER2+ and triple negative breast cancer (TNBC; ER-/PR-/HER2-) which represent biologically distinct disease entities (Perou *et al.*, 2000; Sørlie *et al.*, 2003).

1.2 Prognosis and current treatment strategies

1.2.1 Prognostic factors for breast cancer

Prognostic indicators for breast cancer include age, tumour grade, tumour stage and the molecular subtype (Harbeck *et al.*, 2019). Breast cancer in younger patients, before the age of 35, a rare (< 5%) event, is more frequently associated with an increased familial risk and lower survival rates (Tao *et al.*, 2019). Breast tumours are categorised based on several factors to provide prognosis including histological grade, tumour stage, histological type and the molecular subtype (Koh and Kim, 2019b). Each of these factors are combined in the pathological assessment by clinicians to provide a clearer prognosis for patients using scoring systems such as the Nottingham Prognostic Index, adopted in the UK, Europe and Australia, and Adjuvant, commonly used in the US (Hearne *et al.*, 2015). These validated algorithms render good general estimates of patient prognosis and can aid in discussing therapy options with patients by providing numerical estimates of treatment efficacy (Phung, Tin Tin and Elwood, 2019; Lipkus *et al.*, 2010). With advances in screening programmes the stage at diagnosis has significantly decreased and therefore prognostication is increasingly dependent on tumour biology; histological type, grade and molecular subtype (Harbeck *et al.*, 2019).

The intrinsic subtypes are considered to be the most important criteria for treatment decisions (Harbeck *et al.*, 2019). These molecular subtypes display significant differences in disease-free survival with the basal-like/triple-negative subtype having the poorest prognosis (Prat *et al.*, 2015). HER2+ breast tumours more frequently display grade 2 to 3, medium to high proliferation, larger tumours and are associated with poor prognosis (Leone *et al.*, 2019). Molecular classification has allowed the stratification of ER+ tumours into the two distinct

subtypes; luminal A and luminal B, which have very different clinical outcomes, whereby luminal B tumours typically display high grade and proliferation with shorter survival rates (Ahn *et al.*, 2015; Prat *et al.*, 2015).

1.2.2 Current treatment for breast cancer

The appropriate breast cancer treatment strategy is selected based on the histological, clinicopathological features and the molecular subtype (Ehinger *et al.*, 2017). Treatment strategies for breast cancer include surgery, radiation and systemic therapies used singly or in combination (Nounou *et al.*, 2015). Systemic therapy includes the use of chemotherapy, endocrine therapy for hormone receptor-positive disease, chemotherapy, anti-HER2 therapy for HER2-positive disease and poly (ADP-ribose) polymerase (PARP) inhibitors for *BRCA1/2* mutation carriers (Harbeck *et al.*, 2019).

1.2.2.1 Surgery

Surgical management of breast cancer is a critical treatment modality, which includes lumpectomy, where just the tumour and some surrounding breast tissue is removed, or a mastectomy, where the whole breast is removed (Guarneri and Conte, 2004). There has been profound advances in oncoplastic surgical techniques to improve breast conservation in many clinical situations that had earlier led to primary mastectomy (Haloua *et al.*, 2013). Depending on the tumour burden surgery can also be preceded by neoadjuvant therapy, such as radiotherapy, to shrink the tumour and enhance breast conservation (Franceschini *et al.*, 2015). Surgery is usually followed by adjuvant therapy to target residual cancer cells that are not removed during surgery to ensure full recovery and minimize the risk of metastases (Dhankhar *et al.*, 2010).

1.2.2.2 Radiotherapy

Radiation therapy improves disease-free and overall survival for patients with early breast cancer with lymph node involvement (Darby *et al.*, 2011). This treatment modality is mediated by irreparable damage to the DNA of malignant cells resulting in cell death (Harris *et al.*, 2008). Additionally, radiotherapy augments the antitumoral immune response to target both primary tumours and distant sites of metastatic disease through the release of tumour antigens and cytokines into the tumour microenvironment (Harris *et al.*, 2008). Hence, radiotherapy plays a crucial role in the management of advanced metastatic breast cancer, alleviating symptoms from bone, brain and soft tissue metastases (Jutzy *et al.*, 2018).

1.3.3 Systemic therapy

Systemic therapies, both chemotherapy and targeted approaches, are highly effective particularly for early breast cancer in significantly reducing breast cancer mortality (Prat *et al.*, 2015). The appropriate treatment strategy is dependent on the molecular subtype, tumour burden and the risk of recurrence (Albain *et al.*, 2012; Tong *et al.*, 2018). Standard chemotherapy regimens include an anthracycline, such as Doxirubicin and a taxane, such as Cyclophosphamide given preferentially in sequence with care to avoid excessive toxicity (Von Minckwitz *et al.*, 2012). The clinical benefit for chemotherapy is particularly significant for TNBC and is also recommended for HER2-positive breast cancers, and in high-risk luminal tumours, depending on the individual risk of recurrence (Albain *et al.*, 2012). Overall, chemotherapy regimens reduce breast cancer mortality risk by one-third (Albain *et al.*, 2012). However, chemotherapy induces cytotoxic and genotoxic side effects due to non-specific damage to cells therefore,

there has been a massive effort to develop more advanced treatments with fewer side effects.

There has been a significant emergence of various novel targeted therapies for the molecular subtypes of breast cancer (Tong *et al.*, 2018). For example, for hormone receptor positive breast cancer endocrine therapy is the mainstay for treatment, which works by blocking the effects of hormone, such as tamoxifen, or lowering hormone levels, such as aromatase inhibitors (Waks and Winer, 2019). Since endocrine therapies utilise different mechanisms of action these are often used in combination to increase efficacy and reduce drug resistance (Boehnke Michaud, Jones and Buzdar, 2001). Novel agents, such as Pictilisib, are in development to reverse tumour-resistance to endocrine therapies, which can be induced by tumour cells through the upregulation of other signalling pathways (Schöffski *et al.*, 2018). The targeted therapies currently in clinical use are herein discussed further according to the molecular subtype of breast cancer.

1.3 Breast cancer molecular subtypes

1.3.1 Luminal breast cancer

Luminal A breast cancers are characterized by high expression of luminal epithelial genes, low expression of Ki-67 and a distinct methylation profile of more than 40 genes (Perou *et al.*, 2000). Luminal B breast cancers are characterized by higher Ki-67 and lower expression of several luminal-related genes (such as *ESR1* or *FOXA1*), genomic instability and a higher frequency of *TP53* gene mutations (Sørlie *et al.*, 2003; Creighton, 2012). Luminal B tumours are associated with a worse prognosis and a higher risk of relapse than luminal A breast cancers (Ahn *et al.*, 2015). Both luminal A and luminal B are characterised by the expression of the hormone receptors ER and PR, whilst a proportion of

luminal B tumours also express HER2 (Kondov *et al.*, 2018). During normal breast development, the hormones oestrogen and progesterone are largely responsible for coordinating the changes that occur in women through the activation of various transcription factors responsible for cell differentiation and growth (Sreekumar, Roarty and Rosen, 2015). However, constitutive activation of oestrogen signalling pathways along with acquired mutations of oncogenes leads to the uncontrolled proliferation of mammary epithelial cells and the development of malignant tumours (Feitelson *et al.*, 2015).

Oestrogenic signalling is mediated by ligand binding to ERs which dimerise and translocate to the nucleus where they bind to ER elements (EREs) to promote the activation of target genes (Osborne *et al.*, 2001) (Figure 1.2). There are two main isoforms of ERs; ERα and ERβ, which have different transcriptional activity (Huang *et al.*, 2014). ERα specifically has been demonstrated to be up-regulated in 75% of breast cancer cases (Nadji *et al.*, 2005). Conversely, normal breast tissue typically expresses ERβ and the level of expression has been demonstrated to decline with the progression of breast tumours (Huang *et al.*, 2014). ERα is a transcription factor for genes associated with cell survival, proliferation, and tumour growth, including insulin-like growth factor-1 receptor (IGF1-R), cyclin D1, anti-apoptotic BCL-2 protein, vascular endothelial growth factor (VEGF) (Lipovka and Konhilas, 2016). On the other hand, ERβ has been found to be an important modulator of proliferation, as well as an inhibitor of cell motility and invasion in breast cancer cells (Lazennec *et al.*, 2001). Hence, the loss of ERβ expression has been suggested to be a leading event to the development of hormone positive breast cancer.

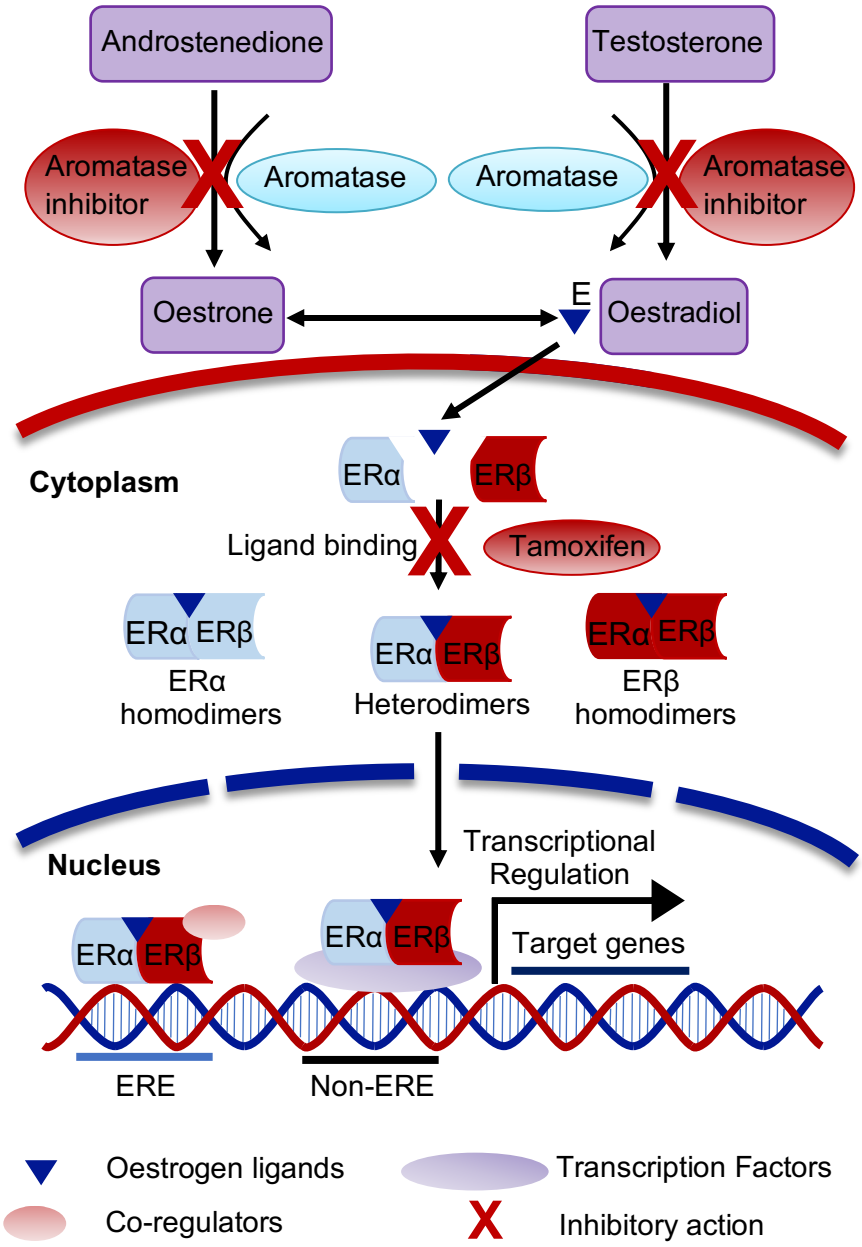

Figure 1.2 Oestrogen receptor (ER) signalling pathway and mechanism of action of aromatase inhibitors and tamoxifen. Breast cancer cells have relatively high ERα expression and low ERβ expression. These two types of nuclear hormone receptors form homo- or heterodimers upon ligand binding. These ER dimers translocate into the cell nucleus where they bind to the ER response element (ERE) of target genes and recruit co-regulators to achieve the regulation of transcriptional activity. Targeted therapy for hormone receptor positive breast cancer includes Tamoxifen which prevents the binding of oestrogens to the ERs hence inhibiting the transcription of ER target genes. Aromatase inhibitors prevent the production of oestrogens by inhibiting the aromatase enzyme complex from converting androstenedione and testosterone to oestrogens such as oestrone and oestradiol. Adapted from Johnston and Dowsett (2003) and Feng *et al.* (2018).

Targeted therapy for ER+ breast cancer includes tamoxifen, an ER antagonist, that prevents oestrogen mediated transcriptional activation of proliferative and anti-apoptotic genes (Shagufta and Ahmad, 2018; Johnston and Dowsett, 2003) (Figure 1.2). Intratumoural oestrogen levels can also be increased by tumour cells which activate stromal fibroblasts to express aromatase, a key enzyme in oestrogen biosynthesis, which enhances oestrogenic signalling (Yamaguchi, 2007). Aromatase inhibitors prevent the conversion of androgens, such as testosterone, to oestrogen, and as a result reduces oestrogen production and ER activation (Johnston and Dowsett, 2003) (Figure 1.2). Clinical use of these targeted endocrine therapies has significantly reduced mortality rates, by about 25-30% (Haque and Desai, 2019). Despite this, not all ER-positive breast tumours respond to endocrine therapy, and even those that are initially responsive eventually become resistant as the disease progresses (Louie and Sevigny, 2017).

Emerging evidence suggests signalling through growth factor pathways contributes to endocrine therapy resistance by ligand-independent activation of ER (Osborne *et al.*, 2001). ER+ breast tumours which also overexpress growth factors such as HER2 and EGFR have been demonstrated to be more likely to become resistant to hormone therapy (Yang, Barnes and Kumar, 2004; Yamaguchi, 2007). Nuclear ERα has several different phosphorylation sites such as Ser118 and Ser167 for kinases such as MAPK and PI3K/Akt which are HER2 downstream signalling molecules (Le Goff *et al.*, 1994). Clinical trials with the use of PI3K/AKT inhibitors combined with aromatase inhibitors revealed promising results for ER+/HER2+ tumours with PI3K/AKT hyperactivation (Lee, Loh and Yap, 2015). Therefore, targeting growth factor pathways, in addition to ER, is a

developing strategy to prevent the development of resistance to endocrine therapy.

1.3.2 HER2-positive breast cancer

HER2+ breast cancer is characterised by the absence of ER and PR expression, *HER2* amplification and the low expression of luminal and basal clusters (Kondov *et al.*, 2018; Perou *et al.*, 2000). HER2 (also known as ERBB2) is a member of the epidermal growth factor (EGFR) family of receptor tyrosine kinases (RTK) including HER1, HER3 and HER4 (Yarden and Pines, 2012). Activation of HER2 signalling occurs through homo- or heterodimerization with other receptors from the EGFR family, which results in the autophosphorylation of tyrosine residues within the cytoplasmic domain of the receptor and initiates a variety of signalling pathways, such as mitogen-activated protein kinase (MAPK) and PI3K activation (Figure 1.3) (Iqbal and Iqbal, 2014).

HER2 signalling is an important mediator of cell proliferation and differentiation during normal breast development. However, *HER2* amplification leads to overexpression of the receptor which is associated with the development of HER2+ breast cancer. When overexpressed, HER2 exists in a constitutively open conformation, leaving it intrinsically capable of interacting with available RTK binding partners even in the absence of ligand (Cho *et al.*, 2003). The HER2-HER3 heterodimer is the most potent stimulator of downstream signalling pathways resulting in increased cell proliferation, survival, differentiation, angiogenesis, and invasion of the breast cancer cells (Holbro *et al.*, 2003; Iqbal and Iqbal, 2014).

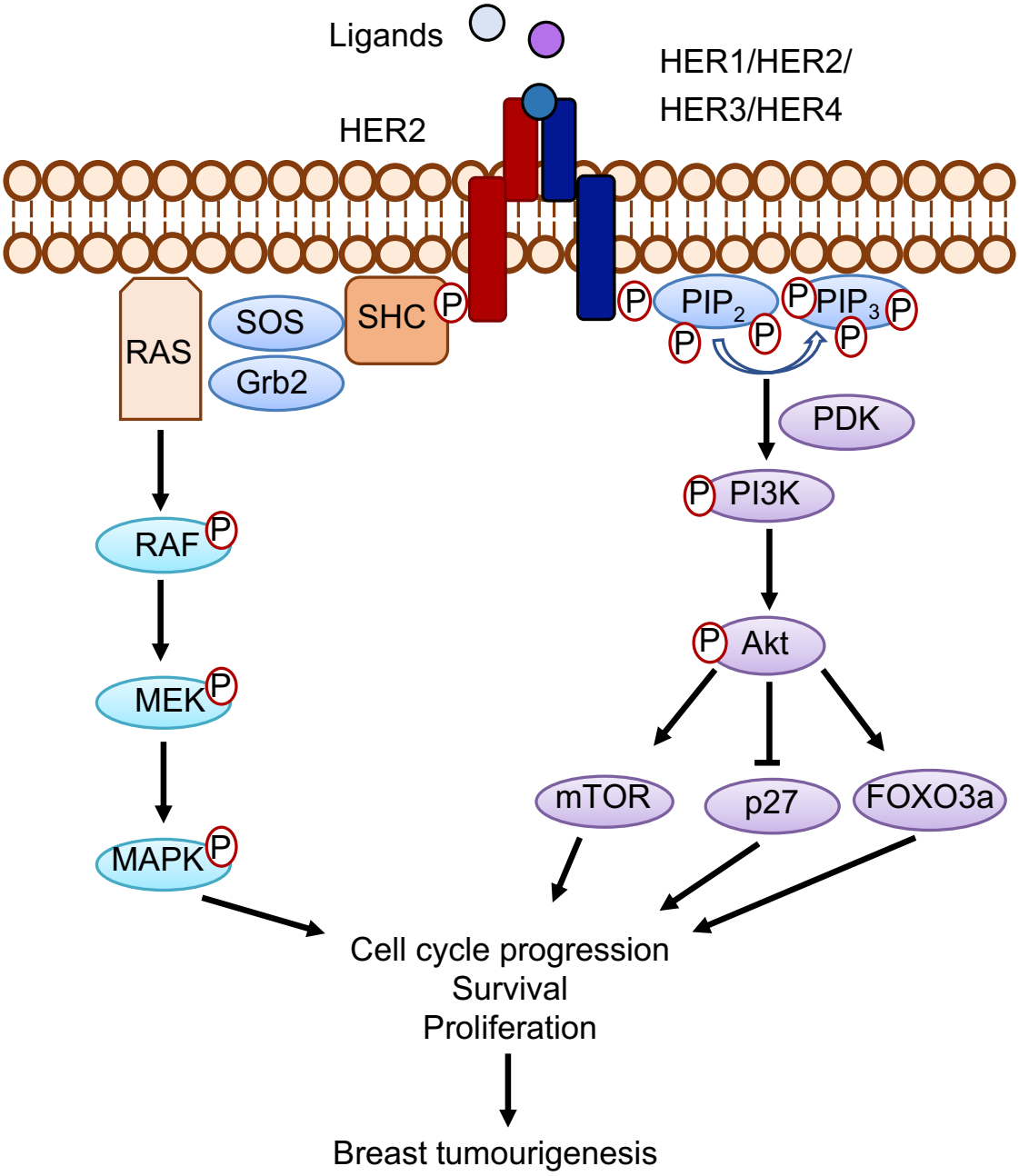

Figure 1.3 HER2 signalling pathway. HER2 is located on the cell membrane which dimerises with members of the epidermal growth factor (EGFR) family of receptor tyrosine kinases (RTK) including HER1, HER3 and HER4 (Yarden and Pines, 2012). Upon ligand binding and receptor homo- or hetero-dimerization phosphorylation of the tyrosine kinase domain in the cytoplasm initiates downstream oncogenic signalling pathways such as phosphoinositide-3 kinase (PI3K)/serine/threonine-specific protein kinase (Akt) pathway and the Ras/ the mitogen-activated protein kinase (MAPK) pathway leading to cell cycle progression and proliferation (Feng *et al.*, 2018). Grb2, growth factor receptor-bound 2; mTOR, mammalian target of rapamycin; Nrf2, nuclear factor (erythroid-derived 2)-like-2; PDK, phosphoinositide-dependent protein kinase; PIP_2, phosphatidylinositol (4,5) bisphosphate; PIP_3, phosphatidylinositol (3,4,5) trisphosphate; FOXO3a, forkhead box O3; Shc, Src homology 2 domain containing transforming protein; SOS, son of sevenless.

HER2+ breast cancer accounts for 15-20% of all diagnosed cases and is associated with a more aggressive disease, higher recurrence rate and shorter disease-free survival (Slamon *et al.*, 1987; Iqbal and Iqbal, 2014). HER2 status of15-20% by immunohistochemistry (IHC) or an amplified *HER2* gene copy number by fluorescence *in situ* hybridisation (FISH), identifies women that are likely to respond to HER2 targeted therapies (Wolff *et al.*, 2013), such as trastuzumab (Kast *et al.*, 2017). Trastuzumab is a monoclonal antibody which consists of two antigen-specific sites that bind to the juxtamembrane portion of the extracellular domain preventing the dimerization and activation of HER2 (Vu and Claret, 2012). This results in the inhibition of the downstream MAPK and PI3K signalling cascades, which induces the suppression of cell growth and proliferation (Vu and Claret, 2012). Pertuzamab, which inhibits the dimerization of HER2 and HER3 receptors, is also used in combination with trastuzumab for HER2+ breast cancer to enhance the inhibition of HER2 signalling (Capelan *et al.*, 2013). Such novel targeted treatments have greatly improved the prognoses of patients with HER2+ metastatic breast cancer (Kast *et al.*, 2017). However, multiple mechanisms of resistance have been demonstrated to decrease the efficacy of targeted therapies, such as the truncation or masking of the extracellular domain of HER2, which inhibit the binding of monoclonal antibodies (Vu and Claret, 2012). Increased expression of insulin-like growth factor 1 receptor (IGF-1R) has also been implicated in trastuzumab resistance (Vu and Claret, 2012). The up-regulation of IGF-1R was found to promote resistance in trastuzumab-sensitive SKBR3, HER2+ breast cancer cells, through the down regulation of the cell cycle inhibitor $p27^{Kip1}$, whilst the addition of IGF-binding protein-3 (IGFBP-3) restored trastuzumab-induced growth inhibition (Lu *et al.*, 2001). Hence, understanding the molecular mechanisms by which tumour cells

confer resistance to therapy may reveal novel promising targets to enhance therapeutic benefit and improve prognosis further.

1.3.3 Triple-negative breast cancer

TNBC is characterised by tumours that lack the expression of ER and PR, the absence of HER2 overexpression, and high expression of Ki-67 (Kondov *et al.*, 2018). TNBC accounts for 10-20% of diagnosed breast cancers, frequently affects younger women and is more prevalent in African-American women (Lehmann *et al.*, 2011). At diagnosis, TNBC tumours are more likely to be T2 or T3, to have already metastasised to the lymph nodes and are more biologically aggressive than other subtypes of breast cancer (Dent *et al.*, 2007). TNBCs are less likely to be detected by screening methods such as mammography which has been suggested to be due to rapid growth rate or higher breast tissue density of women with TNBC leading to late detection (Dent *et al.*, 2007). Currently, TNBC patients have the worst prognosis compared with other breast cancer subtypes as there is a lack of specific therapy available for their treatment as there is for hormone receptor and HER2+ positive breast cancer.

The major issue for the development of targeted therapy against TNBC is the lack of specific oncogene drivers due to wide heterogeneity for this subtype (Shah *et al.*, 2012). Molecular profiling for TNBC has identified oncogenic drivers associated with TNBC such as the expression of HER1/EGFR in approximately 50% of cases and *BRCA1/2* mutations in up to 20% of cases (Nakai, Hung and Yamaguchi, 2016; Gonzalez-Angulo *et al.*, 2011). Of the TNBC subtypes approximately 70% are classified as basal-like tumours which demonstrate the worst prognosis of this subtype (Dawson, Provenzano and Caldas, 2009; Bertucci *et al.*, 2008). Basal-like tumours are characterised by the expression of distinctive genes of the epithelial cells in the basal or outer layer of the mammary gland such

as cytokeratin 5 and 17 and EGFR (Lehmann *et al.*, 2011). Upregulation of basal markers such as cytokeratin 5 and 17 leads to the deregulation of genes involved in the cell cycle, DNA damage response pathways and alteration of growth factor signalling, leading to increased cell proliferation and tumour progression (Dai *et al.*, 2015).

Currently, radiation therapy and chemotherapy using cytotoxic agents are typically used for TNBC management (Lebert *et al.*, 2018). Neoadjuvant chemotherapy for localised early-stage TNBC results in higher rates of pathological complete response compared with hormone-positive breast cancer (28% vs. 6.7%) (Von Minckwitz *et al.*, 2012). In particular, patients with defective DNA repair mechanisms such as those with *BRCA1/BRCA2* mutations, are highly responsive to chemotherapy (Lebert *et al.*, 2018). PARP inhibitors offer targeted therapy for tumours with *BRCA1/BRCA2* mutations, which interferes with the repair of single-stranded DNA breaks leading to cancer cell apoptosis (Rouleau *et al.*, 2010). Although TNBC shows favourable clinical response to chemotherapy overall survival rate is lower compared with the other breast cancer subtypes, which is largely attributed to a more biologically aggressive disease (Wahba and El-Hadaad, 2015). EGFR signalling in TNBC has also been indicated to play an important role in tumour progression, however results of clinical studies of EGFR-targeted therapy in breast cancer have been disappointing (Masuda *et al.*, 2012). Resistance to EGFR-targeted therapies has been suggested to be promoted by the upregulation of down-stream signalling independent of EGFR activation (Nakai, Hung and Yamaguchi, 2016). Perturbed growth factor signalling in breast cancer is largely attributed to altered cell metabolism (DeBerardinis and Chandel, 2016), elucidating the involvement of metabolic proteins in growth factor signalling may identify novel therapeutic targets which this thesis aims to explore further.

1.4 Tumour metabolism re-programming

Altered cellular metabolism is a fundamental adaptation of cancer during disease development (DeBerardinis and Chandel, 2016). Many metabolic pathways have been reported to be dysregulated in breast cancer leading to metabolite addiction, such as dependency on glutamine for tumour growth, which can be exploited in cancer therapy (El Ansari *et al.*, 2018; Long, Li and Zhang, 2016). This reprogramming of cell metabolism in breast cancer is initiated by the activation of oncogenes such as *c-Myc* which enhances glutaminolysis or the inactivation of tumour suppressor genes such as *p53* which suppresses glycolysis (Dang, 2009). TNBC and HER2+ subtypes typically overexpress *c-Myc* whilst ER+ breast tumours display increased fatty acid metabolism which is mediated by the upregulation of sterol response element binding protein 1 (SREBP1) (Xu, Chen and Olopade, 2010; Chen, Brown and Russo, 2009). Hence, genetic modulation in tumour cells gives rise to differential metabolic dependence between subtypes of breast cancer. Understanding the altered metabolic dependencies in tumour cells and thus identifying targetable metabolic vulnerabilities provides a novel approach for targeted therapy (Long, Li and Zhang, 2016).

Despite early preconception that cancer cells completely bypass the tricarboxylic acid (TCA) cycle and primarily utilize aerobic glycolysis, substantial evidence demonstrates that tumour cells, particularly those with dysregulated oncogene and tumour suppressor expression, also rely heavily on the TCA cycle which is supported by functional mitochondrial activity in tumour cells (Anderson *et al.*, 2018). The TCA cycle is comprised of a series of biochemical reactions occurring in the mitochondria, which provides energy, macromolecules, and redox balance to the cell (Anderson *et al.*, 2018). In the presence of oxygen, differentiated non-proliferating cells metabolise glucose to pyruvate via glycolysis, pyruvate is then

converted to acetyl-Coenzyme A (Acetyl-coA) in the mitochondrion which is catabolised by the TCA cycle and then enters oxidative phosphorylation (OXPHOS), which leads to the net production of 36 ATPs compared with 2 ATPs generated by glycolysis alone (Zheng, 2012). However, Warburg, Wind and Negelein (1927) notably observed tumour tissue to exhibit high lactate production even in the presence of adequate oxygen despite aerobic glycolysis to produce less ATP than OXPHOS (Figure 1.4).

Lactate secreted into the tumour microenvironment as a result of increased aerobic glycolysis acidifies the extracellular space which increases activation of vascular endothelial growth factor (VEGF), which supports tumour angiogenesis, and hypoxia-inducible factor 1 (HIF1) even under normoxia (Giatromanolaki *et al.*, 2006; Doherty and Cleveland, 2013). HIF1 activation by lactate acts as a positive feedback loop further enhancing tumour cell dependency on aerobic glycolysis (Mishra and Ambs, 2015). Elevated levels of ATP synthesised by OXPHOS inhibits phosphofructokinase 1 (PFK1), the rate-limiting enzyme in glycolysis, which suppresses glycolysis (Zheng, 2012). Hence, exceedingly high levels of ATP by OXPHOS is unfavourable for cancer cell growth. Additionally, although glycolysis yields less ATP than OXPHOS, the rate of ATP generation by glycolysis is higher than by OXPHOS, which is more suited to the energy demands of proliferative malignant cells (Pfeiffer, Schuster and Bonhoeffer, 2001). Enhanced glycolysis supplies tumour cells with biochemical precursors required for the synthesis of larger molecules such as lipids, proteins, and nucleic acids (Heiden, Cantley and Thompson, 2009). For example, glucose-6-phosphate fuels the pentose phosphate pathway to synthesise nucleic acids (Patra and Hay, 2014). Also, 3-phosphoglycerate in glycolysis feeds into the serine synthesis pathway,

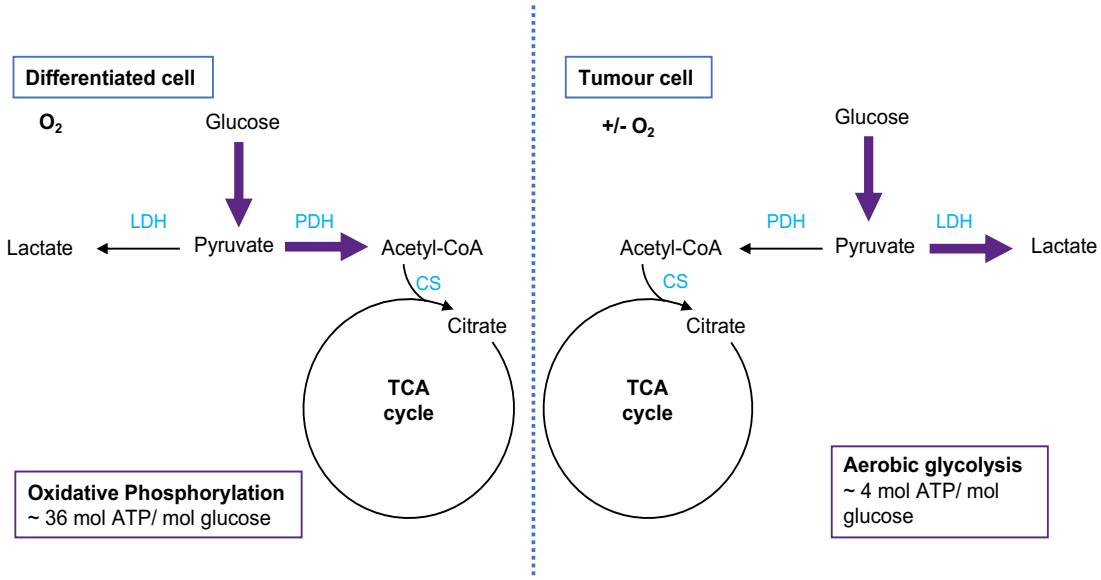

Figure 1.4 Schematic representation of oxidative phosphorylation and aerobic glycolysis in normal and tumour cells. Normal differentiated cells under aerobic conditions first metabolise glucose to pyruvate which then undergoes oxidative phosphorylation in the mitochondrion. As oxygen is required as the final electron acceptor to oxidise glucose, under hypoxic conditions cells are unable to undergo oxidative phosphorylation and lactate is generated in the cytosol (anaerobic glycolysis). Anaerobic glycolysis allows the recycling of NAD+ to NADH to allow glycolysis to continue (Zheng, 2012). In contrast, cancer cells heavily rely on glycolysis and divert most of the pyruvate to lactate production both under normoxia and hypoxia, despite lower ATP production compared with oxidative phosphorylation (Pfeiffer, Schuster and Bonhoeffer, 2001). Acetyl-coA, acetyl-coenzyme A; ATP, adenosine triphosphate; CS, citrate synthase; LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; TCA cycle, tricarboxylic acid cycle.

which metabolises glycine (Yang and Vousden, 2016), which goes into the one carbon pathway to produce nucleotides and proteins.

1.5 Isocitrate dehydrogenase enzymes

The isocitrate dehydrogenase (IDH) enzymes are responsible for the oxidative decarboxylation of isocitrate to produce α-ketoglutarate and CO_2 (Figure 1.5A) (Stoddard, Dean and Koshland, 1993). This reaction occurs in a two-step process which involves the oxidation of isocitrate to the intermediate oxalosuccinate and the reduction of $NAD(P)^+$ to NAD(P)H (Hurley *et al.*, 1991). This is followed by the decarboxylation of the carboxyl group beta, forming α-ketoglutarate and CO_2. The IDH enzymes exist in three isoforms, IDH1 is localised in the cytoplasm whilst IDH2 and IDH3 are mitochondrial enzymes. The IDH1 and IDH2 enzymes are homodimeric proteins with 70% structural homology which use NADP+ as a cofactor whilst IDH3 is heterotetrametric (IDH3A, IDH3B, and IDH3G) and NAD^+ dependant (Figure 1.5) (Ramachandran and Colman, 1980). IDH3 is the primary isoform involved in the TCA cycle where it catalyses the irreversible conversion of isocitrate to α-ketoglutarate while reducing NAD+ to NADH (Figure 1.5C). This isoform is regulated by substrate availability; ADP and citrate activate IDH3 whilst ATP, NADH and NADPH inhibit its activity (Gabriel, Zervos and Plaut, 1986). α-Ketoglutarate produced by IDH3 is further metabolized to succinate in the TCA cycle, and the NADH is used during OXPHOS to generate ATP (Barnes, Kuehn and Atkinson, 1971).

Similarly, in the mitochondria isocitrate can also be reversibly catalysed to α-ketoglutarate by IDH2 as part of the TCA cycle, to produce NADPH, which is utilised to reduce glutathione (GSH) that participates in the defence against

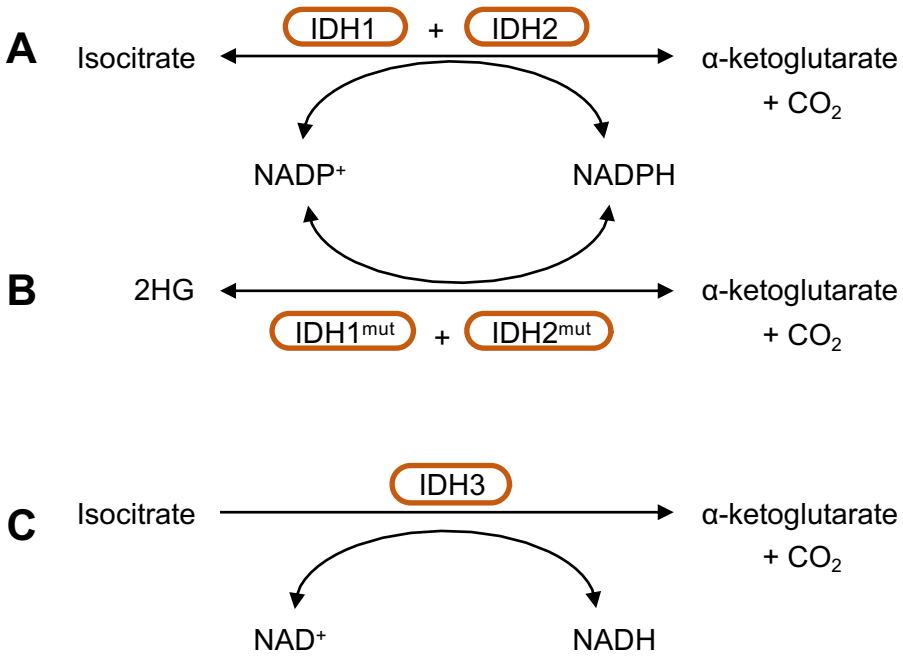

Figure 1.5. Metabolic reactions of the isocitrate dehydrogenase enzyme isoforms. (**A**) IDH1 and IDH2 catalyse the reversible decarboxylation of isocitrate and α-ketoglutarate using NADP+ as a cofactor (**B**) *IDH1* and *IDH2* mutation leads to neomorphic activity to convert α-ketoglutarate to 2-hydroxyglutarate (2HG) (**C**) IDH3 catalyses the irreversible conversion of isocitrate to α-ketoglutarate while reducing NAD+ to NADH (Stoddard, Dean and Koshland, 1993; Gabriel, Zervos and Plaut, 1986; Bhagavan *et al.*, 2015).

reactive oxygen species (ROS) and repair of mitochondrial oxidative damage (Calvert *et al.*, 2017; Lu and Holmgren, 2014). This has been evidenced in glioma cells whereby *IDH*-mutant cells were found to have increased ROS levels and reduced GSH (Shi *et al.*, 2015). IDH1 reversible oxidative carboxylation maintains levels of α-ketoglutarate in the cytosol which can be shuttled to the mitochondria via the membrane transporter SLC25A11 (Kabe *et al.*, 2006). *IDH1/2* mutations have been found to result in neomorphic activity to metabolize α-ketoglutarate to form the oncometabolite R-2-hydroxyglutarate (2HG) in gliomas (Figure 1.5B) (Dang *et al.*, 2009). In gliomas, R132H is the most common *IDH1* mutation, and R172K is the most common *IDH2* mutation (Yan *et al.*, 2009). However, mutations in *IDH3* do not play a role in cancer as unlike IDH1 or IDH2, IDH3 does not catalyse the reverse reductive carboxylation reaction of α-ketoglutarate to isocitrate (Krell *et al.*, 2011). Therefore, for IDH3 the gain of function effect observed with *IDH1/2* mutations which leads to the production of 2HG is not applicable. Moreover, loss of function of IDH3 disrupts the TCA cycle and has a detrimental effect on cell growth causing the cell to apoptose rather than to proliferate and become malignant (Al-Khallaf, 2017).

In *IDH1/2* mutant gliomas, 2HG accumulates and inhibits α-ketoglutarate-dependent chromatin-modifying enzymes, including histone demethylases and the ten-eleven translocation (TET) family of 5-methlycytosine (5mC) hydroxylases (Noushmehr *et al.*, 2010). This oncometabolite instigates epigenetic modulation of proto-oncogenes leading to carcinogenesis in glioblastomas (McBrayer *et al.*, 2018). 2HG levels have been found to be increased 100-fold in breast tumours compared with adjacent noncancerous tissue (Terunuma *et al.*, 2014). In contrast to gliomas, there is little evidence of *IDH1* mutations in breast cancer, with only

one reported case discussed in the literature (Raynaud *et al.*, 2010; Kim *et al.*, 2014). Instead, *Myc*-mediated 2HG production has been reported as the key instigator of 2HG accumulation induced by increased glutaminase (GLS1) expression (Terunuma *et al.*, 2014). Levels of 2HG were found to be significantly increased in ER- tumours and breast cancer cell lines (Raynaud *et al.*, 2010). Interestingly, the only case of *IDH1* mutation was found to be reported in ER+ breast cancer (Kim *et al.*, 2014) indicating *IDH1* mutations to be a rare event in breast cancer. Wild-type *IDH2* and to a lesser extent *IDH1* were found to induce 2HG production in TNBC MDA-MB-231 cells (Smolková *et al.*, 2015). Therefore, increased expression of the IDH enzymes, in the absence of *IDH* mutations in breast cancer, can be proposed to play an important role in breast cancer progression given the oncogenic role of the 2HG metabolite. The expression of the IDH1 and IDH2 enzymes will be assessed in human breast cancer tissue in chapter 3.

1.6 Phosphoglycerate dehydrogenase

Phosphoglycerate dehydrogenase (PHGDH) is the first enzyme branching from glycolysis in the first committed step of the three-step serine biosynthesis pathway (Figure 1.6) (Snell, 1985). PHGDH catalyses the oxidation of the glycolytic intermediate 3-phosphoglycerate (3PG) to 3-phosphohydroxypyruvate (3PHP) using NAD+ as a cofactor (Achouri *et al.*, 1997). Phosphoserine aminotransferase (PSAT) then transaminates 3-PHP to phosphoserine (pSer) which is dephosphorylated to serine by phosphoserine phosphatase (PSPH) (Snell, 1985) (Figure 1.6A). The PHGDH reaction is reversible which under normal physiological conditions, the direction from 3PHP to 3PG is thermodynamically favoured (Fan *et al.*, 2015).

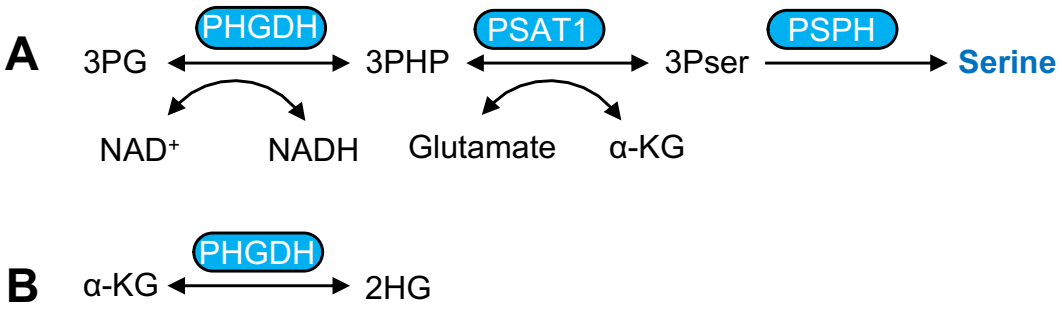

Figure 1.6. Serine biosynthetic metabolic pathways and neomorphic function of phosphoglycerate dehydrogenase (PHGDH). (**A**) Serine is synthesized from the glycolytic intermediate (3PG) by the sequential enzymatic activity of PHGDH, PSAT1, and PSPH. (**B**) In tumour cells PHGDH is proposed to display neomorphic metabolism of α-ketoglutarate (α-KG), which is a structural analogue of 3PHP to the oncometabolite 2-hydroxyglutarate (2HG) (Fan *et al.*, 2015). 3PG, 3-phosphoglycerate; 3PHP, 3-phosphohydroxypyruvate; Pser, phosphoserine; PSAT1, phosphoserine aminotransferase1; PSPH, phosphoserine phosphatase.

PHGDH is frequently amplified in breast tumours and melanomas (Possemato *et al.*, 2011; Locasale *et al.*, 2011). However, the mechanism by which *PHGDH* amplification supports cancer growth has not yet been fully elucidated. The simplest hypothesis is that it increases *de novo* serine synthesis which is a critical precursor for the biosynthesis of nucleotides and protein necessary for cell growth (Mattaini, Sullivan and Heiden, 2016). However, *PHGDH* knockdown does not significantly reduce the intracellular concentration of serine, and the growth inhibition caused by *PHGDH* knockdown cannot be rescued by the addition of exogenous serine (Chen *et al.*, 2013). Hence, increased PHGDH in breast cancer is proposed to play a functional role independent of its role in serine biosynthesis to promote tumour progression.

Possemato *et al.*, (2011) postulated that maintaining a replenished α-ketoglutarate pool in cancer cells is likely a key function of PHGDH as knockdown of this enzyme causes a drop in α-ketoglutarate levels. The transamination reaction catalysed by PSAT is downstream of PHGDH in the serine biosynthesis which converts glutamate to α-ketoglutarate hence elevated levels of PHGDH can drive this reaction. However, other studies have reported this flux of α-ketoglutarate to be relatively small compared with other sources such as IDH metabolism (Locasale *et al.*, 2011). Instead, Fan *et al.* (2015) have reported an additional enzymatic functional role for PHGDH beyond 3PG/3PHP oxidation/reduction whereby PHGDH was demonstrated to catalyse the reduction of α-ketoglutarate to 2HG in the *PHGDH*-amplified TNBC (MDA-MB-468) cell line (Figure 1.6B).

1.7 The human branched-chain aminotransferase proteins

Human branched-chain aminotransferases (hBCAT) are key metabolic enzymes that catalyse the reversible transamination of the essential branched-chain amino acids (BCAAs), leucine, isoleucine and valine to form their respective branched-chain α-ketoacids (BCKA) and glutamate (Ichihara and Koyama, 1966). Although the three BCAAs are often treated as one the hBCAT enzymes have substrate preferences of isoleucine ≥ leucine > valine ≥ glutamate (Hall *et al.*, 1993). Moreover, metabolite formation differs between the BCAAs whereby catabolism of leucine and isoleucine leads to the production of acetyl-coA whilst valine catabolism leads to the production of succinyl-coA (Figure 1.7).

The hBCAT proteins follow a 'ping-pong' reaction, whereby the enzyme and cofactor revert back to their initial state after release of the temporary amino group (Hall *et al.*, 1993; Berg, Tymoczko and Stryer, 2002). During the first half of the reaction, pyridoxal-5-phosphate (PLP), a vitamin B_6 co-factor, acts as a temporary acceptor of the α-amino group being donated from the BCAA and becomes pyridoxamine-5-phosphate (PMP) which releases the respective BCKA. The PMP-enzyme subsequently donates the amino group to α-ketoglutarate to produce glutamate restoring the PLP form of the enzyme (Berg, Tymoczko and Stryer, 2002).

The second reaction of BCAA metabolism is catalysed by the branched-chain α-keto acid dehydrogenase enzyme (BCKD) complex (Harris *et al.*, 1986). The BCKD complex catalyses the irreversible decarboxylation of the branched-chain amino ketoacids (BCKA), leading to the synthesis of branched-chain acyl-CoAs,

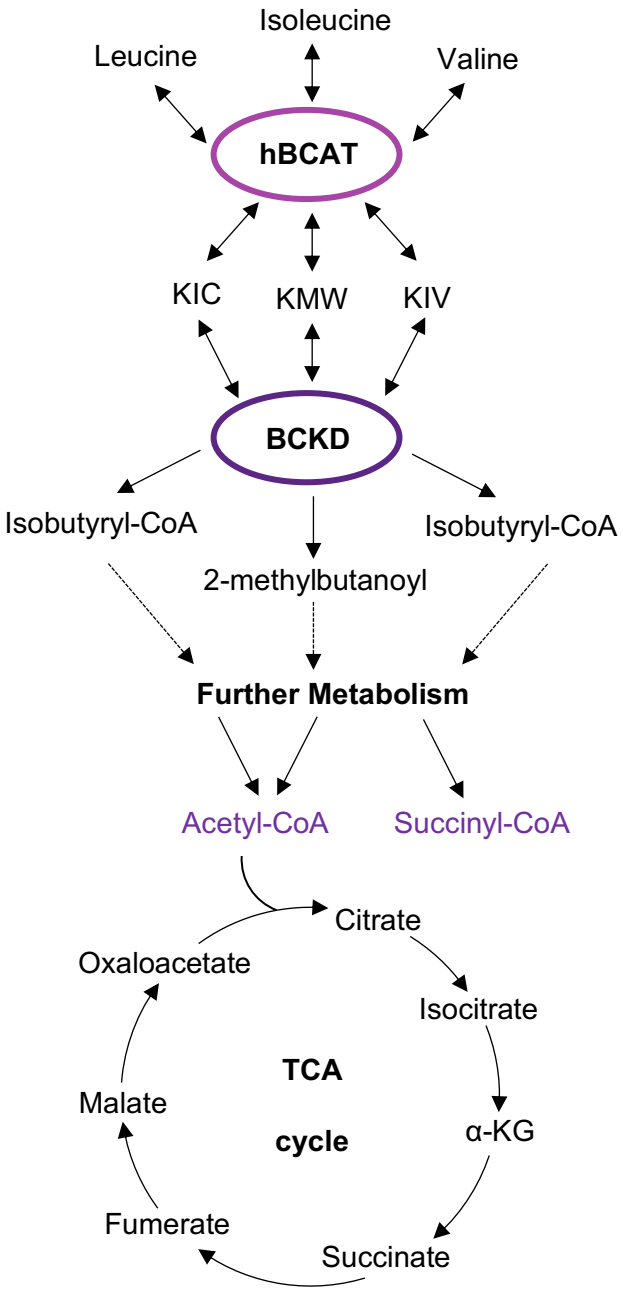

Figure 1.7. Metabolism of the branched-chain amino acids. The BCAAs are first reversibly transaminated to their respective conjugate α-keto acid by the hBCAT proteins. These are then further metabolised by the BCKD complex into the CoA products. Following further metabolism, these metabolites enter the TCA cycle as either acetyl-CoA (for leucine and isoleucine catabolism) or succinyl-CoA (for valine catabolism). Adapted from Hutson, Sweatt and LaNoue (2005). Abbreviations: α-KG, α-Ketoglutarate; KIC, ketoisocaproate; KMV, ketomethylvalerate; KIV, ketoisovalerate; BCKD, branched-chain α-keto acid dehydrogenase; TCA cycle, tricarboxylic acid (TCA) cycle.

which are further metabolized to generate cellular energy through the tricarboxylic acid (TCA) cycle. The BCKD complex contains three enzymes: a branched-chain α-keto acid decarboxylase (E1), a dihydrolipoyl transacylase (E2), and a dihydrolipoyl dehydrogenase (E3) (Harris *et al.*, 1997). The activity of this complex is tightly controlled by covalent modification with phosphorylation of the E1α subunits by a specific kinase (BDK) causing inactivation when BCAAs are required for protein synthesis (Shimomura *et al.*, 1990). The BCKD complex is then reactivated, when BCAAs are in excess, through dephosphorylation by a specific phosphatase (BDP) (Damuni *et al.*, 2006).

1.7.1 hBCAT expression in tumours

The hBCAT proteins exist in two main isoforms. The mitochondrial hBCAT isoform (hBCATm protein, *BCAT2* gene) is widely expressed in most tissues, whereas the cytosolic hBCAT (hBCATc protein, *BCAT1* gene) is restricted to highly specialised tissues including brain and placenta (Hall *et al.*, 1993). These hBCAT proteins share 58% homology in their primary amino acid sequence (Davoodi *et al.*, 1998; Hutson *et al.*, 1998). Emerging studies have identified upregulation of hBCATc to be associated with increased cell proliferation, cell cycle progression in several malignancies including gliomas (Tönjes *et al.*, 2013; Conway *et al.*, 2016) ovarian, colorectal and breast cancer (Wang *et al.*, 2015; Yoshikawa *et al.*, 2006; Thewes *et al.*, 2017). Similarly, upregulation of hBCATm has been demonstrated to promote proliferation of oestrogen receptor positive (MCF7) cells (Antanavičiūtė *et al.*, 2017). The regulation of these proteins differs whereby *BCAT1* is c-Myc regulated and *BCAT2* is a Kruppel-Like factor 15 (Klf15) regulated gene (Ben-Yosef, Eden and Benvenisty, 1998; Benvenisty *et al.*, 1992; Shimizu *et al.*, 2011). SREBP1 (sterol response element binding protein 1) has been identified to regulate *BCAT2* in pancreatic ductal adenocarcinoma (Dey *et al.*, 2017) whilst

upregulation of *BCAT1* in breast cancer has been found to be due to histone H3K79 methylation by disruptor of telomeric silencing 1-like (DOT1L) in TNBC MDA-MB-231 cells (Oktyabri *et al.*, 2016). Both genes can also be transcribed by activating transcription factor 4 (ATF4) in response to cellular stresses such as starvation, and oxidative stress (Bröer and Bröer, 2017; Budczies *et al.*, 2013).

In gliomas, upregulation of hBCATc proteins has been demonstrated to correlate with increased grade and aggressiveness (Tönjes *et al.*, 2013) whilst low expression of hBCATm was associated with improved prognosis (Conway *et al.*, 2016), indicating hBCAT proteins to play an intrinsic role in tumour progression. Indeed, suppression of hBCATc in glioma cells, was found to reduce glutamate production resulting in reduced cell proliferation and invasion (Tönjes *et al.*, 2013). Expression of the hBCAT metabolic proteins was demonstrated to be limited to wild-type *IDH* gliomas and were therefore identified as a potential novel therapeutic target in these poor prognosis tumours (Tönjes *et al.*, 2013). On the contrary, gliomas with mutant-*IDH* result in neomorphic production of 2HG which has been demonstrated to inhibit hBCATc expression (Dang *et al.*, 2009; Noushmehr *et al.*, 2010; Mayers and Vander Heiden, 2013; McBrayer *et al.*, 2018). Similarly, in breast cancer, shRNA-mediated knockdown of *BCAT1* reduced proliferation, migration and invasion of TNBC cells, indicating an intrinsic role to tumour progression (Thewes *et al.*, 2017). However, there is little evidence of *IDH1* mutations in breast cancer (Raynaud *et al.*, 2010; Kim *et al.*, 2014), instead *Myc*-mediated 2HG production has been reported as the key instigator of 2HG accumulation induced by increased glutaminase (GLS1) expression in breast cancer (Terunuma *et al.*, 2014). Interestingly, despite significantly elevated 2HG levels compared with adjacent noncancerous tissue (Terunuma *et al.*, 2014), hBCATc expression has been demonstrated to be upregulated in TNBC tissue

(Thewes *et al.*, 2017). Given their correlated links in gliomas it is timely to investigate the association between IDH and hBCAT expression in breast cancer, which may point to crosstalk between these metabolic pathways offering insight into novel pathways that govern tumorigenesis.

1.7.2 The hBCAT CXXC redox sensitive motif

A novel function of the hBCAT proteins is that they are regulated by different redox environments (Davoodi *et al.*, 1998; Conway *et al.*, 2008). This is important in cancer as the redox environment is commonly disturbed in cancer (Hecht *et al.*, 2016). The hBCAT proteins have been characterised to have a redox-sensitive CXXC motif (Cys315-Gln336-Val337-Cys318 for hBCATm, Cys335-Val336-Val337-Cys338 for hBCATc), which was shown to be important for the binding of hBCATm to the E1 subunit of BCKDC, hence facilitating substrate channelling to BCKDC (Hull *et al.*, 2012). Conversely, under reduced conditions glutamate dehydrogenase (GDH) facilitates the oxidative deamination of glutamate to regenerate BCKAs (Islam *et al.*, 2010). Both isozymes of BCAT catalyse reversible transamination of BCAAs, and it is this redox centre that permits this reversibility.

The oxidation state of the CXXC motif is essential for protein-protein interactions between hBCAT and other proteins, where the reactive thiols of cysteine are particularly sensitive to oxidation (Barford, 2004). Modifications with thiol specific reagents or oxidation of the thiols to a disulphide bond results in a loss of hBCAT activity (Conway *et al.*, 2002, 2008). Site-directed mutagenesis identified C315 and C318 cysteines for hBCATm and Cys335 and Cys338 for hBCATc to represent the most reactive thiols under reducing conditions. S-thiolation of hBCATc under oxidizing conditions, followed by de-glutathionylation via the

GSH/Grx repair pathway validated the thiols of the CXXC motif as key targets for the physiologically vital reducing systems of the cell. It has been suggested that these reducing mechanisms may be working together with hBCATc as a redox sink conserving GSH, or that the protein itself is regulated during oxidative stress via S-glutathionylation (Conway *et al.*, 2008). Through modification of reduced (GSH) to oxidized (GSSG) glutathione ratio (GSH:GSSG), the overall redox potential for hBCATc was found to be lower than that of hBCATm, which allows decreased sensitivity of hBCATm to the redox state thus preserving aminotransferase activity in the oxidising mitochondrial environment (Coles, Hancock and Conway, 2012). The differential redox potentials between the hBCAT CXXC motifs was suggested to reflect different biochemical behaviours as well as their different sub-cellular locations.

1.7.3 The novel role of hBCAT in deregulated tumour signalling pathways

During tumorigenesis oxidative stress is induced as the metabolic activity of cancer cells increases as either an overproduction of reactive oxygen species (ROS) or insufficient antioxidant activity (Gorrini, Harris and Mak, 2013). However, excessive oxidative stress can induce apoptosis (Hole *et al.*, 2013), hence to counteract excessive ROS accumulation tumour cells increase their antioxidant capacity (Chandel and Tuveson, 2014). Overexpression of *BCAT1* has been demonstrated to decrease cell apoptosis in prostate cancer cells (Zhu, Shao and Peng, 2017), however the underlying mechanism has yet to be elucidated. As the hBCAT proteins have been demonstrated to regulate the expression of redox regulating proteins (Conway *et al.*, 2008), it can be hypothesised that the redox-sensitive hBCAT protein may play an intrinsic role in the evasion of apoptosis through modulation of the redox status in tumour cells. Moreover, hBCATm has

been demonstrated to play a protective role in Alzheimer's disease, through interaction with the protein-folding protein disulfide isomerase (PDI), which was regulated through S-glutathionylation under oxidative stress (Hindy *et al.*, 2014). This study highlighted a novel redox chaperone role for hBCATm through interaction with PDI which mediates an adaptive response to changes in the cellular redox environment to promote cell survival (Nakamura, 2005).

1.8 Insulin and IGF-1 signalling axis

Aberrant growth factor signalling such as amplified insulin/IGF-1 signalling in breast cancer has been demonstrated to play an intrinsic role in oncogenesis and tumour progression in addition to contributing to resistance to targeted therapies (Christopoulos, Msaouel and Koutsilieris, 2015; Vander Heiden and DeBerardinis, 2017). The insulin/insulin-like growth factor (IGF) system is a complex network essential for regulation of cell growth, proliferation and survival (Lewitt and Boyd, 2019). Ordinarily these pathways are transiently activated in response to growth factor stimulation, however up-regulation of both insulin/IGF-1 and their receptors in breast cancer can lead to constitutive signalling (Tsujimoto, Kajio and Sugiyama, 2017; Law *et al.*, 2008; Obr *et al.*, 2018). Insulin/IGF-1 signalling plays a central role in tumour cell proliferation, survival, invasion, and metastasis in breast cancer (Christopoulos, Msaouel and Koutsilieris, 2015).

1.8.1 Insulin

Insulin is a polypeptide hormone consisting of 51 amino acids, which is secreted from pancreatic β-cells into the blood and plays a major role in regulating glucose uptake and metabolism (Sanger, 1960; Leroux *et al.*, 1975). In response to increased glucose levels, insulin increases glucose absorption by promoting the

translocation of the glucose transporter proteins from the cytoplasm to the cell membrane (Leroux *et al.*, 1975). Insulin acts as an anabolic protein by increasing the uptake and incorporation of amino acids into protein, inhibits protein degradation, stimulates lipogenesis, and suppress lipolysis (Leroux *et al.*, 1975; Qaid and Abdelrahman, 2016). Insulin may be considered a storage hormone by promoting movement of glucose directing absorbed amino acids into peripheral tissues (Qaid and Abdelrahman, 2016).

In the breast, insulin has been demonstrated to be important in the maintenance of milk secretion during lactation mediated by increased lipid synthesis (Jones, Ilic and Williamson, 1984). In addition to its metabolic effects, insulin also promotes cell proliferation and migration and inhibits apoptosis (Saltiel and Kahn, 2001). High levels of circulating insulin as well as increased expression of insulin receptor (IR) has been associated with increased breast cancer risk (Tsujimoto, Kajio and Sugiyama, 2017; Law *et al.*, 2008). Insulin is involved in tumour progression rather than initiation and neoplastic transformation of the breast epithelial cell (Rose and Vona-Davis, 2012). Hyperinsulinemia increases the secretion of adipokines, such as leptin, from tumour-associated adipose tissue to enhance cancer cell proliferation, survival and angiogenesis (Rose and Vona-Davis, 2012). Insulin may also indirectly promote breast cancer development, via the upregulation and modulation of the bioavailability of IGF-1, especially in the tumour microenvironment (Kang, LeRoith and Gallagher, 2018).

1.8.2 IGF-1

IGF-1 is a small peptide consisting of 70 amino acids and shares 70% structural similarity to insulin (Laron, 2001). The structural similarity to insulin explains the ability of IGF-1 to bind (with low affinity) to the IR (Kim and Accili, 2002). IGF-1 is

produced primarily in the liver under direct stimulation of growth hormone (GH) (Laron, 2001), which is essential for mammary gland development as demonstrated in mice lacking GH receptor (GHR) leads to a severely compromised ductal network (Marshman and Streuli, 2002). IGF-1 is expressed mainly in stromal cells of the mammary connective tissue (i.e. fibroblasts and adipocytes) whilst the IGF-1R is primarily expressed in the epithelial cells, supporting the vital role of stromal–epithelial for full mammary gland development (Sreekumar, Roarty and Rosen, 2015). Importantly, IGF-1 acts as a mediator of mammary branching and ductal formation, which is implicated in the pathogenesis of breast cancer (Macias and Hinck; Christopoulos, Msaouel and Koutsilieris, 2015).

IGF-1 plays a major mitogenic role in regulating cell proliferation, differentiation, migration and survival and also a metabolic role to increase the cellular uptake of glucose and amino acids and stimulate glycogen and protein synthesis (Zapf, Schmid and Froesch, 1984; Dimitriadis *et al.*, 1992). The family of six IGF binding proteins (IGFBP1-6) are major regulators of the bioavailability of IGF-1, which transport IGF-1 in the circulation, facilitate their movement out of the vascular compartment and elongate the half-life of the hormones (Rajpathak *et al.*, 2009; Bruning *et al.*, 1995). IGFBPs, preferentially IGFBP-3, bind IGF-1 with high affinity but do not bind to insulin (Bruning *et al.*, 1995). Elevated IGF-1 and decreased IGFBP-3 levels are observed in the serum of women presenting with early stage breast cancer indicating increased levels of available IGF-1 may contribute to breast cancer development (Bruning *et al.*, 1995). Additionally, increased expression of IGF-1R in breast cancer tissue compared with normal or benign

tissue indicates the IGF-1 signalling pathway plays a key role in tumour progression (Obr *et al.*, 2018).

1.8.3 The insulin and IGF-1 receptors

IR and IGF-1R are homologous receptor tyrosine kinases (RTKs) and share almost 84% structure similarity (Chitnis *et al.*, 2008). These two cell membrane receptors are composed of two extracellular spanning α subunits and two transmembrane β subunits connected by disulphide bonds to form homodimers (Figure 1.8) (Laron, 2001). The intracellular domain of the β subunit contains a tyrosine kinase domain, which constitutes the signal transduction mechanism (Kavran *et al.*, 2015). In addition to the homodimer receptors, the α and β chains from IGF-1R and IR can dimerize with each other to form functional heterodimer receptors referred to as hybrid receptors (Pandini *et al.*, 1999; Rajpathak *et al.*, 2009).

Binding of IGF-1 and insulin to the IGF-1R, IR and IGF-1/IR hybrids induces a conformational change in the catalytic domain of the β-subunit to promote phosphorylation of tyrosine residues 1158, 1162 and 1163 in the kinase regulatory loop (Hubbard, Mohammadi and Schlessinger, 1998; Girnita *et al.*, 2014). Autophosphorylation releases the regulatory loop from its inhibitory position, which allows the phosphorylation and recruitment of specific substrates, in particular insulin receptor substrate (IRS)-1, IRS-2 and Src homology 2 domain containing transforming protein (Shc) to the cell membrane (Hubbard, Mohammadi and Schlessinger, 1998; Girnita *et al.*, 2014; Boucher, Kleinridders and Ronald Kahn, 2014). The activation of these substrates generates recruitment sites for downstream signalling proteins containing phosphor-tyrosine-recognition domains, such as the Src homology 2 (SH2) domain or the

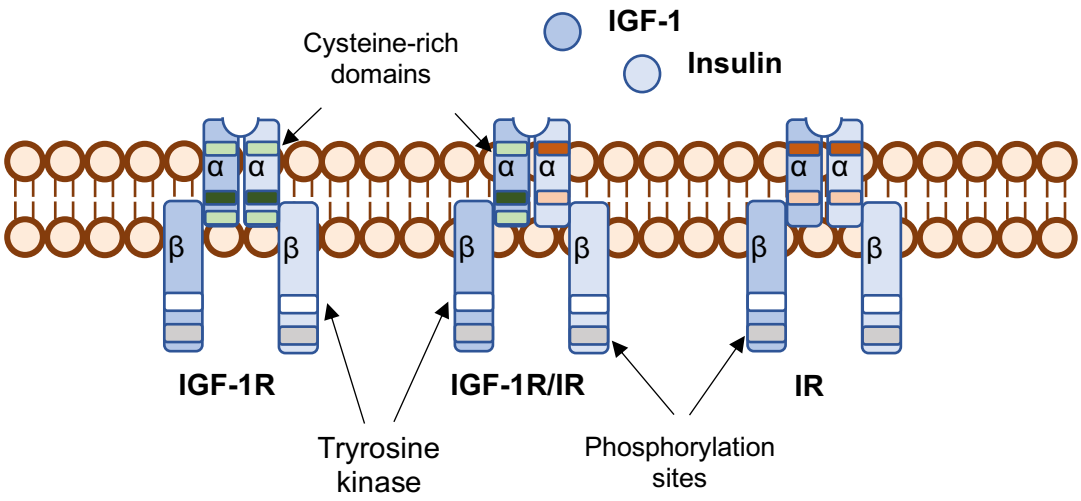

Figure 1.8. Structure of the IGF-1 and insulin receptors. The receptors of the insulin/IGF-1 system are comprised of two half receptors, each with an extracellular ligand-binding domain and an intracellular tyrosine kinase domain (Bowers *et al.*, 2015). The insulin-like growth factor 1 receptor (IGF-1R) and insulin receptor (IR) receptors are highly similar in structure and both belong to the tyrosine kinase receptor family (Rajpathak *et al.*, 2009). Both IGF-1R and IR homodimers and IGF-1R/IR heterodimers can form (Bowers *et al.*, 2015). Ligand binding stimulates the kinase activity of these receptors via transphosphorylation of their beta subunits (Bowers *et al.*, 2015).

phosphotyrosine-binding (PTB) domain (Girnita *et al.*, 2014; Hakuno and Takahashi, 2018). This results in the activation of two major signalling pathways; the PI3K/AKT/mammalian target of rapamycin (mTOR) pathway, which is mainly responsible for metabolic activities (Saxton and Sabatini, 2017), and the Ras/MAPK pathway, which is involved in regulating cellular growth, proliferation and migration (McCubrey *et al.*, 2007).

1.8.4 The RAS/MAPK signalling cascade

IGF-1/IR activation and subsequent tyrosine-phosphorylated IRS1, IRS2 and Shc leads to the activation of the RAS/MAPK signalling cascade (Figure 1.9). The pathway is initiated by recruitment of Grb2 which consists of a SH2 domain flanked by N- and C-terminal Src homology 2 (SH3) domains (Giubellino, Burke and Bottaro, 2008). Grb2 is activated by binding directly to phosphorylated IRS or Shc through its SH2 domain, while the SH3 domains interact with son of sevenless (Sos), a guanine nucleotide exchange protein (Hakuno and Takahashi, 2018; Girnita *et al.*, 2014). Sos catalyses the release of guanosine diphosphate (GDP) and subsequent binding of guanosine triphosphate (GTP) to membrane-bound Ras (Mendoza, Er and Blenis, 2011). Activated (GTP-bound) Ras in turn interacts with and translocates the serine/threonine protein kinase Raf to the plasma membrane, where Raf becomes activated (Girnita *et al.*, 2014). Raf phosphorylates and activates the dual specificity MAPK, MEK, which in turn stimulates the ERK subset of MAPKs: extracellular-related kinase (ERK)1 and ERK2 (Girnita *et al.*, 2014).

The MAPKs, ERK1/2 are serine/threonine kinases which are the main effectors of the Ras/MAPK signalling cascade to regulate cell growth, proliferation and

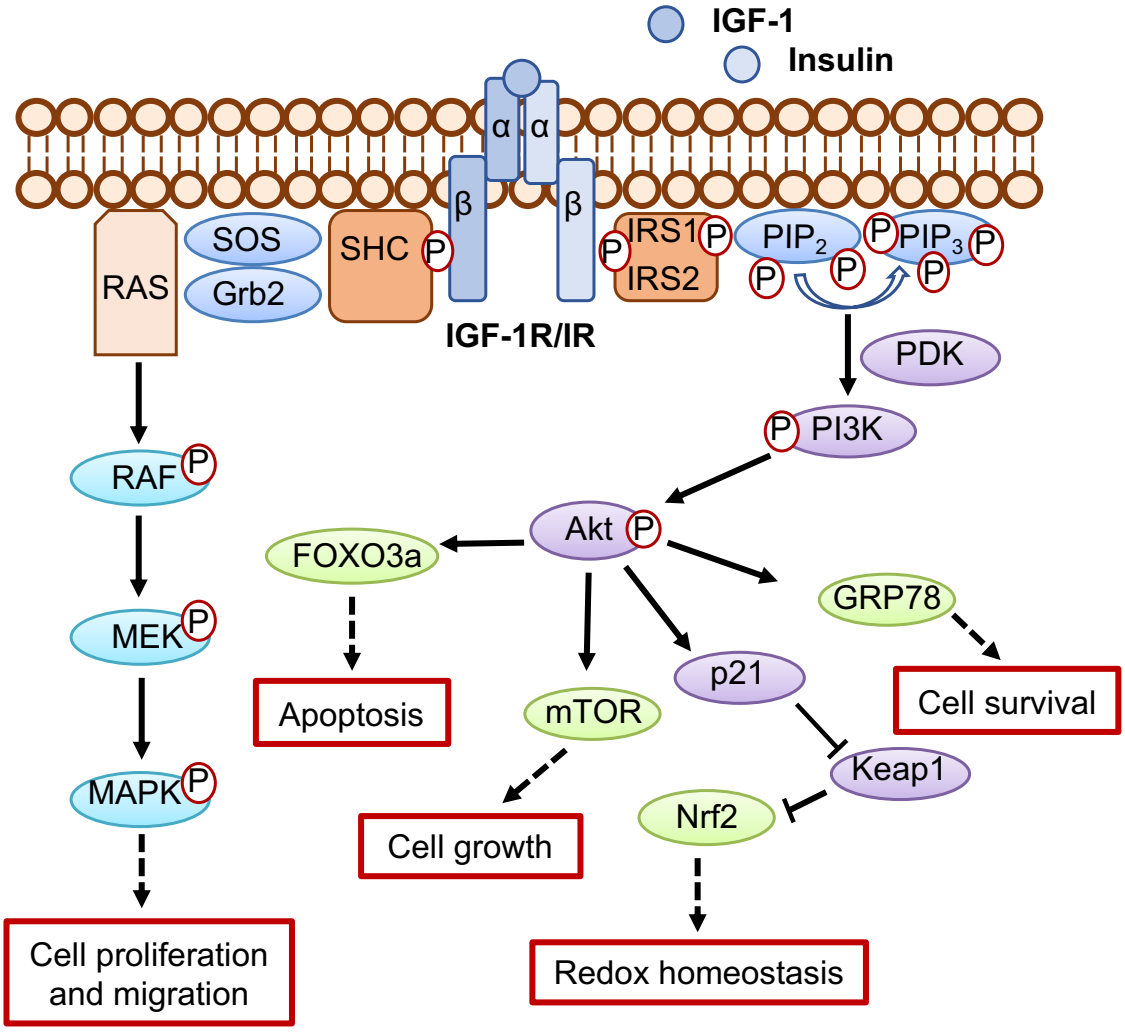

Figure 1.9. IGF-1/insulin signalling cascade. Activation of insulin-like growth factor 1 receptor (IGF-1R) and insulin receptor (IR), mediated by ligand binding of IGF-1 or insulin activates phosphoinositide-3 kinase (PI3K)/serine/threonine-specific protein kinase (Akt) pathway and the Ras/mitogen-activated protein kinase (MAPK) pathway signalling cascades (Girnita *et al.*, 2014). This initiates the phosphorylation of various signalling effector proteins coordinating cell growth, proliferation, migration, survival, apoptosis and redox homeostasis as indicated (Feng *et al.*, 2018). Grb2, growth factor receptor-bound 2; GRP78, glucose regulated protein 78; IRS, insulin receptor substrate; Keap1, kelch-like ECH-associated protein 1; mTOR, mammalian target of rapamycin; Nrf2, nuclear factor (erythroid-derived 2)-like-2; PDK, phosphoinositide-dependent protein kinase; PIP_2, phosphatidylinositol (4,5) bisphosphate; PIP_3, phosphatidylinositol (3,4,5) trisphosphate; FOXO3a, forkhead box O3; Shc, Src homology 2 domain containing transforming protein; SOS, son of sevenless.

migration (McCubrey *et al.*, 2007). The phosphorylation of MAPK results in an activation of its kinase activity to phosphorylate several cytoplasmic and nuclear targets, including kinases, phosphatases, transcription factors and cytoskeletal proteins (Dhillon *et al.*, 2007). In the cytoplasm, MAPK regulates microtubule dynamics by phosphorylating microtubule-associated proteins to mediate cell motility and migration (Girnita *et al.*, 2014). Cytoplasmic targets for MAPK include p90 ribosomal S6 kinase (RSK), which phosphorylates several cytoplasmic targets and transcriptional regulators such as myosin phosphatase–targeting subunit 1 (Samson *et al.*, 2019; Cargnello and Roux, 2011). MAPK nuclear targets include ETS Like-1 protein (Elk1), a ternary complex factor transcription factor, which is involved in the expression of immediate-early (IE) genes, such as *c-Fos* to promote cell survival, cell division, and cell motility (Vickers *et al.*, 2004).

1.8.5 The PI3K/Akt/mTOR signalling cascade

Activation of the IGF-1/IR signalling cascade leads to IRS1 and IRS2 phosphorylation at multiple tyrosine residues to create docking sites for the p85 regulatory subunit of PI3K class I, leading to activation of the catalytic subunit p110 of PI3K (Luo *et al.*, 2005). This leads to the formation of PIP_3, which binds to the pleckstrin-homology (PH) domain of Akt disrupting its interaction with the catalytic domain, which is subsequently phosphorylated by phosphoinositide-dependent protein kinase-1 (PDK1) (Um, D'Alessio and Thomas, 2006). Activated Akt subsequently phosphorylates multiple substrates including p21, transcription factor forkhead box O3 (FOXO3a) and glucose regulated protein 78 (GRP78) (Girnita *et al.*, 2014).

Redox homeostasis is regulated by the phosphorylation of p21 by Akt which increases the stability of nuclear factor (erythroid-derived 2)-like-2 (Nrf2) (Koundouros and Poulogiannis, 2018). Nrf2 and its repressor protein kelch-like ECH-associated protein 1 (Keap1) mediate cellular response to oxidative stress through the regulation of antioxidant genes such as thioredoxin (Trx) (Nakaso *et al.*, 2003). Akt activation of p21 disrupts the interaction between Keap1 and Nrf2, reducing Nrf2 phosphorylation, preventing its nuclear export and ubiquitination (Koundouros and Poulogiannis, 2018). PI3K/Akt activation of Nrf2 promotes a cell protective role in response to changes in the redox state which is commonly perturbed in cancer (Gorrini, Harris and Mak, 2013). Akt activation also induces the nuclear translocation of FOXO3a which regulates several genes involved in apoptosis, such as Bcl-2-like protein (Bim) and cell proliferation such as cyclin-dependent kinase inhibitor 1B ($p27^{Kip1}$) (Liu *et al.*, 2018). Moreover, PI3K/Akt activation stabilises GRP78, which acts as a molecular chaperone protein, to prevent binding to pro-apoptotic factors thereby evading apoptosis (Dai *et al.*, 2010). GRP78 plays a key role in cell survival and is upregulated in response to endoplasmic reticulum (ER) stress to suppress the activity of the pro-apoptotic factors and thus inhibit apoptosis (Fu, Li and Lee, 2007). Akt activation can also directly phosphorylate and inhibit several pro-apoptotic proteins such as Bcl-2-associated death promoter (BAD), the pro-apoptotic effector protein glycogen synthase kinase-3β (GSK-3β) and caspase 9 (Cardone *et al.*, 1998; Girnita *et al.*, 2014).

Activated Akt also induces phosphorylation and degradation of tuberous sclerosis complex protein 2 (TSC2), which acts in a complex with TSC1 as a GTPase activating protein for the Ras homolog enriched in brain (Rheb) to form inactive

GDP-bound Rheb (Nobukuni, Kozma and Thomas, 2007)Therefore, TSC2 degradation permits GTP bound Rheb to directly interact with mTOR complex 1 (mTORC1) which consists of regulatory-associated protein of mTOR (raptor), proline-rich Akt substrate 40 (PRAS40), G protein beta protein subunit-like (GβL) and DEP domain-containing mTOR-interacting protein (DEPTOR) (Saxton and Sabatini, 2017). Activation of mTOR leads to phosphorylation of several target proteins related to ribosomal biogenesis and cell growth, including ribosomal protein S6 kinase 1 (S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) (Saxton and Sabatini, 2017).

Nutrient levels of BCAAs particularly leucine, substrates of hBCAT, regulate the mTOR pathway (Son *et al.*, 2019) which is a key signalling kinase downstream of the EGFR pathways including HER2 and the insulin/IGF-1 signalling cascades which induces protein synthesis, supporting tumour growth (Christopoulos, Msaouel and Koutsilieris, 2015; Laplante and Sabatini, 2013). Hence, overexpression of hBCATc in breast cancer (Thewes *et al.*, 2017) can be proposed to promote increased cell proliferation through the regulation of the mTOR pathway. Understanding the underlying mechanisms by which metabolic proteins may converge with components of these signalling cascades, such as mTOR, may offer novel therapeutic targets. As discussed, the hBCAT proteins are potentially involved in several processes including deregulated cell metabolism, redox status and aberrant mTOR signalling, offering multiple benefits to tumour cells for cell growth and survival in the unfavourable conditions found in the tumour stroma. Together, this highlights the importance of understanding the role of the hBCAT proteins in these signalling cascades, which will be investigated further in this thesis.

1.9 Overall aims

1.9.1 General hypothesis:

Metabolic reprogramming is an important cancer hallmark to support cell survival and growth. Key metabolic enzymes involved in BCAA metabolism (hBCATc and hBCATm), isocitrate dehydrogenase (IDH1 and IDH2) and serine metabolism (PHGDH) are upregulated in breast cancer. The different breast cancer molecular subtypes lead to the activation of oncogenic pathways and subsequently differential metabolic liabilities. However, the expression patterns and the role of these metabolic proteins in breast cancer remain to be elucidated. As these metabolic pathways have been demonstrated to converge in gliomas, it was hypothesised that there is crosstalk between these metabolic pathways and that these are favoured differentially between breast cancer subtypes.

In breast cancer IGF-1/insulin signalling has also been demonstrated to contribute to tumourigenesis through the activation of growth signalling effectors mediated by two chief pathways: Akt/mTOR and RAS/MAPK. Therapeutic targets targeting the IGF-1 and insulin receptors lack efficacy, highlighting the need for novel therapeutic targets for the modulation of these signalling cascades. As nutrient levels of BCAAs particularly leucine, substrates of hBCATc, regulate the mTOR pathway hBCATc was hypothesised to play a role in the regulation of proliferation and migration of TNBC cells through the IGF-1 and insulin signalling pathways.

1.9.2 Overall aims and objectives

The aim of this study is to identify the role of metabolic dependencies in breast cancer and to assess the role of the metabolic proteins in signalling pathways associated with tumour progression.

Aim 1: Characterisation of metabolic proteins in breast cancer

To assess the expression levels and cellular localisation of metabolic enzymes involved in BCAA metabolism (hBCATc and hBCATm), isocitrate dehydrogenase (IDH1 and IDH2) and serine metabolism (PHGDH) in a cohort of human breast cancer tissue samples. The expression of these metabolic proteins will be correlated with breast cancer subtypes, clinicopathological features and disease-free survival.

Aim 2: The role of hBCATc in the IGF-1/insulin signalling cascades

To determine whether hBCATc plays a role in IGF-1 and insulin mediated proliferation and migration siRNA-mediated *BCAT1* knockdown models will be used. The role of hBCATc in the regulation of the two principal cell signalling pathways; Akt/mTOR and RAS/MAPK and relevant downstream signalling effectors will be investigated using shRNA-mediated *BCAT1* knockdown and overexpression models.

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Chapter 2

2.0 Materials and Methods

Mai Ahmed Shafei

2.0 Materials and Methods

2.1 Materials

Annexin V-FITC Kit was purchased from Biolegend (San Diego, CA, USA). Lentiviral pULTRA vector reference no. 24129, was supplied by Addgene (Cambridge, MA). Seahorse XFe24 FluxPak and Seahorse XF Glycolysis Rate Assay Pack were purchased from Agilent Technologies (Cheshire, UK). Direct-zol™ RNA MiniPrep and TriZol reagent were purchased from Cambridge bioscience (Cambridge, UK). Glass 18 mm diameter rounded coverslips were purchased from Cellpath (Newtown, UK). Annexin V Binding Buffer, Dulbecco's Modified Eagle Medium (DMEM), Earle's Balanced Salt Solution (EBSS), Ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), foetal bovine serum (FBS), Gibco® Opti-MEM™, Gibco® Trypsin-EDTA, glacial acetic acid, glycine, hydrogen chloride, Insulin-Transferrin, Lipofectamine™ RNAiMAX, magnesium chloride, methanol, NuPAGE lithium dodecyl sulfate (LDS) sample buffer 4 X, poly-l-lysine, propidium iodide staining solution, sodium bicarbonate, sodium chloride, sodium dodecyl sulphate (SDS), sodium hydroxide, Spectra ladder, trichloric acid (TCA), Superfrost Plus slides, Tris, Tween® 20, were purchased from Fisher Scientific (Loughborough, UK). Thincert™ cell culture inserts (8 µm) for 24 well plates were purchased from Greiner Bio-One (Stonehouse, UK). Histoclear was purchased from National Diagnostics (Atlanta, GA, USA). Polyvinylidene fluoride (PVDF) membrane (IPFL00010) was purchased from Merck (Watford, UK). pSUPER plasmid was purchased from Oligoengine (Seattle, WA). Phosphate buffered saline (PBS) was purchased from Oxoid (Hampshire, UK). Ultima gold liquid scintillation cocktail was purchased from Perkin Elmer (Beaconsfield, UK). BM chemiluminescence blotting substrate and complete mini EDTA-free protease

inhibitor cocktail tablets were purchased from Roche (West Sussex, UK). 30% Acrylamide, β-mercaptoethanol, bovine serum albumin (BSA), bromophenol blue dye, Cholera Toxin from *Vibrio cholerae*, Coomassie blue G dye, Crystal Violet, DMEM/Nutrient Mixture F-12, DPX-mountant, Ham glucose, Harris's Haematoxylin, horse serum, hydrocortisone, insulin, L-glutamine, MISSION® lentiviral packaging mix, polyethylenimine (PEI) rapamycin, sodium cacodylate, TEMED and Triton^{TM} X-100 were purchased from Sigma-Aldrich (Dorset, UK). ImmPACT DAB peroxidase (HRP) substrate, Vectashield hardset mounting medium with DAPI and Vectastain elite ABC kit were purchased from Vector Labs (Peterborough, UK). Ethanol was purchased from VWR (Leicestershire, UK). Marvel was purchased locally.

2.1.2 Cell lines

MCF10a: basal breast benign cells,and breast cancer cell lines BT20: basal A TN, MCF7: luminal ER+/PR+, MDA-MB-231: basal B TN, MDA-MB-361: luminal ER+/PR+/HER2+, MDA-MB-453: luminal ER-/PR-/HER2+, SKBR3: luminal ER-/PR-/HER2+ were obtained from ATCC ^{TM} (Manassas, VA). Human embryonic kidney cells; HEK-293T cells used for lentiviral particle production were obtained from Takara Bio (Otsu, Japan).

Table 2.1: List of antibodies, dilution and source

Antibody (raised in)	Dilution (method)	Manufacturer Code	Manufacturer
α-tubulin (mouse)	1:5,000 (WB)	Ab7291	Abcam (Cambridge, UK)
β-actin (mouse)	1:5,000 (WB)	Ab8226	Abcam (Cambridge, UK)
Grb2 (rabbit)	1:3,000 (WB)	Ab32037	Abcam (Cambridge, UK)
IDH1 (rabbit)	1:1,000 (WB) 1:400 (IHC)	Ab113232	Abcam (Cambridge, UK)
IDH2 (mouse)	1:3,000 (WB) 1:200 (IHC)	Ab184196	Abcam (Cambridge, UK)
mTOR (rabbit)	1:500 (WB)	Ab134903	Abcam (Cambridge, UK)
Nrf2 (rabbit)	1:3,000 (WB)	Ab62352	Abcam (Cambridge, UK)
PHGDH (rabbit)	1:5,000 (WB) 1:600 (IHC)	Ab125865	Abcam (Cambridge, UK)
Phospho-mTOR (rabbit)	1:500 (WB)	Ab137133	Abcam (Cambridge, UK)
mTOR (rabbit)	1:500 (WB)	Ab134093	Abcam (Cambridge, UK)
SOS1 (rabbit)	1:1,000 (WB)	Ab140621	Abcam (Cambridge, UK)
hBCATc (mouse)	1:50 (ICC)	611271	BD Biosciences (New Jersey, US)
Phospho-Akt (rabbit)	1:500 (WB)	9271	Cell Signaling (Danvers, MA)
Akt (rabbit)	1:500 (WB)	9272	Cell Signaling (Danvers, MA)

FOXO3a (rabbit)	1:1,000 (WB)	2497	Cell Signaling (Danvers, MA)
IGF1-Rβ (rabbit)	1:1,000 (WB)	9750	Cell Signaling (Danvers, MA)
MAPK (rabbit)	1:1,000 (WB) 1:100 (ICC)	4695	Cell Signaling (Danvers, MA)
Phospho-MAPK (rabbit)	1:1,000 (WB) 1:100 (ICC)	4370	Cell Signaling (Danvers, MA)
hBCATc (rabbit)	1:3,000 (WB) 1:200 (IHC)	Custom synthesis	Insight Biotechnology (Wembley, UK)
hBCATm (rabbit)	1:3,000 (WB) 1:800 (IHC)	Custom synthesis	Insight Biotechnology (Wembley, UK)
Donkey anti-Mouse IgG (H + L) IRDye® 800CW	1:5,000 (WB)	92532212	LI-COR Biosciences (Lincoln, USA)
Goat anti-Rabbit IgG (H + L) IRDye® 800CW	1:5,000 (WB)	92532213	LI-COR Biosciences (Lincoln, USA)
Goat anti-Mouse Alexa Fluor 488 Secondary	1:250 (ICC)	10544773	Invitrogen (Paisley, UK)
Goat anti-Rabbit Alexa Fluor 568 Secondary	1:500 (ICC)	10463022	Invitrogen (Paisley, UK)
GRP78 (mouse)	1:400 (WB)	SC-1050	Santa Cruz (Heidelberg, Germany)

GAPDH (mouse)	1:5,000 (WB)	SC-47724	Santa Cruz (Heidelberg, Germany)
SHC (mouse)	1:1,000 (WB)	SC-967	Santa Cruz (Heidelberg, Germany)
Mouse IgG HRP-conjugate (horse)	1:3,000 (WB)	PI-2000	Vector (Peterborough, UK)
Rabbit IgG HRP-conjugate (goat)	1:3,000 (WB)	PI-1000	Vector (Peterborough, UK)

2.2 Methods:

2.2.1 Immunohistochemistry

2.2.1.1 Patient Characteristics

Archival human breast cancer tissue samples from surgical resections of breast tumours were obtained from the Bristol Royal Infirmary under ethical approval from NHS Health Research Authority and UWE Ethics Committee (Ref. 11/SW/0127). To re-confirm phenotype all of the cases were IHC stained for ER, PR and HER2. The patient cohort consisted of a total of 83 breast cancer cases which were classified into subtypes; HER2+ subtype (18 cases), luminal A (19 cases), luminal B (10 cases) and TNBC (36). Patients' clinical pathological characteristics included hormone receptor status, tumour stage, lymph-node metastasis and survival. HER2+ breast cancer and TNBC demonstrated higher histopathological grading ($p < 0.001$) compared to the other subtypes (Table 2.2).

2.2.1.2 Immunohistochemical Staining and Scoring

Formalin-fixed, paraffin-embedded (FFPE) tissue were serially sectioned at 4 µm using a microtome (Leica RM2235) and mounted on Superfrost Plus slides. Tissue sections were deparaffinised in histoclear and rehydrated using a series of ethanol concentrations and dH_2O. Endogenous peroxidase was quenched in 3% v/v hydrogen peroxide for 10 minutes at room temperature. Antigen retrieval was performed by heating sections in 10 mM citrate buffer pH 6.0 boiled at 95°C for 30 minutes using a water bath and then allowing the sections to cool to room temperature in the buffer. Non-specific binding sites were blocked with 5% horse serum in Tris-buffered saline (TBS; 200 mM sodium chloride, 2 mM tris, pH 7.5) for 1 hour at room temperature and sections were incubated with primary antibody in blocking serum overnight at 4°C.

Table 2.2 Clinicopathological characteristics of the patient cohort according to breast cancer subtypes

Parameters	**Total (n=83) (%)**	**HER2 (n= 18) (%)**	**Luminal A (n= 19) (%)**	**Luminal B (n= 10) (%)**	**TN (n= 36) (%)**	***p*-value**
Age (year, mean±SD)	61.04±13.1	60.44±10.0	61.06±14.0	55.59±16.3	62.85±13.0	
ER						**< 0.001**
Negative	52 (64.2)	15 (88.2)	1 (5.3)	1 (10.0)	35 (100.0)	
Positive	29 (35.8)	2 (11.8)	18 (94.7)	9 (90.0)	0 (0.0)	
PR						**< 0.001**
Negative	58 (71.6)	15 (88.2)	5 (26.3)	3 (30.0)	35 (100.0)	
Positive	23 (28.4)	2 (11.8)	14 (73.7)	7 (70.0)	0 (0.0)	
HER2						**< 0.001**
Negative	57 (70.4)	2 (11.8)	15 (78.9)	5 (50.0)	35 (100.0)	
Positive	24 (29.6)	15 (88.2)	4 (21.0)	5 (50.0)	0 (0.0)	
Tumour type						0.159
Lobular	9 (10.8)	0 (0.0)	3 (15.8)	0 (0.0)	6 (16.7)	
Ductal	68 (81.9)	18 (100)	15 (78.9)	8 (80.0)	27 (75.0)	
Mixed/Other	6 (7.2)	0 (0.0)	1 (5.3)	2 (20.0)	3 (8.3)	
Histological Grade						**< 0.001**
I/II	42 (50.6)	7 (38.9)	18 (94.7)	6 (60.0)	11 (30.6)	
III	41 (49.4)	11 (61.1)	1 (5.3)	4 (40.0)	25 (69.4)	
Tumour Stage						0.208
T1	43 (52.4)	8 (44.4)	14 (73.7)	4 (40.0)	17 (48.6)	
T2	34 (41.5)	10 (55.6)	3 (15.8)	5 (50.0)	16 (45.7)	
T3	5 (6.1)	0 (0.0)	2 (10.5)	1 (10.0)	2 (5.7)	
Nodal Stage						0.074
N0	54 (65.9)	9 (50.0)	15 (78.9)	5 (50.0)	25 (71.4)	
N1	18 (22.0)	7 (38.9)	2 (10.5)	3 (30.0)	6 (17.1)	
N2	7 (8.5)	2 (11.1)	2 (10.5)	0 (0.0)	3 (8.6)	
N3	3 (3.7)	0 (0.0)	0 (0.0)	2 (20.0)	1 (2.9)	
TNM Stage						**0.03**
I	35 (42.7)	3 (16.7)	13 (68.4)	4 (40.0)	15 (42.9)	
II	35 (42.7)	13 (72.2)	4 (21.1)	3 (30.0)	15 (42.9)	
III	12 (14.6)	2 (11.1)	2 (10.5)	3 (30.0)	5 (14.3)	
Tumour size (cm)						0.162
≤ 2	46 (57.5)	7 (41.2)	13 (68.4)	8 (80.0)	18 (52.9)	
> 2	34 (42.5)	10 (58.8)	6 (31.6)	2 (20.0)	16 (47.1)	

ER, oestrogen receptor; HER2, Human Epidermal Growth Factor Receptor 2; TN, triple negative

Sections were washed twice with TBS and incubated with biotinylated secondary antibody in TBS for 1 hour followed by avidin-biotin complex in TBS incubation for 30 minutes (Vectastain ABC kit). Slides were subsequently developed with 3,3'-diaminobenzidine (ImmPACT DAB Peroxidase (HRP) Substrate) for 10 minutes. Sections were counter-stained with Haematoxylin (25% w/v Harris's Haematoxylin) for 2 minutes and washed in running water for 5 minutes. Slides were dehydrated with in a series of increasing concentrations of ethanol, cleared in two changes of histoclear for 5 minutes in each and mounted using Distyrene, a plasticizer, and xylene (DPX). Secondary antibody only controls were included.

The sections were examined on a light microscope and scored using the IRS scoring system as described in Table 2.3. The percentage of positive cells was defined on a scale between 0-4, as 0% (0), <10% (1), 10–50% (2), 51–80% (3), >80% (4) and the staining intensity evaluated on a scoring scale ranging from no reaction (0), weak (1), moderate (2) to intense reaction (3). The IRS class was calculated by multiplying the quantitative expression with the intensity score giving rise to a point range between 0 and 12. IRS classification was categorised on a scale between 0 and 3 from negative (0-1), weak positive expression (2-3), moderate positive expression (4-8) and strong positive expression (9-12). Independent scoring was performed by myself and Arwa Flemban (PhD colleague) to validate the scoring assigned. Representative images were captured on a Nikon Eclipse 80i (Nikon UK, Kingston Upon Thames, UK).

Table 2.3 IRS and nuclear expression scoring system

Percentage of positive cells	Intensity of staining	IRS class (0-12)
0 = no positive cells	0 = no reaction	0-1 = negative
1 = <10% of positive cells	1 = mild reaction	2-3 = mild
2 = 10-50% positive cells	2 = moderate reaction	4-8 = moderate
3 = 51-80% positive cells	3 = intense reaction	9-12 = strongly positive
4 = >80% positive cells		

IRS - points	IRS classification (0-3)
0–1	0 = negative
2-3	1 = positive, weak expression
4-8	2 = positive, moderate expression
9-12	3 = positive, strong expression

Nuclear - score	Nuclear expression (0-3)
0	No nuclear staining
1	<10% of tumour cells have nuclear staining
2	10-50% of tumour cells have nuclear staining
3	≥51% of tumour cells have nuclear staining

IRS classification for protein expression is assessed by multiplying the scores of the percentage of positive cells and the immune reaction to give a combined IRS class on a scale between 0 and 3 (Kaemmerer *et al.*, 2012). The level of nuclear protein expression was categorised on a scale between 0 and 3, (Volante *et al.*, 2007).

2.2.2 Mammalian cell culture

All cell lines were cultured to 60-80% confluence in DMEM containing 10% FBS and 2.5 mM L-glutamine on Corning T-75 flasks in a humidified incubator at 37°C with 5% carbon dioxide. The exception to this was the MCF-10A cells, which were maintained in DMEM/F12 medium containing 5% horse serum, 2.5 mM L-glutamine, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin and 10 µg/mL insulin at 60-80% confluence in Corning T-75 flasks in a humidified incubator at 37°C with 5% carbon dioxide.

2.2.3 siRNA transfection

The siRNA sequences for *BCAT1* (Sense 5' CAUUAUCUACUGCUUCACAUU and antisense 5' UGUGAAGCAGUAGAUAAUGUU) were previously designed and validated by Tom Forshaw (Conway lab) (primers were synthesised by Eurofins Genomics, Germany). Cells were transfected with 20 nM *BCAT1* siRNA using Lipofectamine RNAimax or transfection solution prepared in Opti-MEM for 72 hours.

2.2.4 Lentiviral shRNA transfection

The pULTRA-*BCAT1* and overexpression plasmids were kindly provided by Tom Forshaw and Fred Hudd (Conway lab). In brief, for *BCAT1* knockdown, an H1 promoter-*BCAT1* shRNA sequence (CCCAATGTGAAGCAGTAGATATTCAAGA GATATCTACTGTTCACATTGGGTTTTT) cassette was assembled in the pSUPER plasmid (VEC-PBS-0002, Oligoengine, Seattle, WA) by ligation into the *BglII* and *XhoI* restriction sites. This was then amplified by PCR and the new plasmid assembled by ligation into the *SnaBI* restriction site of the pULTRA lentiviral vector (Addgene 24129, Cambridge, MA). For overexpression of *BCAT1,* the human gene was PCR amplified from pET28a (Novagen, Madison, WI)

bacterial overexpression vectors (Davoodi *et al.*, 1998) and the plasmid assembled by ligation into the BamHI and EcoRI restriction sites of pULTRA. Unmodified pULTRA plasmid with no insertions was included as a control. The pULTRA vector design included enhanced green fluorescent protein (GFP) expression, which was used to confirm uptake of the plasmid into transfected cells. These plasmids were subsequently transformed into DH5 competent cells and plasmid purified using a PureLink HiPure Plasmid Miniprep Kit (Fisher Scientific, UK) following manufacture guidelines. This work was carried out kindly by the Conway lab and subsequent steps performed by myself.

HEK293T cells, used to produce lentiviral particles, were grown in Sigma DMEM medium at a seeding density of 1 $x10^6$ cells per T25 flask and grown to 60% confluence. A solution of 2 µg polyethylenimine (PEI) in sterile serum-free Sigma DMEM was prepared, mixed by inversion, sterile filtered and incubated at room temperature for 2 minutes. Separate tubes were prepared containing 2 µg of the respective pULTRA-*BCAT1* plasmids and 20 µL MISSION® lentiviral packaging Mix, an optimised formulation containing two plasmids expressing the key packaging genes and a heterologous viral envelope gene necessary to produce viral particles in HEK293T (Albrecht *et al.*, 2015). The PEI-DMEM was mixed again by inversion and diluted 1:1 with each of the DNA-MISSION® lentiviral packaging mixtures. The solution was mixed and incubated at room temperature for 30 minutes.

The HEK293T cells were then washed twice with warmed PBS, carefully to prevent the cells from detaching. The transfection mix was added to the cells and the medium changed with 10% FBS Sigma DMEM, supplemented with 1 mM

sodium butyrate to enhance viral yield after 4 hours. The lentiviral particles were harvested 48 hours post-transfection of the HEK293T cells. The supernatant on the HEK293T cells was collected, sterile filtered and stored in aliquots at -80 °C. The HEK293T cells were replenished with fresh Sigma DMEM and lentiviral particles harvested 24 hours later (72 hours post-transfection) as previously described.

For viral transfection of the MDA-MB-231 cells, harvested lentiviral particles were incubated with the cells for at least 8 days. To improve transfection efficiency 8 μg/mL polybrene was added to the shRNA transfected cells. Transfection efficiency was assessed by fluorescent microscopy as indicated by GFP activation.

2.2.5 Cell treatments

Prior to cell treatments, cells were incubated in serum free DMEM for 24 hours. To assess insulin/IGF-1 signalling mechanisms, cells were treated with 100 ng/mL IGF-1 for 24 hours and 100 nM insulin for 24 hours following optimisation using a range of concentrations as described in section 4.3.4. Following cell treatments, cells were harvested using trypsin, cell pellets washed with PBS and protein extracted.

2.2.6 Protein extraction of tumour cell lines

Cells were washed with ice cold PBS and harvested using trypsin. Cell pellets were washed with PBS and resuspended in RIPA buffer (10 mM TRIS, 150 mM NaCl, 1 mM EDTA and 1 mM EGTA), 1 x protease inhibitor and 0.1% Triton X or 2% SDS), sonicated for 1 minute and allowed to sit on ice for 30 minutes. The cell pellet extraction buffer mixture was centrifuged at 15,000 x g at 4°C for 10 minutes. The supernatants were aliquoted and stored at -80°C.

2.2.7 Protein quantification using Amido Black assay

If SDS was used during the protein extraction, the amido black method was used for protein quantification. The extracted protein samples were diluted and standards prepared using BSA (0-10 µg/µl) in ultrapure water. To all samples and protein standard solutions 10% Tris-HCl (1M Tris, 1% SDS, pH 7.5) and 20% of 60% trichloric acid (TCA) were added, samples vortexed and incubated at room temperature for 5 minutes. The Buchner flask and nitrocellulose membrane were assembled and submitted to vacuum. The nitrocellulose membrane was prepared by washing three times with 6% TCA solution, prepared protein standards and samples were added to a 4-square surface area of the membrane and rinsed intermittently between every 4 to 5 samples with 6% TCA solution. The membrane was stained with amido black stain (100 mM amido black, 45% methanol, 10% glacial acetic acid) for 2 minutes, washed with deionised water and destained (90% methanol, 2% glacial acetic acid) for 1 minute; with the cycle repeated three times. Following destain, each 4-square surface area was evenly cut and placed into a labelled microcentrifuge tube with eluent solution (25 mM NaOH, 0.05 mM EDTA, 50% ethanol), vortexed with subsequent incubation for 30 minutes at room temperature with rocking. The change in absorbance at λ 620 nm was monitored using a Fluostar Optima spectrophotometer to generate a standard curve and protein concentration estimated for each cell lysate.

2.2.8 Protein quantification using Bradford Assay

The extracted protein samples were diluted and standards prepared using BSA. Measurements were assessed in triplicate within a 96 well plate. Bradford working solution was prepared from 1.5% stock solution (330 mg Coomassie blue G, 66.6% phosphoric acid, 33.3% ethanol), 4% phosphoric acid, 1.9% ethanol and 92.6% deionised water. To each well Bradford working solution was added and

the absorbance at λ 620 nm read using a Fluostar Optima spectrophotometer. A standard curve was generated to estimate the protein concentration of each cell lysate.

2.2.9 Protein expression using Western blot analysis

2.2.9.1 Gel electrophoresis.

Samples were prepared by combining 20 µg/µl protein, 4% β-mercaptoethanol and LDS sample buffer and adjusting total volume to 25 µl with deionised water. Prepared samples were then denatured at 95°C in a hot block for 10 minutes. Proteins were separated on a 4-12% polyacrylamide gel, comprised of a 4% acrylamide stacking gel (4% 37.5:1 acrylamide: bis-acrylamide, 25 mM Tris-HCl pH 6.8, 0.1% SDS, 0.1% APS and 0.1% TEMED) and a 12% acrylamide resolving gel (12% 37.5:1 acrylamide: bis-acrylamide, 375 mM Tris-HCl pH 8.8, 0.1% SDS, 0.1% APS and 0.06% TEMED). The gel tank was filled with 1 x running buffer (25 mM tris-HCl pH 6.8, 192 mM glycine, 0.1% SDS) and run at 100 V for 20 minutes and then 170 V for 60 minutes.

2.2.9.2 Wet transfer Western blot.

Proteins were transferred onto activated (100% methanol, 10 minutes) PVDF membrane pre-soaked in transfer buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% methanol). The proteins were transferred at 30 V for 18 hours at 4°C for high molecular weight proteins and at 50 V for 2 hours at 4°C for low molecular weight proteins. After the proteins had transferred to the membrane, the membrane was removed and washed in Tris buffered saline tween (TBST) (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 3 x 10 minute washes before being blocked with blocking solution (5% marvel milk powder in TBST) for 1 hour at room temperature. The blocking solution was then removed and replenished with

primary antibody prepared in fresh blocking solution at the indicated concentrations in Table 2.1 and incubated for 18 hours at 4°C. The membrane was washed with TBST for 3 x 10 minute washes at room temperature and incubated with secondary antibody diluted (1:1,000 for anti-mouse and 1:3,000 for anti-rabbit) in fresh blocking solution for 1 hour at room temperature in the dark. The membrane was washed with TBST for 3 x 10 minute washes at room temperature. Luminata Forte ECL solution was used to visualise the bands using a Licor odyssey® FC system. Images were captured and densitometries obtained using the Image Studio™ software.

2.2.10 ^{3}H Thymidine Incorporation Proliferation Assay

Radioactively labelled (tritium) thymidine was used to measure the proliferation of MDA-MB-231 cells by the incorporation of [^{3}H] thymidine into the DNA of dividing cells. Cells were seeded at a seeding density of 2 x10^{4} cells in 24 well plates. After 24 hours serum starvation and following 24 hours treatment with insulin (100 nM) or IGF-1 (100 ng/mL), *BCAT1*-siRNA (20 nM) transfected cells were labelled with 0.1 µCi/mL [^{3}H] thymidine, incubated with 5% trichloroacetic acid at 4°C for 10 minutes, followed by a 1 hour incubation with 1 M sodium hydroxide. The resulting suspension was added to a vial containing 2 mL ultima gold liquid scintillation cocktail and incorporated counts were measured using a Beckman Scintillation Counter LS6500. Data were recorded as disintegrations per minute (DPM). All assays were done in triplicate and results were analysed using an unpaired t-test.

2.2.11 Apoptosis assay

To determine the effect of *BCAT1* siRNA knockdown on cell apoptosis, MDA-MB-231 cells were seeded at a density of 1x10^{6} cells per T-25 flask and treated with 20 nM *BCAT1* siRNA for 72 hours. Cells were detached and washed twice with ice cold PBS and resuspended in 1X binding buffer. Annexin V-FITC and

propidium iodide (PI) were added into the binding buffer and incubated for 10 min at room temperature in the dark. The following controls were included to ensure proper analysis; unstained control and two positive controls of cells incubated in a heat block for 10 seconds at 60°C, one was stained with Annexin V-FITC and one stained with PI. One untreated control was also included to determine to ensure that the cells were not subjected to damage during experimental work.

Analysis was performed on a BD Accuri™ C6 Flow Cytometer (BD Biosciences, UK) and data was analysed using Accuri™ C6 Plus software (BD Biosciences, UK) to identify the subpopulations of the apoptotic cells. Cells were gated to exclude debris and cellular doubling, which was conducted on unstained controls from the analysis of >10,000 events. A single cell population was gated based on appropriate size on the basis of forward scatter (FCS-A) versus side scatter (SSC-A). Increased fluorescence was compared to unstained controls. Compensation for Annexin V-FITC/PI signal overlap was conducted using positive Annexin V-FITC control, positive PI control and mixed control (Annexin V-FITC and PI positive). Three independent experiments were performed and two-way ANOVA using Tukey honestly significant differences for statistical analysis.

2.2.12 Migration and Invasion assay

Transwell inserts containing polycarbonate filters with 8 μm pores were used in the assay in 24-well plates. The inserts were coated with 10μg/ml Collagen-I to measure migration and Matrigel® to assess invasion 24 hours prior to cell seeding at room temperature. Following cell treatment, cells were seeded in the upper chambers of the inserts at a density of $1x10^5$ in serum-free DMEM in a humidified incubator at 37°C with 5% carbon dioxide. DMEM containing 10% FBS was placed in the lower chambers. Following 24 hours, the cells on the upper surface

of the membrane were removed using an ethanol coated cotton swab. Cells on the lower chamber were fixed in 4% paraformaldehyde and stained with 0.2% Crystal Violet. For each insert, representative images in 3 evenly distributed x40 fields of view were captured using a light microscope (Zeiss AX10). To quantify migration of cells, 0.1% SDS in PBS was added to the lower chambers and absorbance intensity measured on a plate reader at an excitation of 590 nm. Each experiment was repeated in triplicate.

2.2.13 Immunocytochemistry

For each experiment controls with medium alone and secondary antibody controls were included. Sterile 18 mm diameter rounded coverslips were treated with 0.01% poly-l-lysine for 20 minutes, washed 3 times with distilled water and one placed per well in a 12 well plate. MDA-MB-231 cells were seeded at a density of $5x10^4$ cells per well onto coverslips. After treatment, med/treatments were removed and each well washed twice with PBS. In the fume hood, 4% paraformaldehyde in PBS was added to each well for 20 minutes to fix the cells to the coverslips. Fixing solution was then removed and washed twice with PBS containing 0.01% Triton X-100 to permeabilise cells. The cells were then placed in blocking serum, (10% FBS, 1% BSA and 1% fish gelatine) for 1 hour at room temperature. Primary antibodies were prepared in blocking serum and applied to the cells overnight at 4 °C. The following day, secondary antibodies were prepared in blocking serum. Cells were then washed twice with PBS and the secondary antibody solution added for 1.5 hours in the dark at room temperature. Slides were sterilised with 70% ethanol and labelled. Following 2 PBS washes, coverslips, cell side down, were mounted onto labelled slides using hard-set mounting medium with DAPI. Slides were left in the fridge in the dark ready for

imaging. Labelled cells were imaged using a Zeiss Axiovert 200 confocal microscope.

To quantify nuclear translocation of phospho-MAPK and MAPK Mander's correlation coefficient (Mx) of the overlap of the 568 (red) channel with the 405 (blue) channel were derived using Velocity (Perkin-Elmer). Prior to analysis, the immunofluorescence z-stack images were deconvolved which was used to enhance fluorescent signal and reduce background noise (Biggs, 2010). Confocal analysis using Mx is a robust method for the quantification of the overlap of pixels in two distinct microscopy channels, whilst accounting for different thresholds of signal intensities for the different channels (Dunn, Kamocka and McDonald, 2011). The Mx value signifies the level of co-localisation whereby the signal intensity is quantified with 0 indicating no co-localisation and 1.0 indicating complete co-localisation.

2.2.14 Seahorse Glycolytic Rate assay

The XF^e24 Extracellular Flux analyzer (Seahorse Biosciences, Billerica, MA, USA) was used to measure the rate of glycolysis in the MDA-MB-231 cells. The cellular oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) were measured and expressed as pmol/min and mpH/min, respectively. Glycolytic rates for basal conditions and compensatory glycolysis following mitochondrial inhibition were assessed, to account for contribution of CO_2 to extracellular acidification derived from mitochondrial/TCA cycle activity.

Seeding density was optimised at 4 $x10^4$ cells per well to ensure basal OCR and ECAR was within the recommended range of 50-400 pmol/min and 20-120 mpH/min respectively. Cells transfected with 20 nM *BCAT1* siRNA or with

RNAimax were seeded in a 24 well plate. After 24 hours cells were incubated with serum free DMEM 24 hours before cell treatments with 100 ng/mL IGF-1 for 24 hours. The Agilent Seahorse XF^{e}24 Sensor Cartridge was hydrated at 37°C in a non-CO_2 incubator using XF Calibrant buffer 24 hours before the assay. XF assay phenol red free medium was prepared by the addition of 2 mM glutamine, 10 mM glucose, 1 mM pyruvate and 5 mM HEPES fresh on the day of the assay. Cells were washed once with warmed assay medium and incubated with assay medium for 60 minutes prior to the assay. Subsequently, freshly prepared solutions of Antimycin A (0.5 μM), a mitochondrial inhibitor and 2-deoxy-D-glucose (50 mM), a glycolysis inhibitor were added to specific ports in the sensor cartridges. The prepared sensor cartridges with calibrant plate were loaded into the Seahorse XF^e Analyser for calibration. Once calibrated, assay medium on the cells was replaced with fresh assay medium and glycolytic rate was measured on the Seahorse XF^e Analyzer.

2.2.15 Statistical analysis

Data were statistically analysed using GraphPad Prism 8.0 software for Mac and values expressed as mean ± standard error of the mean (SEM). Significance levels for comparisons between groups were determined with unpaired two-tailed Student's t-test. Statistical significance was reported when $p < 0.05$. Immunohistochemical data was analysed using SPSS (Version 24; SPSS Inc., Chicago, IL, USA) for Mac. Linear by linear association was used to analyse the relationship between the expression of the metabolic proteins and clinicopathological characteristics. Kaplan–Meier survival curves and log-rank statistics were used to evaluate time to tumour recurrence and overall survival.

Chapter 3

3.0 Characterisation of Metabolic Proteins in Breast Cancer

Mai Ahmed Shafei

3.1 Introduction

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3.2 Aims and Objectives

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3.3 Results

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3.4 Discussion

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Chapter 4

4.0 The role of hBCATc in the IGF-1/insulin signaling cascades

Mai Ahmed Shafei

4.1 Introduction

Driven by oncogenic signals, cancer cells undergo metabolic reprogramming, upregulating metabolite transporters and shuttling nutrients to promote proliferation and migration (Yoshida, 2015). Altered metabolic activity fulfils the increased metabolic demand for proliferating cells to support tumour growth during nutrient limitation and fortification of redox homeostatic systems to counteract the metabolic stress caused by malignant transformation (Boroughs and DeBerardinis, 2015). The Ras/MAPK (also known as RAS/ERK) and PI3K/Akt/mTOR signalling pathways are the cell's chief mechanisms for controlling cell survival, proliferation, metabolism and motility (Mendoza, Er and Blenis, 2011). These pathways are controlled through the import of nutrients into cells by growth factors, such as IGF-1 and insulin, which mediate a cascade of events that regulate numerous cellular processes including cell growth and migration (DeBerardinis and Chandel, 2016).

Activation of the IGF-1/insulin signalling cascades has been associated with increased cell proliferation and metastases in breast cancer (Byron *et al.*, 2006). Increased expression of IGF-1R and IR has been demonstrated to strongly correlate with poor patient clinical outcomes across the different molecular subtypes of breast cancer (Law *et al.*, 2008). Despite these correlations, clinical results of anti-IGF-IR monoclonal antibody trials in breast cancer have been disappointing (Ekyalongo and Yee, 2017), highlighting the need for new therapeutic targets for the modulation of this pathway. There is emerging evidence for cross-talk between BCAA metabolism and pathways associated with insulin resistance (Wubetu *et al.*, 2014; Hagiwara, Nishiyama and Ishizaki, 2012).

Nutrient levels of BCAAs particularly leucine, substrates of hBCAT, regulate the mTOR pathway (Son *et al.*, 2019) where a role for hBCAT proteins is proposed in regulating the PI3K/Akt/mTOR axis. Moreover, glutamate, a product of hBCAT metabolism, also activates mTOR, a serine-threonine kinase that binds with proteins to induce intracellular signalling pathways directly involved in glutaminolysis, nucleotide and protein synthesis, supporting tumour growth (Laplante and Sabatini, 2013). Nutrient status is also sensed by the general control non-derepressible 2 kinase (GCN2) and together with mTOR co-ordinate cell growth, proliferation and cell survival (Anthony *et al.*, 2004; Xiao *et al.*, 2011). Therefore, by upregulating membrane transporters and nutrient uptake, cancer cells support the increased demand of macromolecules needed for cell growth (Hattori *et al.*, 2017). Pull-out studies have recently established that the hBCAT proteins can form thiol-dependent interactions with key signalling pathways such as the PI3K/Akt/mTOR and Ras/ERK pathways (under review) and show that phosphorylation of hBCAT by kinases is redox dependent. Here, in this chapter, for the first time hBCATc was found to regulate cross-talk between the PI3K/Akt/mTOR and the Ras/ERK pathway under IGF-1 and insulin treatment in TNBC, indicating a novel role for this metabolic protein in the regulation of IGF-1/insulin-mediated proliferation and migration.

4.2 Aims and Objectives

4.2.1 Hypothesis

hBCATc regulates proliferation and migration of TNBC cells through the IGF-1 and insulin signalling pathways

4.2.2 Specific Aims

Specific aim 1: To optimise siRNA-mediated knockdown of *BCAT1* in TNBC MDA-MB-231 cells.

Specific aim 2: To assess the role of hBCATc in cell proliferation, migration, invasion and apoptosis in TNBC.

Specific aim 3: To establish whether expression of hBCATc plays a role in insulin and IGF-1 mediated proliferation and migration.

Specific aim 4: To investigate the role of hBCATc in the regulation of the two principal cell signalling pathways; Akt/mTOR and RAS/ERK using shRNA-mediated *BCAT1* knockdown and overexpression constructs.

Specific aim 5: To evaluate the role of hBCATc expression on downstream effectors of the Akt/mTOR and RAS/ERK signalling cascades.

4.3 Results

4.3.1 Confirmation of siRNA knockdown of BCAT1

The siRNA sequences for *BCAT1* were previously designed and validated by Tom Forshaw (Conway lab) using neuroblastoma SH-SY5Y cells. The concentration of *BCAT1* siRNA was optimised using a range of concentrations and the level of knockdown of hBCATc protein expression assessed by Western blot analysis in TNBC MDA-MB-231 cells. A concentration of 12.5 nM *BCAT1*-siRNA was found to be insufficient to induce knockdown, whilst a concentration of 25 nM and 50 nM reduced hBCATc protein expression compared with the transfection control (Figure 4.1A). A lower siRNA concentration of 20 nM for 72 hours was found to be sufficient to significantly reduce hBCATc protein expression by 80% compared to transfection control ($p < 0.0001$) (Figure 4.1B).

4.3.2 Knockdown of *BCAT1* reduced cell proliferation, migration and invasion

Using the optimised conditions for *BCAT1* knockdown, hBCATc modulation of proliferation of TNBC cells was assessed utilising the tritiated thymidine incorporation (TTI) assay. This assay measures the incorporation of radioactive [^{3}H] thymidine into the DNA of dividing cells using a scintillation counter (Taylor, Woods and Hughes, 1957). Knockdown of *BCAT1* was found to significantly reduce the proliferation of MDA-MB-231 cells ($p < 0.0001$) (Figure 4.2A). To investigate the role of hBCATc in tumour invasiveness *in vitro* transwell inserts with 8 µm pores (Greiner Bio-One, Stonehouse, UK) were coated with 10 µg/ml Collagen-I to measure migration and Matrigel® added to the inserts to assess invasion of the MDA-MB-231 cells (Kramer *et al.*, 2013). Migration and invasion of TNBC was significantly reduced following siRNA-mediated knockdown of *BCAT1* ($p < 0.0001$) suggesting hBCATc to play a significant role in these tumour promoting processes (Figure 4.2B and 4.2C).

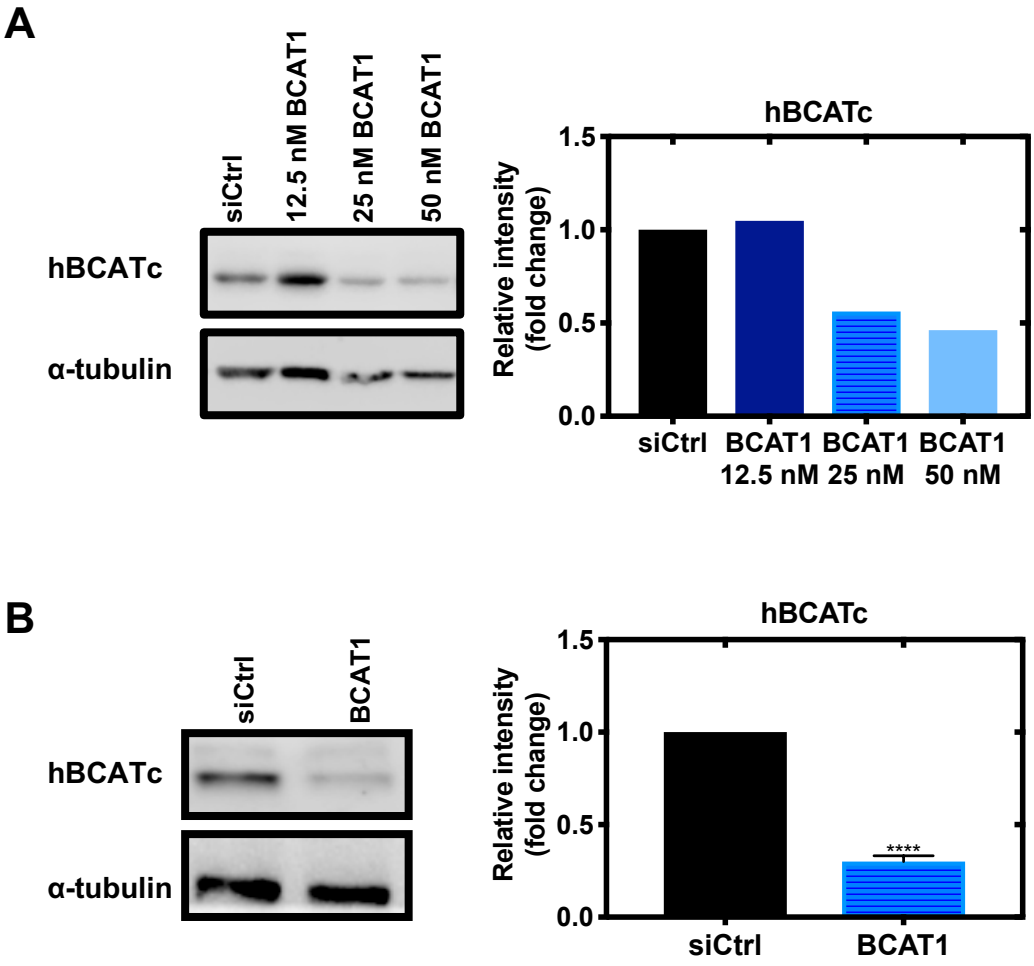

Figure 4.1. Optimisation of siRNA knockdown of BCAT1 in MDA-MB-231 cells. Western blot analysis to assess hBCATc expression (**A**) Cells treated with 12.5, 25 and 50 nM *BCAT1* siRNA for 72 hours (**B**) Cells treated with 20 nM *BCAT1* siRNA transfection for 72 hours. Respective densitometric analysis of fold changes of protein expression relative to α-tubulin are presented to the right of immunoblots. Data representative of mean ±SEM (n=3). Statistical significance was calculated using t-tests, ***p < 0.001.

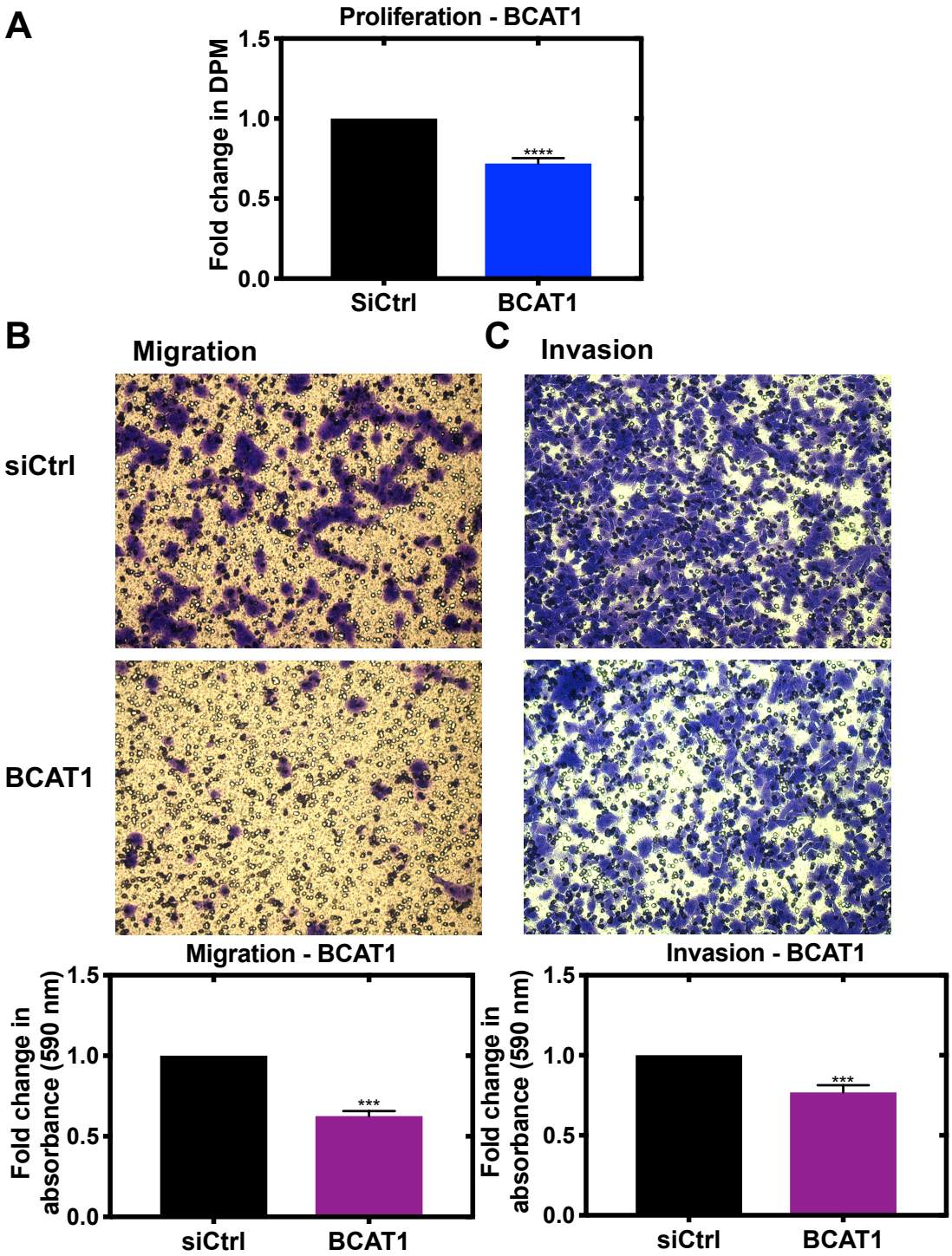

Figure 4.2. Knockdown of BCAT1 significantly reduces proliferation, migration and invasion of MDA-MB-231 cells. Cells were treated with 20 nM *BCAT1* siRNA for 72 hours and the effect on proliferation assessed using the tritiated thymidine incorporation assay, migration was assessed using cells seeded onto 8 µm Transwell inserts (Greiner Bio-One) coated with collagen and after 24 hours, migrated were fixed and stained with 0.2% Crystal Violet, solubilised and absorbance measured and to assess invasion Matrigel added to the inserts as described above (**A**) Fold change in disintegrations per minute (DPM) and representative images of (**B**) migrated cells and (**C**) invaded cells with fold changes in absorbance at 590 nm ±SEM presented below (n=3). Statistical significance was calculated using t-tests,*** p< 0.001 and ****p < 0.0001.

As knockdown of *BCAT1*, which is responsible for the reversible catabolism of BCAA, reduced cell proliferation, the effect of BCAA levels on proliferation were assessed in the TNBC cells. Leucine treatment at 10 mM and 20 mM significantly increased cell proliferation ($p < 0.01$, $p=0.027$) compared to control cells (Figure 4.3). However, at higher concentrations of 20 mM and 40 mM leucine there was a dose-dependent decrease in proliferation relative to 10 mM leucine treatment. Similarly, isoleucine treatment at 20 mM and 40 mM significantly decreased proliferation of cells relative to cells treated with 10 mM isoleucine ($p=0.0201$). Valine treatment at all concentrations resulted in a similar level of increased cell proliferation. These results indicate that levels of BCAAs may play an important role in regulating hBCATc-mediated proliferation.

4.3.3 Knockdown of *BCAT1* induces apoptosis in MDA-MB-231 cells

To assess the effect of hBCATc on apoptosis in MDA-MB-231 cells following siRNA mediated knockdown of *BCAT1*, fluorescein isothiocyanate (FITC)-Annexin V detection was assessed by flow cytometry (Figure 4.4). Probing the cells with FITC-Annexin V in combination with propidium iodide (PI) allows separation of live, early-apoptotic, late-apoptotic and dead cells (Casciola-Rosen *et al.*, 1996). This assay is based on the observation that during early apoptosis, cells translocate phosphatidylserine from the inner surface of the plasma membrane to the cell surface (Rimon *et al.*, 1997). Once on the cell surface, phosphatidylserine can be specifically detected by staining with FITC-labelled Annexin V, thus identifying cells in early apoptosis. PI is a fluorescent dye used in combination with Annexin V which specifically binds to DNA to detect late apoptosis and necrotic cells (Nicoletti *et al.*, 1991). Knockdown of *BCAT1* using siRNA resulted in a significant decrease of live cells ($p < 0.0001$) whilst increasing cells in early

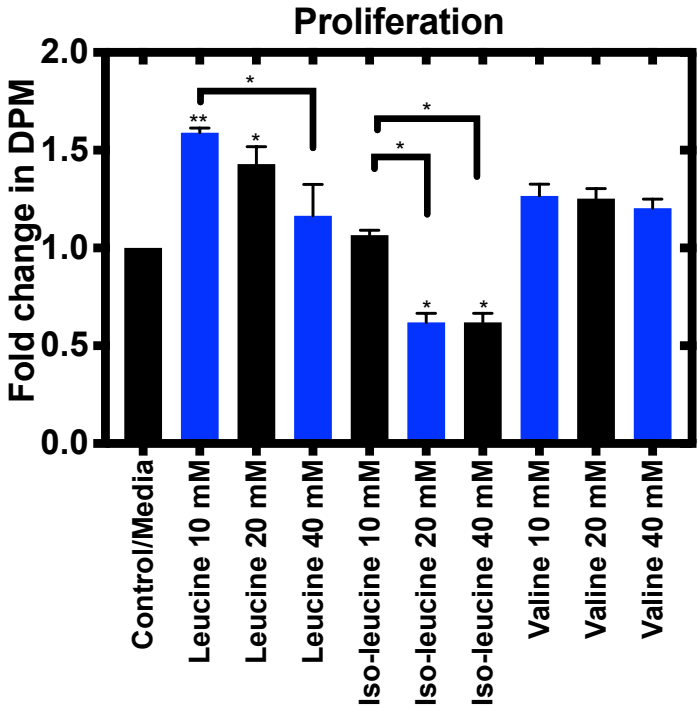

Figure 4.3. Branched-chain amino acid treatment regulates cell proliferation in a dose-dependent manner. Cells were treated with leucine, isoleucine and valine at the indicated concentrations. Tritiated thymidine incorporation (TTI) assay was used to assess changes in cell proliferation following treatments compared with control cells. Data presented as fold changes of mean values of disintegrations per minute (DPM) ±SEM, relative to control (n=3) Statistical significance was calculated by one-way ANOVA using Tukey honestly significant differences,*$p < 0.05$, **$p < 0.01$ and ****$p < 0.0001$.

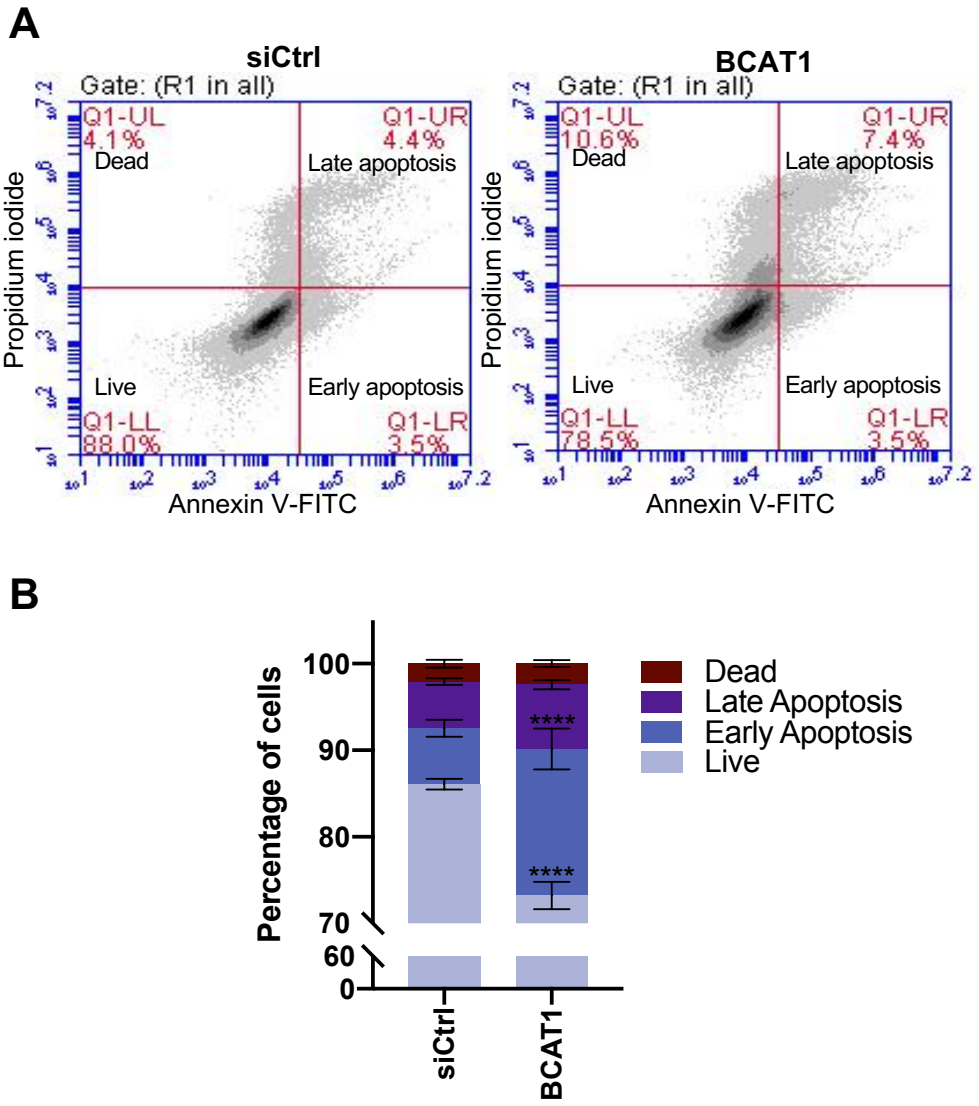

Figure 4.4. *BCAT1* promotes tumour cell survival by evasion of apoptosis. Cells were treated with 20 nM *BCAT1* siRNA for 72 hours or transfection control, and cell apoptosis was determined by FITC-Annexin V and propidium iodide staining using flow cytometry. Unstained cells were gated to exclude debris and cellular doubling from the analysis of >10,000 events and used to delimit background fluorescence. Controls including the combination of cells stained with Annexin V-FITC and cells stained with propidium iodide were used to set the compensation level for differentiating between the two signals (**A**) Representative flow cytometric analysis (**B**) Data representative of mean ±SEM (n=3). Statistical significance was calculated by two-way ANOVA using Tukey honestly significant differences, ****p < 0.0001.

apoptosis ($p < 0.0001$) compared with cells treated with the transfection mix alone as control. The percentage of cells in late apoptosis and dead cells following *BCAT1* knockdown were also increased although this did not reach significance. These results indicate that hBCATc regulates early apoptosis in TNBC.

4.3.4 hBCATc regulates proliferation and migration through the IGF and insulin signalling pathway

The potential role of hBCATc in tumour progression induced by insulin and IGF-1 in tumour growth and metastatic potential of TNBC cells was evaluated using the TTI assay and the Transwell migration assay, respectively. Initially, MDA-MB-231 cells were treated with insulin and IGF-1 at a range of concentrations for 24 hours and cell proliferation assessed. Insulin treatment at a concentration of 50 nM significantly increased cell proliferation ($p < 0.001$), whilst 100 nM insulin treatment showed a moderately higher increase in cell proliferation ($p < 0.0001$) (Figure 4.5A). Although treatment of the TNBC cells with 400 nM insulin showed the highest increase in cell proliferation ($p < 0.0001$), a concentration of 100 nM was selected for further experimental work as increased proliferation observed between these two concentrations was not significantly higher despite being four times the concentration. IGF-1 treatment at a concentration of between 12.5 ng/mL to 50 ng/mL for 24 hours did not show a significant change in cell proliferation as observed in Figure 4.5A. A significant increase in cell proliferation was observed only at a concentration of 100 ng/mL IGF-1 for 24 hours ($p=0.0241$) relative to control which was therefore selected as the optimal concentration for further experimental work.

At the optimised conditions of 100 ng/mL IGF-1 and 100 nM insulin cell proliferation of MDA-MB-231 cells was increased ($p < 0.0001$ and $p= 0.1332$

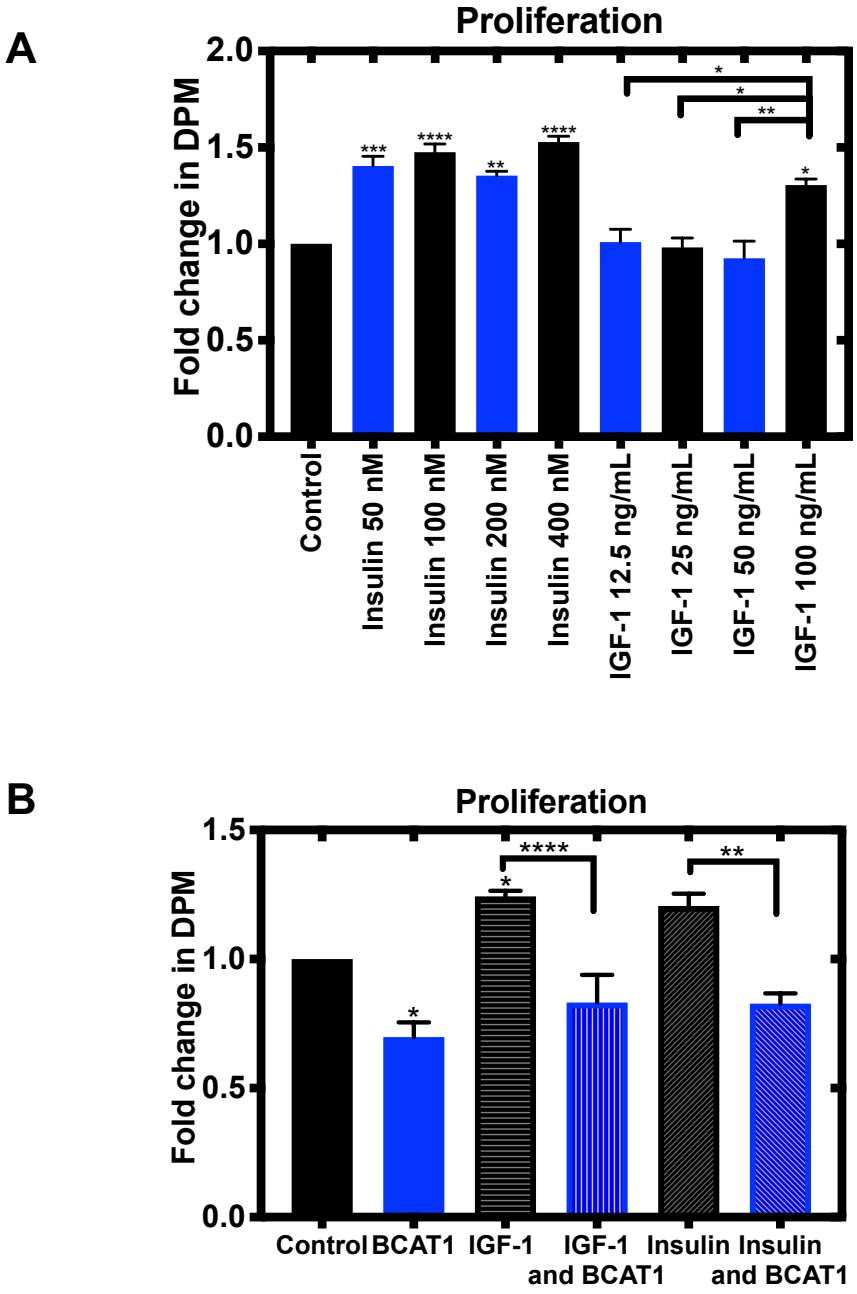

Figure 4.5. Knockdown of BCAT1 significantly reduces insulin and IGF-1-mediated proliferation of MDA-MB-231 cells. (**A**) Cells were treated with insulin and IGF-1 at the indicated concentrations and cell proliferation measured using the thymidine incorporation (TTI) assay. Fold changes of mean values of disintegrations per minute (DPM) ±SEM, relative to control. (**B**) Cells were treated with 20 nM *BCAT1* siRNA, 100 nM insulin and 100 ng/mL IGF-1 accordingly and tritiated thymidine incorporation assay used to assess changes in cell proliferation. Data presented as fold changes of mean values of DPM ±SEM, relative to control. Statistical significance was calculated by one-way ANOVA using Tukey honestly significant differences, *p < 0.05, **p < 0.01, *** p< 0.001 and ****p < 0.0001.

respectively) (Figure 4.5B). However, *BCAT1* knockdown significantly reduced this effect, leading to a significant decrease in cell proliferation with insulin treatment ($p < 0.01$). Similarly, although combined treatment of TNBC cells with IGF-1 and *BCAT1* knockdown did not significantly decrease cell proliferation compared to control, a significant decrease in cell proliferation compared with IGF-1 treatment was observed ($p < 0.0001$). Further, treatment of MDA-MB-231 cells with 100 nM insulin, for 24 hours significantly increased cell migration of TNBC cells ($p < 0.01$) (Figure 4.6). Knockdown of *BCAT1* significantly abrogated IGF-1 and insulin mediated cell migration ($p < 0.001$ and $p < 0.0001$, respectively). These results combined suggest that hBCATc plays an intrinsic role in insulin and IGF-1 mediated cell proliferation and migration.

4.3.5 Changes in expression of hBCATc does not affect glycolysis

Changes in the glycolytic rate were assessed using a XF^e24 Extracellular Flux analyser (Seahorse Biosciences, Billerica, MA, USA) which contains sensitive sensors which measure cellular oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR), using small microchambers on the cells (Romero *et al.*, 2017). Glycolysis and mitochondrial-derived CO_2 (oxidative phosphorylation) are the two main contributors to extracellular acidification and proton efflux rate (PER) in cultured cells. Basal ECAR and OCR were measured followed by sequential injection of rotenone and antimycin A (Rot/AA), a mitochondrial inhibitor and 2-deoxy-D-glucose to assess complementary glycolysis (Figure 4.7A). There was an inhibition of mitochondrial induced compensatory glycolysis as indicated by a rapid increase in ECAR and by a rapid decrease in OCR for all conditions (Figure 4.7B). Changes in ECAR and OCR following Rot/AA injection were similar for cells with shRNA-mediated knockdown

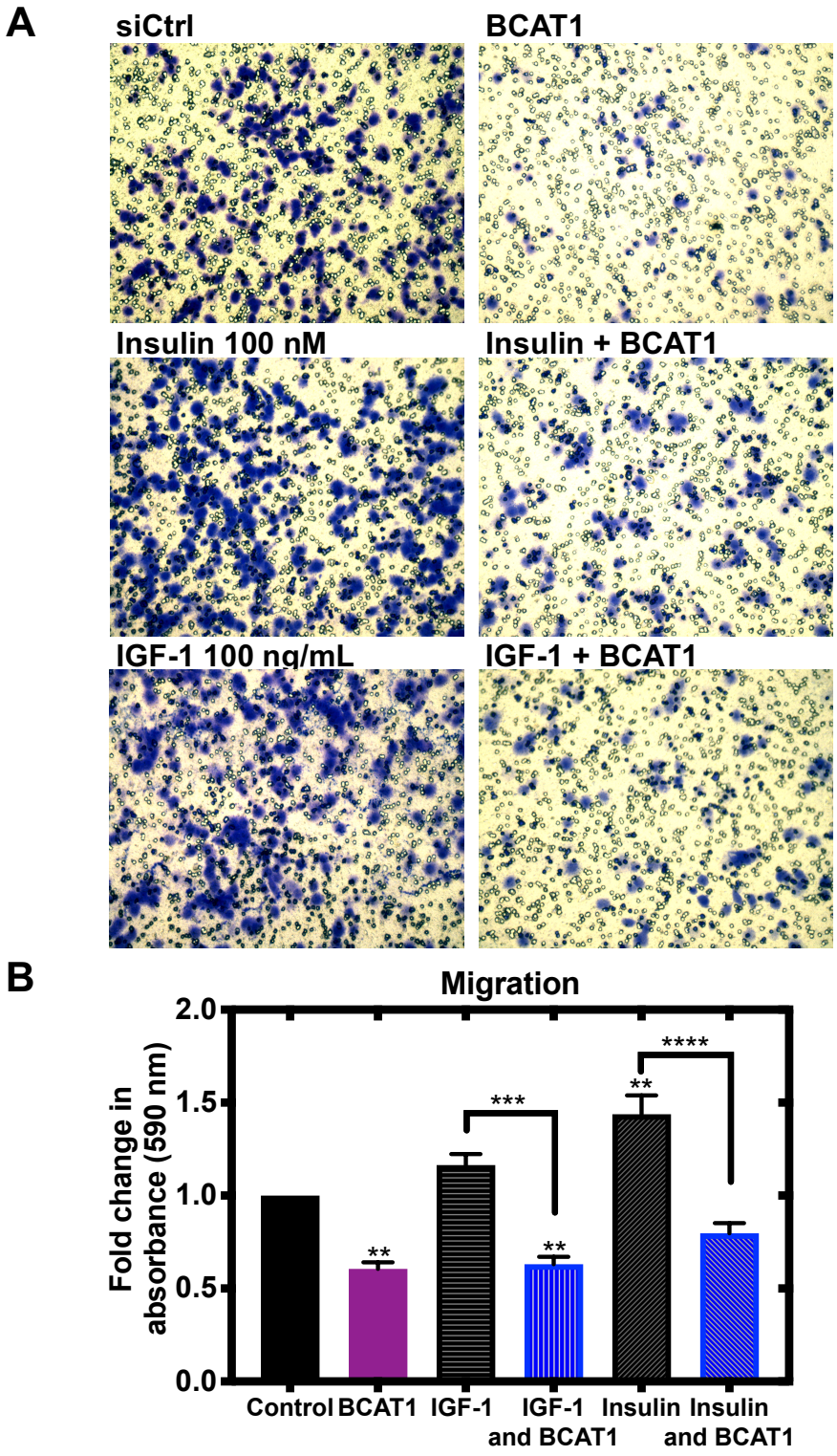

Figure 4.6. Knockdown of BCAT1 significantly reduces insulin and IGF-1-mediated migration of MDA-MB-231 cells. Cells were treated with 20 nM *BCAT1* siRNA, 100 nM insulin and 100 ng/mL IGF-1 accordingly and migration assessed using cells seeded onto 8 µm Transwell inserts (Greiner Bio-One) coated with collagen and after 24 hours, migrated cells were fixed and stained with 0.2% Crystal Violet, solubilised and absorbance measured (**A**) Representative images of migrated cells (**B**) Data presented as mean fold changes of absorbance at 590 nm. Statistical significance was calculated by one-way ANOVA using Tukey honestly significant differences, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

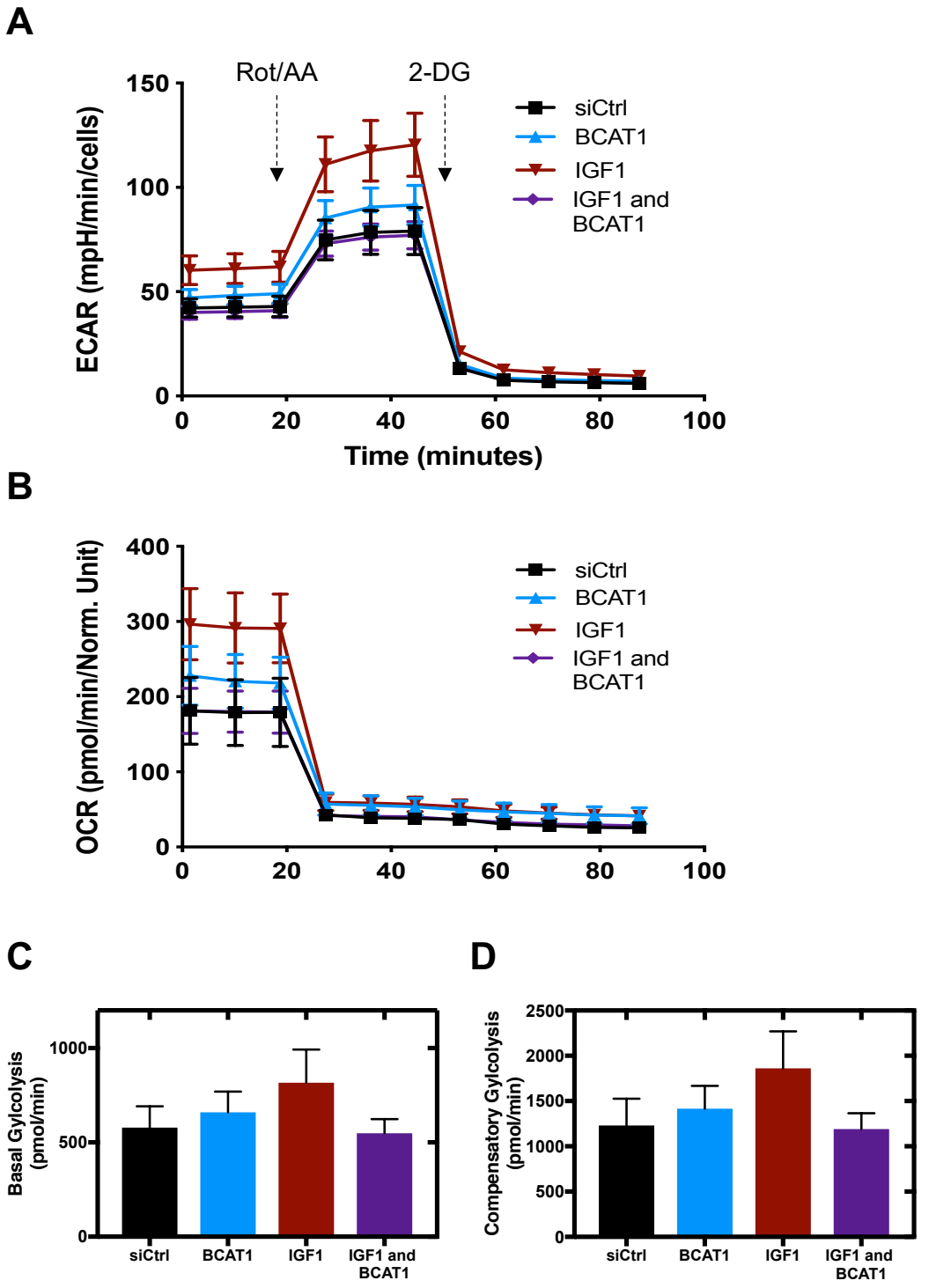

Figure 4.7. hBCATc regulates IGF-1-mediated glycolysis in MDA-MB-231 cells. Cells were treated with 20 nM *BCAT1* siRNA, 100 nM insulin and 100 ng/mL IGF-1 accordingly. Glycolytic rate was assessed using a Seahorse XF^{e}24 analyser. (**A**) Extracellular acidification rate (ECAR) and (**B**) oxygen consumption rate (OCR) were measured followed by sequential injection of rotenone and antimycin A (Rot/AA), a mitochondrial inhibitor and 2-deoxy-D-glucose and (**C**) basal glycolysis and (**D**) compensatory glycolysis assessed. Data representative of mean ±SEM (n=3) statistical significance was calculated by one-way ANOVA using Tukey honestly significant differences.

and overexpression of *BCAT1* compared to cells containing empty vector. Last, addition of 2-deoxy-D-glucose (2-DG) to block glycolysis decreased ECAR back to basal rates in all conditions (Figure 4.7A). Knockdown of *BCAT1* did not affect basal glycolytic rate or compensatory glycolysis (Figure 4.7C and 4.7D). Treatment with IGF-1 resulted in a moderate increase of compensatory glycolysis, which was ameliorated following *BCAT1* knockdown, suggesting hBCATc to play a role in IGF-1-mediated glycolysis.

4.3.6 Development of stable transfection model using shRNA

Transduction with lentiviral vectors is well characterised as a highly efficient method capable of transducing cells to modulate expression of target genes (Blömer *et al.*, 1997). To further understand the role of hBCATc upregulation in TNBC on downstream pathways affecting tumour progression, a stable transfection model for knockdown and overexpression for hBCATc was used for further work (Figure 4.8). This transfection model was optimised by Tom Forshaw (Team Conway). MDA-MB-231 cells were incubated with medium containing pULTRA-empty vector shRNA, pULTRA-*BCAT1* knockdown and pULTRA-*BCAT1* overexpression GFP-tagged shRNA lentiviral particles. Ideally, two independent *BCAT1*-shRNA sequences, targeting different regions in *BCAT1* should have been used as a control for off-target knockdowns in this study (Moore *et al.*, 2010). The target sequences for *BCAT1-shRNA* mediated knockdown were obtained from Tönjes *et al.* (2013) and have been validated in multiple studies (Raffel *et al.*, 2017; McBrayer *et al.*, 2018) as well as in MDA-MB-231 cells (Thewes *et al.*, 2017). Almost 100% of cells were confirmed to uptake the lentiviral particles as confirmed by glowing cells on a fluorescent microscope as compared with control

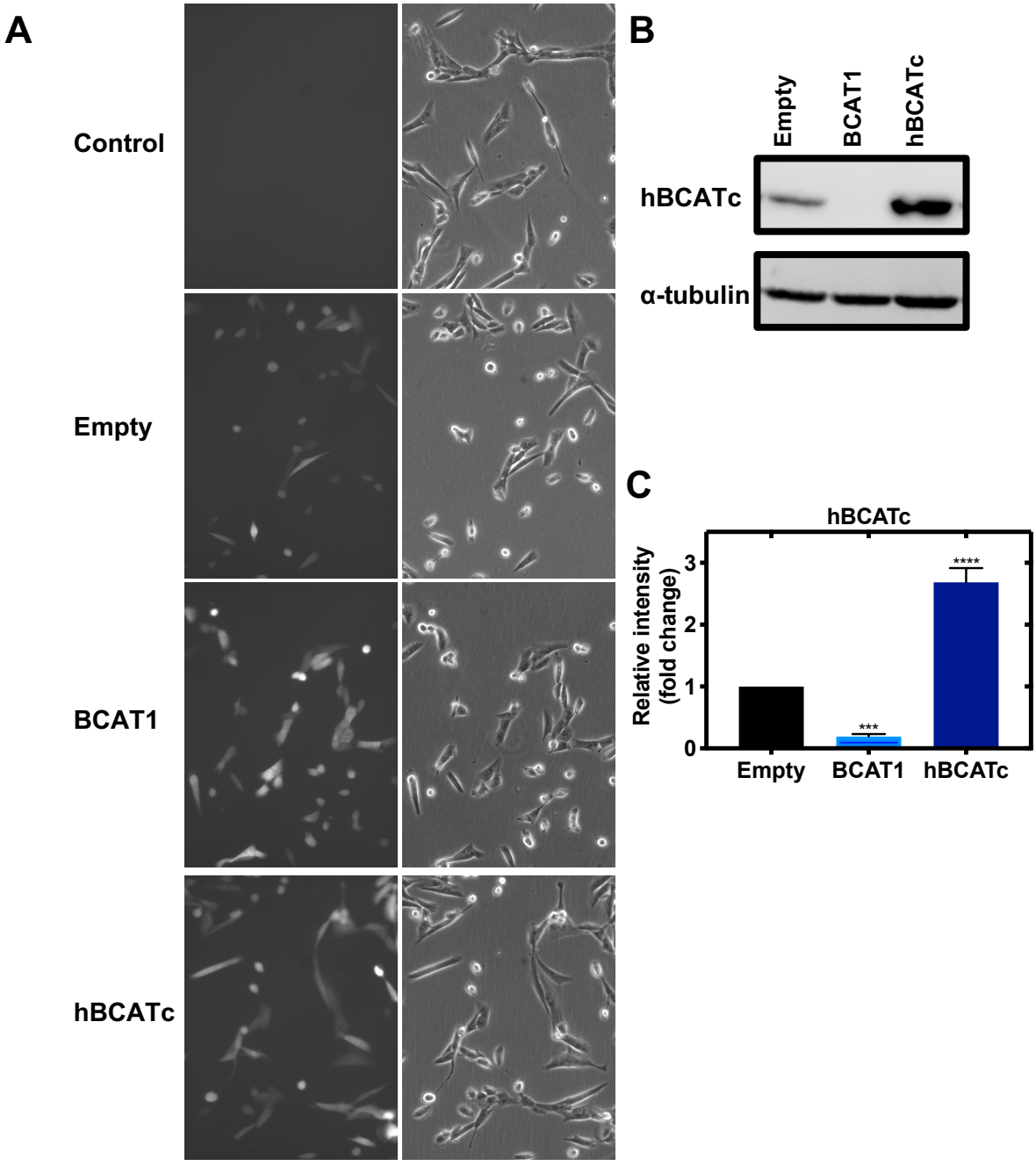

Figure 4.8. shRNA Knockdown of BCAT1 and overexpression confirmation in MDA-MB-231 cells. Cells were stably transfected with shRNA for knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty). (**A**) Representative images following 8 days of incubation with shRNA lentivirus to show transduction efficiency, to the right cells are light microscopy and to the left FITC-filter images. (**B**) Western blot analysis of *BCAT1* knockdown and overexpression. (**C**) Densitometry analysis of fold changes of hBCATc expression relative to α-tubulin normalized to control. Data representative of mean ±SEM (n=3). Statistical significance was calculated using t-tests, *** $p < 0.001$ and **** $p < 0.0001$.

cells which were not exposed to the lentiviral particles (Figure 4.8A). Knockdown and over expression of hBCATc, following transduction with lentiviral particles containing the respective shRNA was confirmed using Western blot analysis (Figure 4.8B and 4.8C). Stable transfection of *BCAT1*-shRNA resulted in 80% decrease of hBCATc protein expression ($p < 0.001$) whilst the overexpression vector increased hBCATc protein expression by 2.68-fold ($p < 0.001$), indicating these shRNA constructs to significantly suppress and overexpress hBCATc protein expression.

4.3.7 hBCATc regulates IGF-1 mediated signalling pathway proteins but attenuates MAPK phosphorylation

To further elucidate the potential role of hBCATc in mediating IGF-1 and insulin induced cell proliferation and migration, following shRNA-mediated *BCAT1* knockdown and overexpression the protein expression of downstream targets of the insulin/IGF-1 signalling pathway were assessed using Western blot analysis. Knockdown and overexpression of *BCAT1* using shRNA constructs was confirmed by Western blot analysis for all biological repeats (Figure 4.9A). Knockdown of *BCAT1* led to significant decrease in the levels of the IGF-1 receptor β subunit (IGF-1Rβ) ($p < 0.001$) (Figure 4.9B). In line with this, although protein expression of growth factor receptor-bound 2 (Grb2) remained unchanged (Figure 4.9D), levels of the p66 isoform of Src homology 2 domain containing transforming protein (Shc) and son of sevenless (SOS) were shown to be significantly increased following hBCATc overexpression ($p=0.0235$ and $p=0.0122$ respectively) (Figure 4.9C and 4.9E). The increased level of these two proteins without a significant change in levels when hBCATc is knocked down indicates that hBCATc may sequester these scaffold proteins in the cytoplasm rather than affect their transcription.

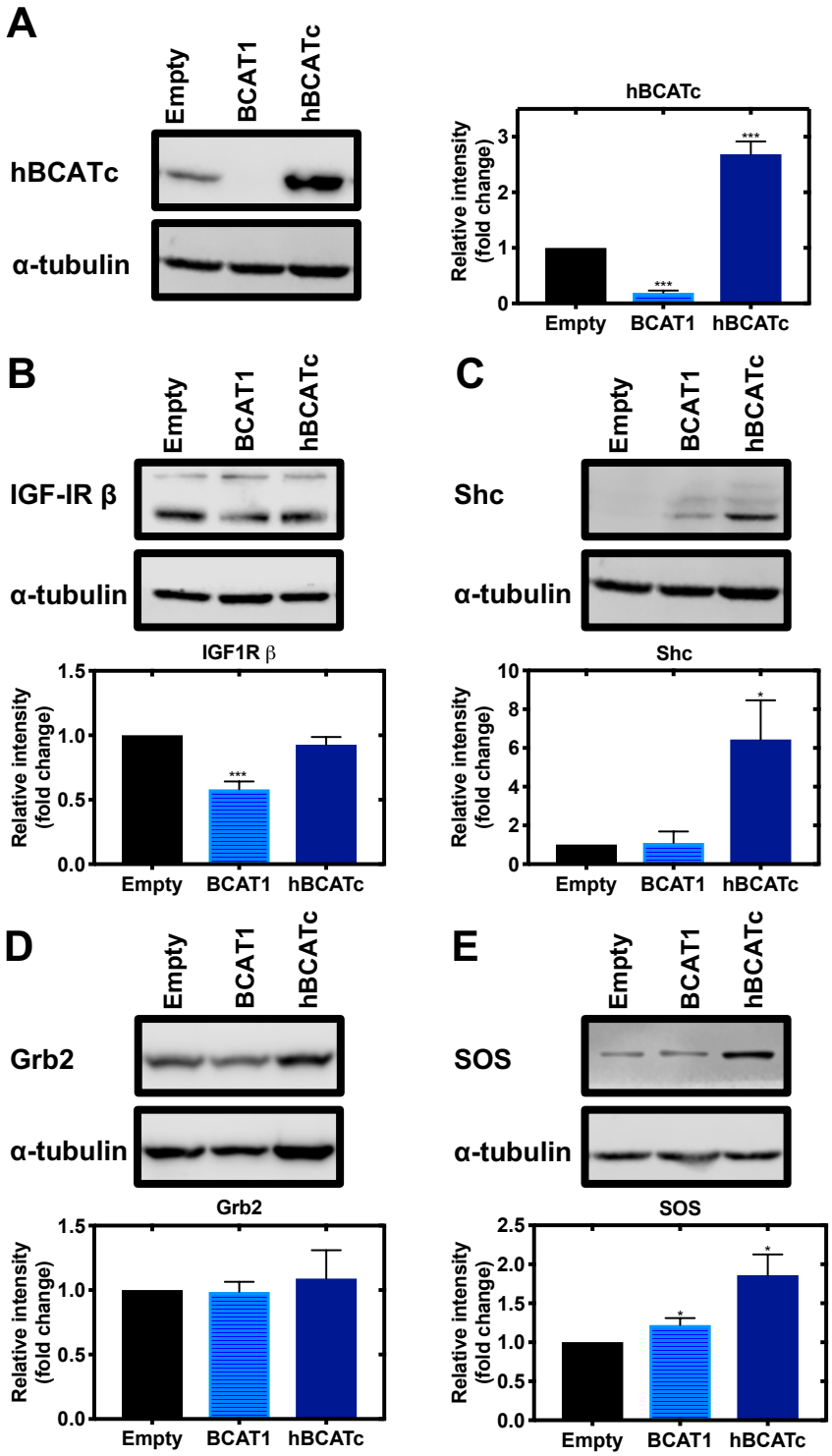

Figure 4.9. Knockdown and overexpression of *BCAT1* regulates IGF-1/insulin signaling cascade in MDA-MB-231 cells. Cells were treated with stable shRNA transfection for *BCAT1* knockdown (BCAT1) and overexpression (hBCATc) for 8 days. (**A**) Western blot analysis confirmed *BCAT1* knockdown and overexpression. Western blot analysis was also used to assess changes in the protein expression of IGF-1 downstream targets (**B**) IGF-IRβ (**C**) Shc (**D**) Grb2 (**E**) SOS. Respective densitometric analysis of fold changes of protein expression relative to α-tubulin are presented. Data representative of mean ±SEM (n=3). Statistical significance was calculated using t-tests,*p < 0.05, ***p < 0.001 and **** p< 0.0001.

Interestingly, knockdown of *BCAT1* resulted in a significant increase in the activation of phospho-MAPK ($p < 0.01$), indicating that hBCATc may play a regulatory role in MAPK activation (Figure 4.10). To explore the role of hBCATc regulation of MAPK activation, *BCAT1*-shRNA knockdown and overexpression TNBC cells were treated with IGF-1 and insulin and Western blot analysis performed. Following IGF-1 and insulin treatment *BCAT1* knockdown significantly increased activation of MAPK ($p < 0.0001$ and $p=0.0294$ respectively) compared with treatment. whilst overexpression of hBCATc significantly reduced IGF-1 and insulin mediated phosphorylation of MAPK ($p < 0.0001$ and $p < 0.001$) compared with IGF-1 and insulin treatment. These results suggest that high levels of hBCATc, observed in breast tumours, increases activation of the IGF-1/insulin signalling cascades through sustained high levels of Shc and SOS, together with inhibition of MAPK activation.

4.3.8 hBCATc mediates insulin induced nuclear translocation of MAPK

In related studies, the Conway group has demonstrated hBCATc to influence the translocation of proteins to the membrane and nucleus. Here, using immunocytochemistry, the effect of hBCATc expression on phospho-MAPK and MAPK translocation was assessed (Figure 4.11 and Figure 4.12). Additionally, co-localisation of hBCATc with phospho-MAPK and MAPK under IGF-1 and insulin treatment was investigated (Figure 4.13 and Figure 4.14). Expression of phospho-MAPK was primarily cytoplasmic, with a low level of punctate expression in the nucleus of TNBC, MDA-MB-231 cells under normal conditions (Figure 4.11). Insulin and IGF-1 treatment alone did not affect nuclear localisation. Following *BCAT1* knockdown nuclear localisation of phospho-MAPK was significantly decreased ($p < 0.0001$). Interestingly, knockdown of *BCAT1*

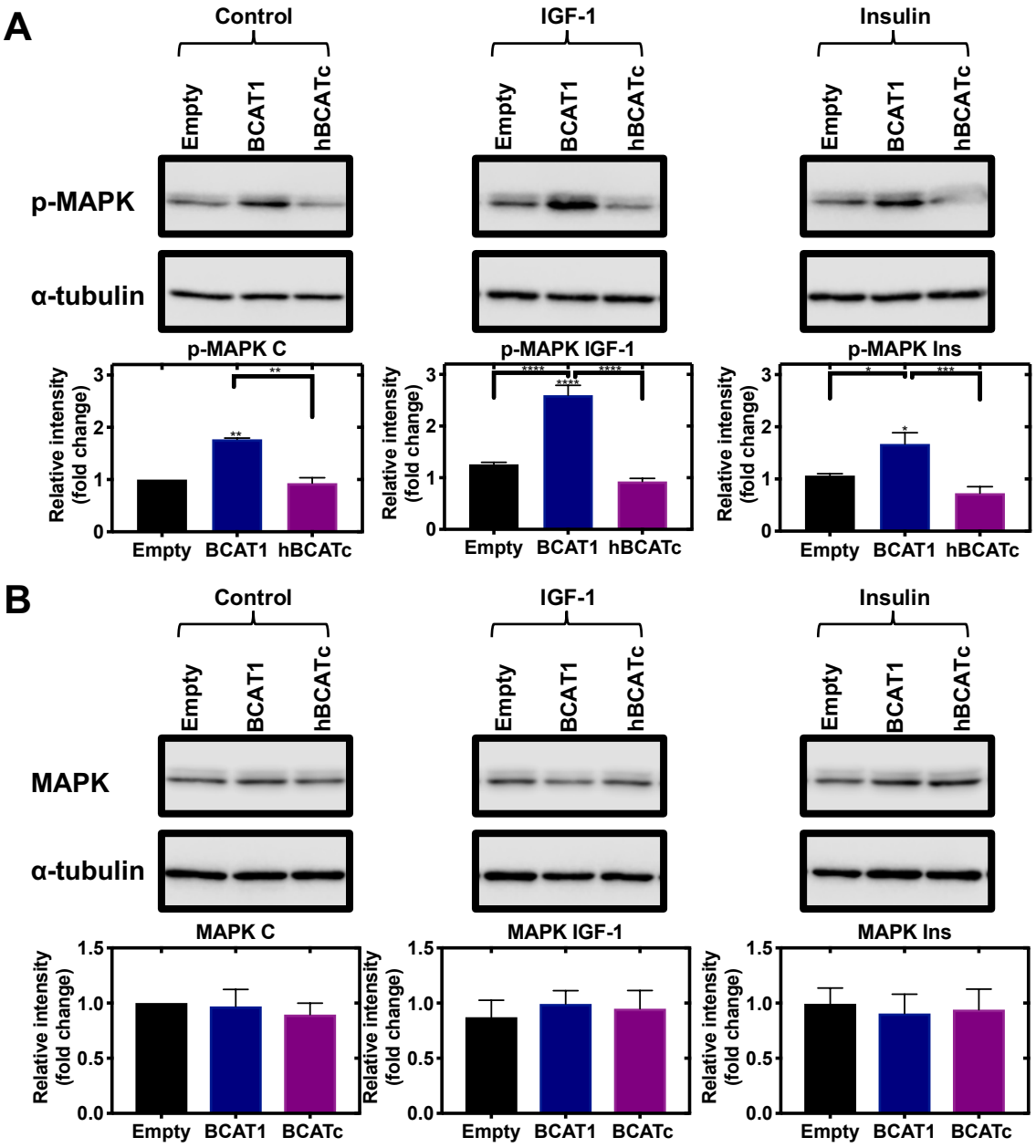

Figure 4.10. Knockdown and overexpression of BCAT1 negatively regulates phosphorylation of MAPK in MDA-MB-231 cells. Cells were treated with stable shRNA transfection for *BCAT1* knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty) for 8 days and treated with 100 nM insulin and 100 ng/mL IGF-1 for 24 hours accordingly. (**A**) Western blot analysis was used to assess changes in the protein expression of phospho-MAPK and (**B**) MAPK. Respective densitometric analysis of fold changes of protein expression relative to α-tubulin are presented below the immunoblots. Data representative of mean ±SEM (n=3) Statistical significance was calculated by one-way ANOVA using Tukey honestly significant differences, *p < 0.05, **p < 0.01 and *** p< 0.001.

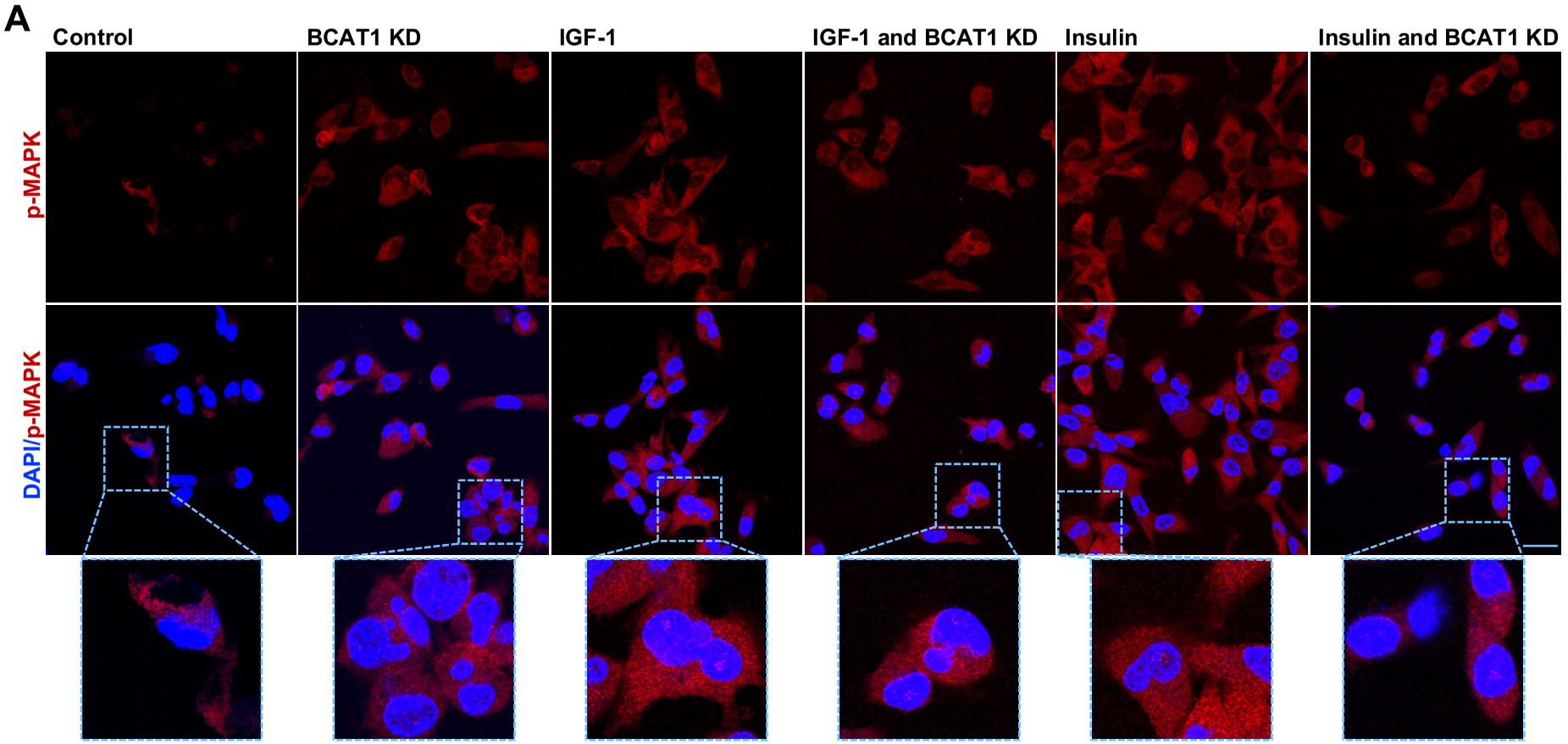

Figure 4.11. Knockdown of BCAT1 induces Insulin-mediated nuclear translocation of phospho-MAPK in MDA-MB-231 cells. MDA-MB-231 cells were cultured on sterile glass coverslips (treated with poly-L-lysine) and treated with 20 nM *BCAT1* siRNA, 100 nM insulin and 100 ng/mL IGF-1 accordingly. Cells were fixed with 4% paraformaldehyde and permeabilised with Triton X. (**A**) Immunofluorescence Z stacked images of MDA-MB-231 cells probed with anti-phospho-MAPK (1:100) overnight at 4C, and then anti-rabbit Alexa Fluor® 568 (1:250) for 1.5 hours. The coverslips were then mounted in VECTASHIELD HardSet antifade mounting medium with DAPI and imaged using a Zeiss Axiovert 200 confocal microscope. (**B**) Respective velocity analysis of Mander's coefficient as compared to control. Data representative of mean ±SEM (n=3) *p < 0.05 and *** p< 0.001. Blue - DAPI; Red – phosho-MAPK.

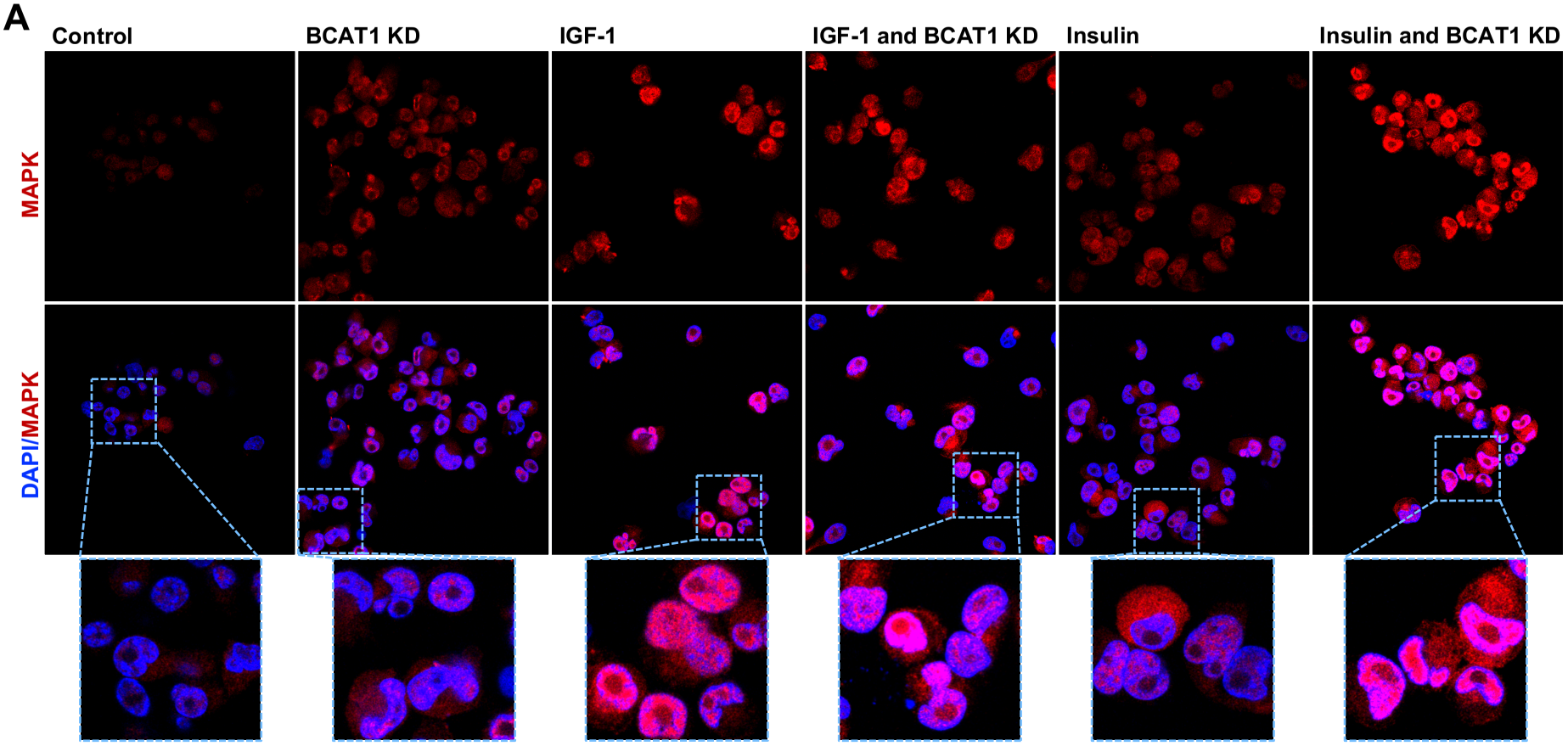

Figure 4.12. Insulin-mediated nuclear translocation of MAPK is significantly increased with BCAT1 knockdown in MDA-MB-231 cells. MDA-MB-231 cells were cultured on sterile glass coverslips (treated with poly-L-lysine) and treated with 20 nM *BCAT1* siRNA, 100 nM insulin and 100 ng/mL IGF-1 accordingly. Cells were fixed with 4% paraformaldehyde and permeabilised with Triton X. (**A**) Immunofluorescence Z stacked images of MDA-MB-231 cells probed with anti-MAPK (1:100) overnight at 4C, and then anti-rabbit Alexa Fluor® 568 (1:250) for 1.5 hours. The coverslips were then mounted in VECTASHIELD HardSet antifade mounting medium with DAPI and imaged using a Zeiss Axiovert 200 confocal microscope. (**B**) Respective velocity analysis of Mander's coefficient as compared to control. Data representative of mean ±SEM (n=3) *p < 0.05 and *** p< 0.001. Blue - DAPI; Red - MAPK.

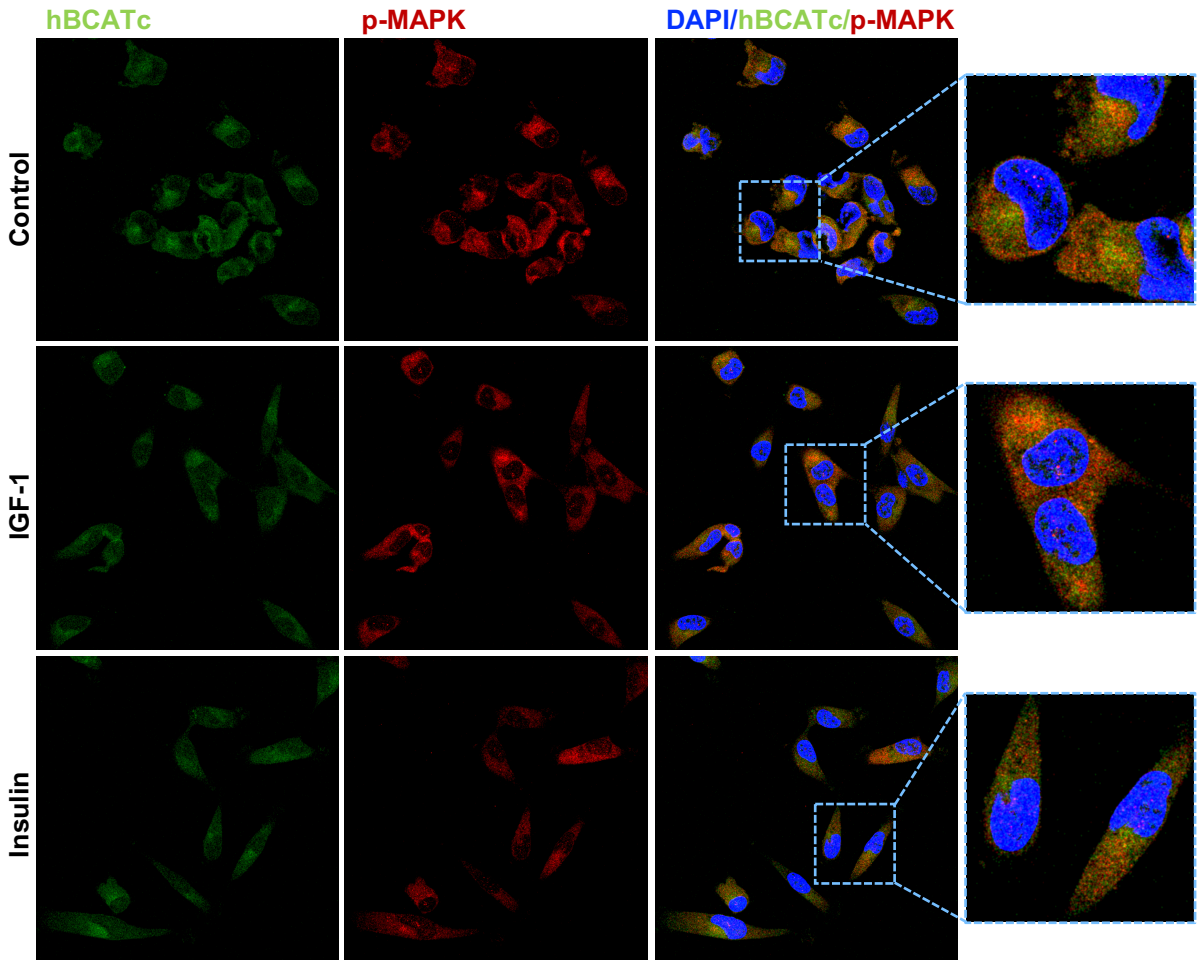

Figure 4.13. Insulin increases phospho-MAPK co-localisation with hBCATc. MDA-MB-231 cells were cultured on sterile glass coverslips (treated with poly-L-lysine) and treated with 20 nM *BCAT1* siRNA, 100 nM insulin and 100 ng/mL IGF-1 accordingly. Cells were fixed with 4% paraformaldehyde and permeabilised with Triton X. Immunofluorescence Z stacked images of MDA-MB-231 cells probed with anti-phospho-MAPK (1:100) and anti-hBCATc (1:50) overnight at 4C, and then anti-mouse Alexa Fluor® 488 (1:100) and anti-rabbit Alexa Fluor® 568 (1:100) for 1.5 hours. The coverslips were then mounted in VECTASHIELD HardSet antifade mounting medium with DAPI and imaged using a Zeiss Axiovert 200 confocal microscope. Blue - DAPI; Green - hBCATc; Red – phospho-MAPK.

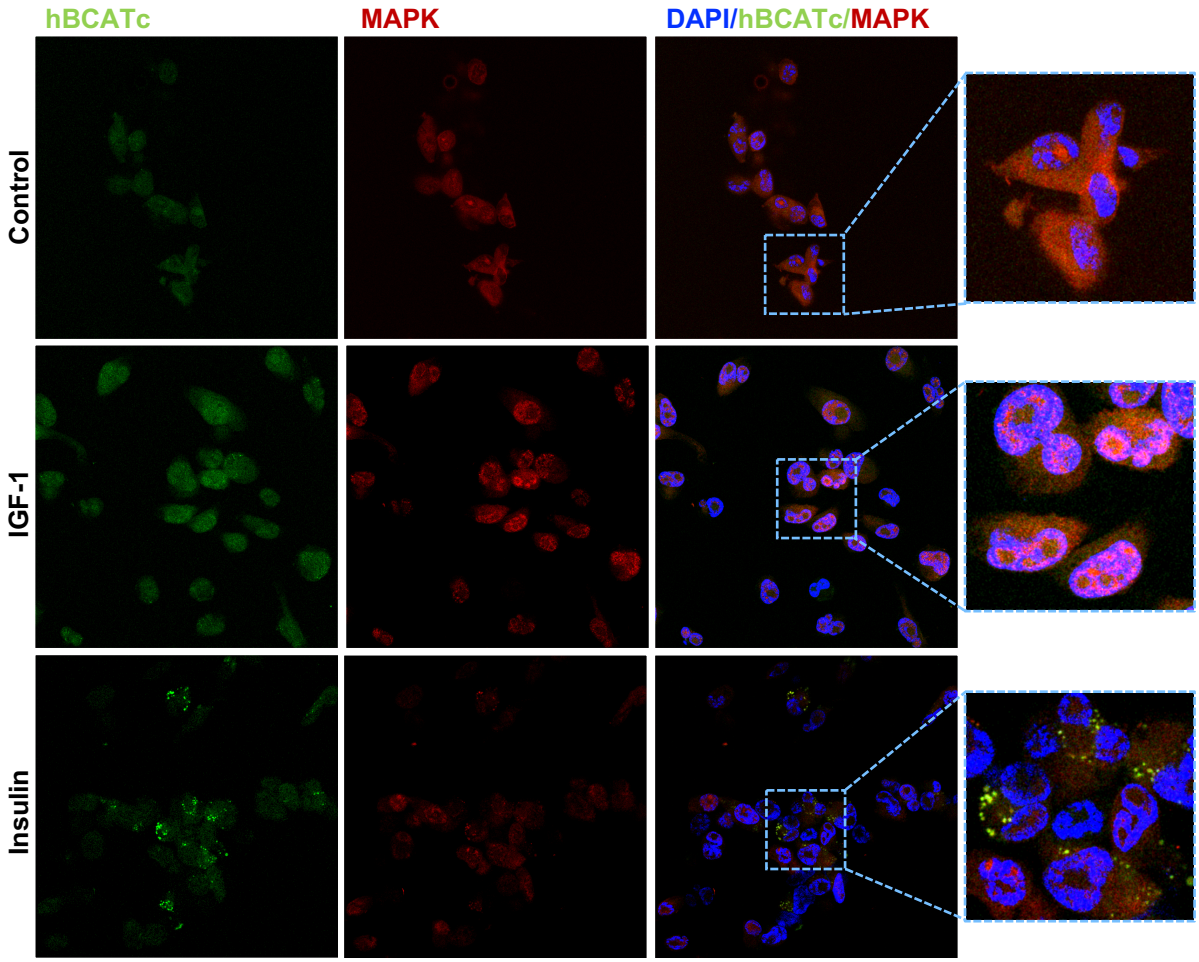

Figure 4.14. Insulin increases MAPK co-localisation with hBCATc. MDA-MB-231 cells were cultured on sterile glass coverslips (treated with poly-L-lysine) and treated with 20 nM *BCAT1* siRNA, 100 nM insulin and 100 ng/mL IGF-1 accordingly. Cells were fixed with 4% paraformaldehyde and permeabilised with Triton X. Immunofluorescence Z stacked images of MDA-MB-231 cells probed with anti-MAPK (1:100) and anti-hBCATc (1:50) overnight at 4C, and then anti-mouse Alexa Fluor® 488 (1:250) and anti-rabbit Alexa Fluor® 568 (1:250) for 1.5 hours. The coverslips were then mounted in VECTASHIELD HardSet antifade mounting medium with DAPI and imaged using a Zeiss Axiovert 200 confocal microscope. Blue - DAPI; Green - hBCATc; Red - MAPK.

combined with insulin treatment led to increased nuclear phospho-MAPK localisation ($p = 0.036$). Insulin treatment increased interaction of phospho-MAPK with hBCATc (Figure 4.13) thus indicating insulin to enhance hBCATc inhibition of phospho-MAPK nuclear translocation.

Similar to the results of Western blot analysis (Figure 4.10B), knockdown of *BCAT1* was found to increase expression of MAPK compared to control as observed in Figure 4.12. Additionally, knockdown of *BCAT1* significantly increased nuclear translocation of MAPK ($p < 0.0001$). IGF-1 and insulin treatment of cells also increased MAPK movement to the nucleus ($p < 0.0001$).

However, IGF-1 treatment combined with knockdown of *BCAT1* did not alter IGF-1-mediated MAPK nuclear translocation, indicating hBCATc expression is not involved in this process. Interestingly, knockdown of *BCAT1* was found to increase insulin-mediated MAPK translocation the nucleus, indicating hBCATc to inhibit MAPK activation of nuclear targets in response to insulin. Concurringly, whilst IGF-1 treatment had no effect on co-localisation of hBCATc with MAPK, insulin treatment increased co-localisation of these proteins compared to control (Figure 4.14). Interestingly, expression of both MAPK and hBCATc differed to the control whereby punctate areas could be observed suggesting hBCATc to interact with MAPK following insulin treatment. Combined these results indicate hBCATc to inhibit MAPK activation, hence the role of hBCATc on the PI3K/Akt/mTOR pathway was next investigated.

4.3.9 hBCATc significantly increases insulin mediated activation of Akt and increases protein levels of downstream targets

Following shRNA-mediated *BCAT1* knockdown and overexpression, levels of proteins associated with the PI3K/Akt/mTOR pathway were assessed using

Western blot analysis. In response to insulin, overexpression of hBCATc caused a significant increase in the phosphorylation and activation of Akt ($p < 0.01$) relative to control and compared with *BCAT1* knockdown ($p < 0.01$) (Figure 4.15A). Increased levels of hBCATc also led to higher expression of Akt in response to insulin ($p < 0.01$) (Figure 4.15B). These findings indicate that overexpression of hBCATc activates Akt in response to insulin. Following IGF-1 and insulin treatment, knockdown of *BCAT1* significantly decreased levels of phospho-mTOR ($p < 0.0137$ and $p < 0.0001$ respectively) (Figure 4.16A) relative to treatments. Additionally, overexpression of hBCATc significantly increased levels of p-mTOR protein expression following IGF-1 and insulin treatment ($p <0.001$ and $p < 0.0001$, respectively) relative to treatments. Therefore, hBCATc is indicated to play a regulatory role for Akt/mTOR activation which is further enhanced upon IGF-1 and insulin treatment.

To assess hBCATc regulation of the PI3K/Akt/mTOR pathway protein expression of downstream targets of this pathway were assessed using Western blot analysis following *BCAT1* knockdown and overexpression. Knockdown of *BCAT1* significantly reduced protein expression of the transcription factor forkhead box O3 (FOXO3a) whilst overexpression of hBCATc significantly increased FOXO3a expression ($p=0.0167$ and $p=0.0386$ respectively) (Figure 4.17A). Similarly, knockdown of *BCAT1* reduced nuclear factor (erythroid-derived 2)-like-2 (Nrf2) protein expression ($p=0.0025$) whilst overexpression of hBCATc increased Nrf2 expression as observed ($p=0.0143$) in Figure 4.17B. Additionally, following hBCATc overexpression protein expression of glucose regulated protein 78 (GRP78), a downstream target of Akt/mTOR axis, was found to significantly increased ($p=0.0435$) (Figure 4.17C). Taken together these results indicate

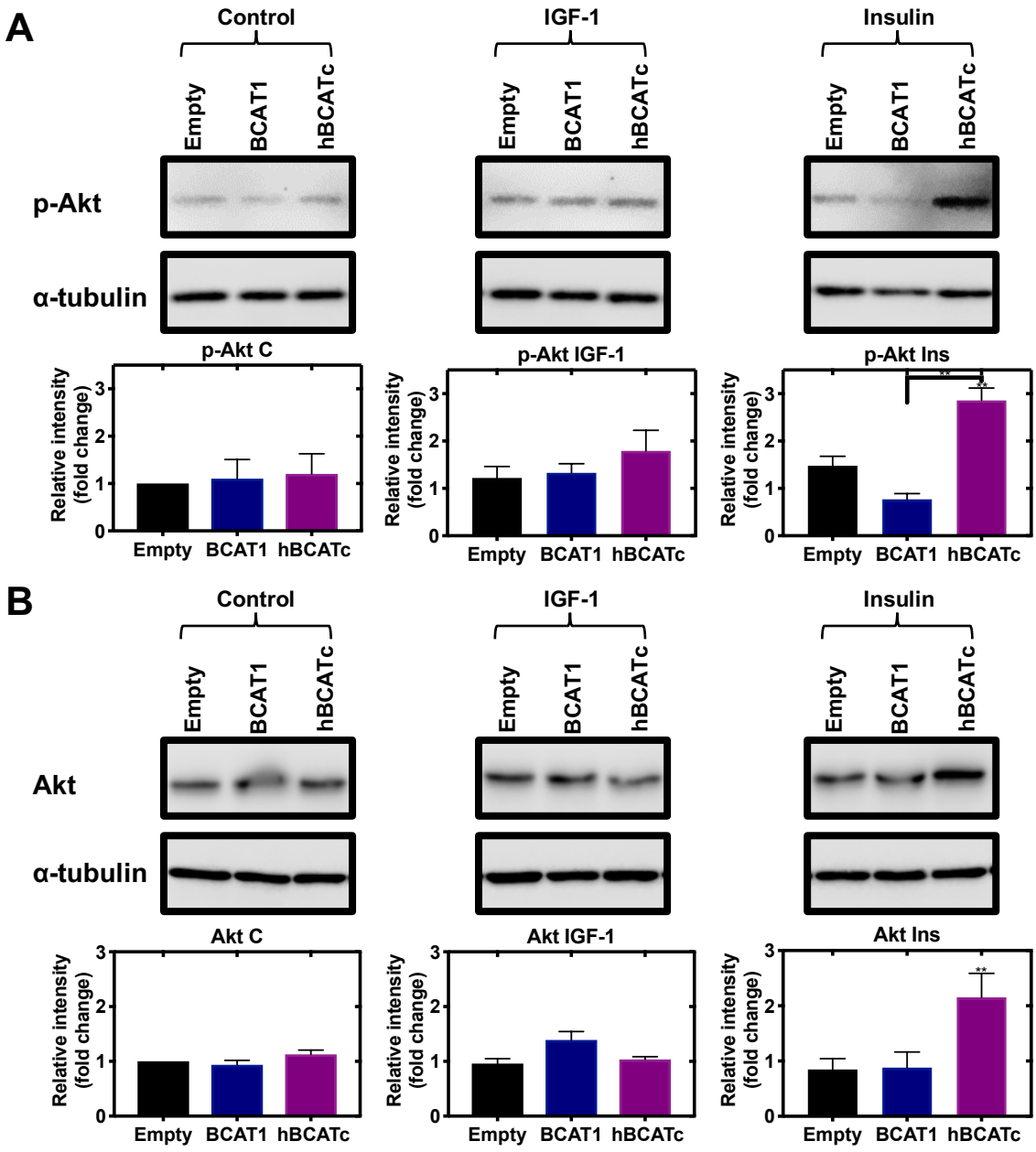

Figure 4.15. Knockdown and overexpression of BCAT1 regulates the Akt axis in MDA-MB-231 cells. Cells were treated with stable shRNA transfection for *BCAT1* knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty) for 8 days and treated with 100 nM insulin and 100 ng/mL IGF-1 for 24 hours accordingly. (**A**) Western blot analysis was used to assess changes in the protein expression of phospho-Akt and (**B**) Akt. Respective densitometric analysis of fold changes of protein expression relative to α-tubulin are presented below the immunoblots. Data representative of mean ±SEM (n=3). Statistical significance was calculated by one-way ANOVA using Tukey honestly significant differences, *$p < 0.05$ and **$p < 0.01$.

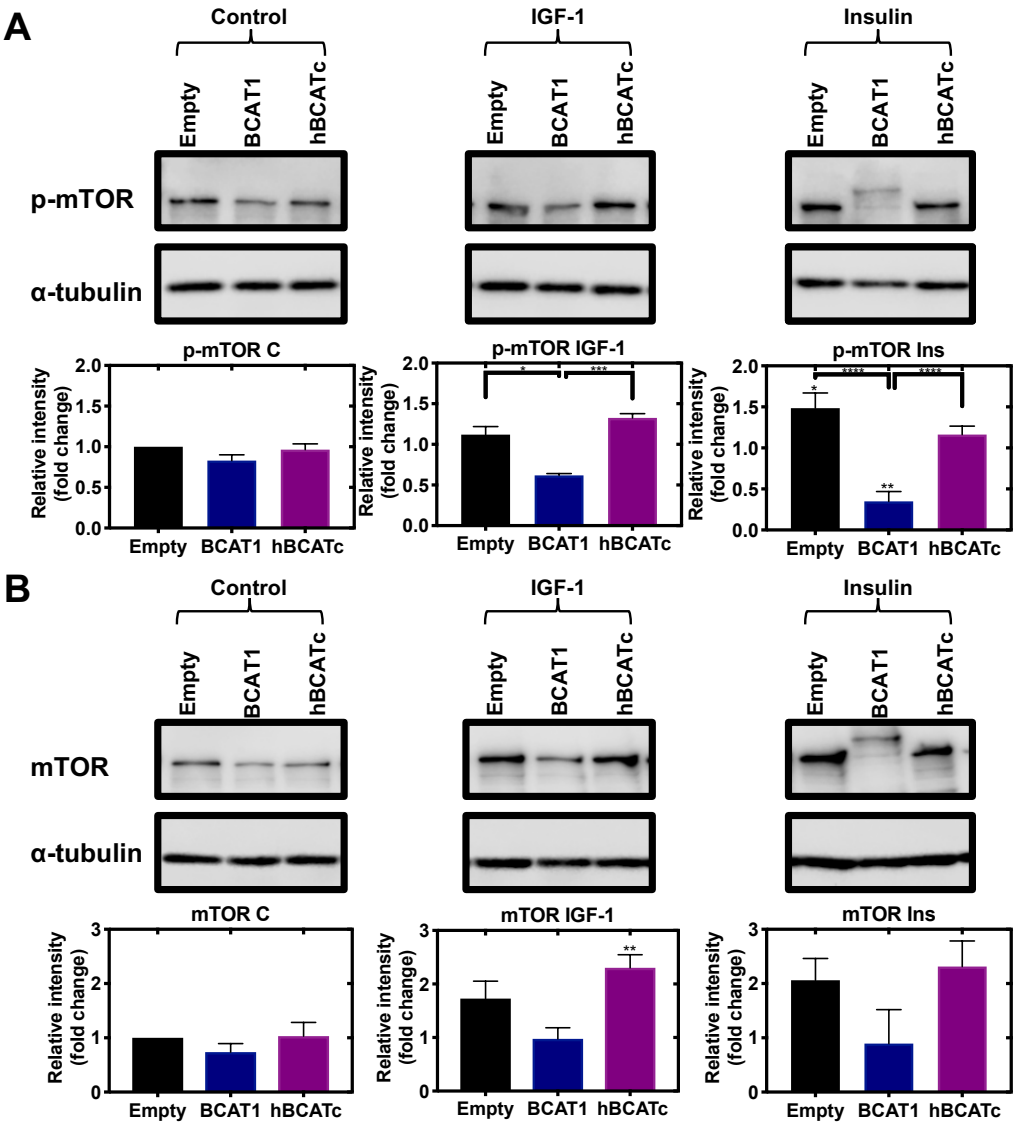

Figure 4.16. Knockdown and overexpression of BCAT1 regulates the mTOR axis in MDA-MB-231 cells. Cells were treated with stable shRNA transfection for *BCAT1* knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty) for 8 days and treated with 100 nM insulin and 100 ng/mL IGF-1 for 24 hours accordingly. (**A**) Western blot analysis was used to assess changes in the protein expression of phospho-mTOR and (**B**) mTOR. Respective densitometric analysis of fold changes of protein expression relative to α-tubulin are presented below the respective immunoblots. Data representative of mean ±SEM (n=3). Statistical significance was calculated by one-way ANOVA using Tukey honestly significant differences, *p < 0.05, **p < 0.01 and **** p< 0.0001.

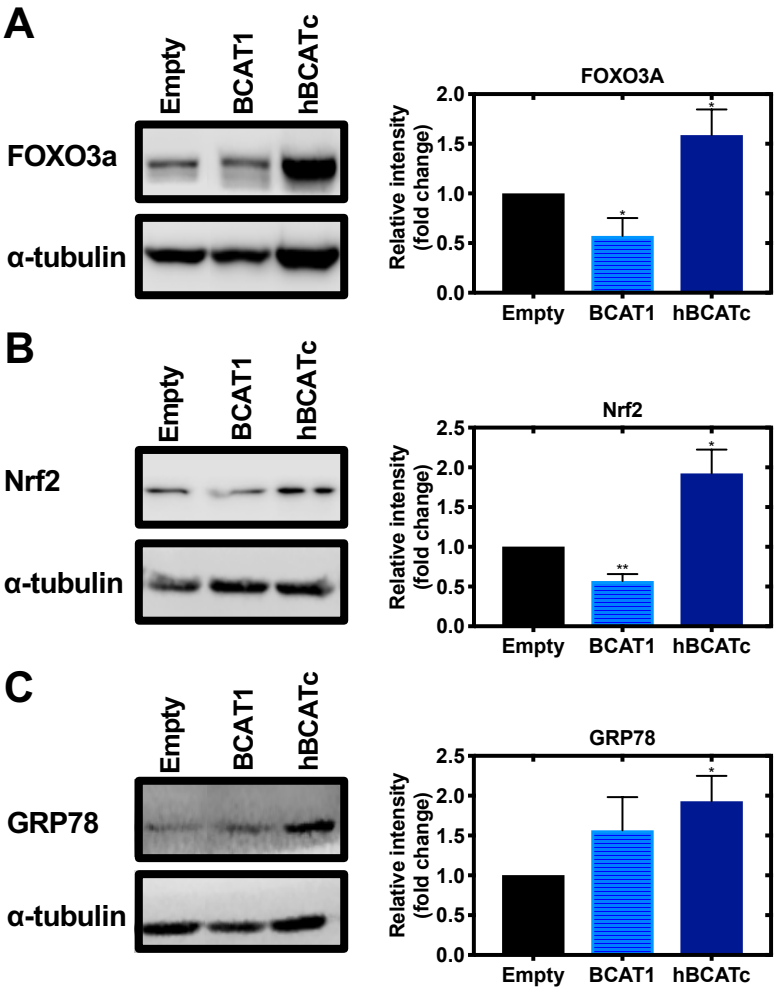

Figure 4.17. Knockdown and overexpression of BCAT1 regulates downstream targets of the Akt axis in MDA-MB-231 cells. Cells were treated with stable shRNA transfection for *BCAT1* knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty) for 8 days. Western blot analysis was used to assess changes in the protein expression of (**A**) FOXO3a (**B**) Nrf2 (**C**) GRP78. Respective densitometric analysis of fold changes of protein expression relative to α-tubulin are presented to the right of the immunoblots. Data representative of mean ±SEM (n=3). Statistical significance was calculated using t-tests, *$p < 0.05$ and **$p < 0.01$.

hBCATc to negatively regulate MAPK whilst upregulating Akt/mTOR activation which is supported by modulation of downstream targets of this pathway.

4.4 Discussion

As reported in chapter 4, hBCATc expression was limited to TNBC, MDA-MB-231, cells from the breast cancer cell panel assessed. Upregulation of hBCATc has been demonstrated to increase cell proliferation, migration and invasion in breast cancer cells (Thewes *et al.*, 2017). To obtain an insight into the underlying metabolic processes that are controlled by hBCATc, transient knockdown of *BCAT1* was induced using siRNA and later shRNA constructs for the knockdown and overexpression of *BCAT1* were developed in the TNBC cells. The IGF-1/insulin pathway facilitates an orchestrated activation of numerous cell signalling events initiated through phosphorylation of IRS1/2 (Boucher, Kleinridders and Ronald Kahn, 2014). For the first time knockdown of *BCAT1* was found to augment IGF-1/insulin-mediated proliferation and migration. Moreover, suppression of hBCATc expression reduced protein levels of downstream targets of the IGF-1/insulin signalling cascade, indicating that hBCATc plays a role in the modulation of cell proliferation and migration through this pathway. Ultimately, hBCATc was found to modulate activation of Akt/mTOR whilst concomitantly suppressing RAS/MAPK signalling.

4.4.1 Knockdown of BCAT1 reduces cell proliferation, migration and invasion

Using Western blot analysis, the concentration of *BCAT1* siRNA was optimised at the lowest concentration sufficient to significantly decrease hBCATc protein expression at 20 nM for 72 hours in the MDA-MB-231 cells (Figure 4.1), to minimise potential toxic effects of using higher concentrations of siRNA which

could induce downstream toxic effects (Olejniczak, Galka and Krzyzosiak, 2009). siRNA knockdown of *BCAT1* demonstrated hBCATc be intrinsically involved in mediating cell proliferation, migration and invasion of MDA-MB-231 cells (Figure 4.2). This is supported by previous findings which demonstrate hBCATc to increase proliferation, metastasis and invasion of tumour cells in breast, colon and hepatocellular cancer (Thewes *et al.*, 2017; Yoshikawa *et al.*, 2006; Xu *et al.*, 2016). Interestingly, BCAA levels were found to influence proliferation in a dose dependant manner whereby treatment with BCAAs initially increased cell proliferation however, at higher concentrations cell proliferation decreased (Figure 4.3). Increased levels of BCAAs, in particular leucine, have been demonstrated to contribute to tumour growth mediated by the activation of mTOR signalling by the acetylation of the mTOR regulator, Raptor (Zhang and Han, 2017; Son *et al.*, 2019). mTOR activation leads to increased lipid and protein synthesis promoting cell growth and proliferation (Zhang, Pavlova and Thompson, 2017; Zhang and Han, 2017). Therefore, these findings indicate that tight regulation of BCAA levels, mediated by hBCATc transamination, is important for tumour cell proliferation mediated through the regulation of mTOR signalling (further detailed in section 4.4.6).

4.4.2 hBCATc regulates apoptosis

The ability for tumour cells to evade apoptosis, thereby leading to uncontrolled proliferation, is one of the key hallmarks of cancer (Hanahan and Weinberg, 2011). Previous reports have demonstrated a role for hBCATc in cell apoptosis which is mediated by the BCKA metabolites, in particular ketoisocaproate (Eden and Benvenisty, 1999). Overexpression of *BCAT1* has also been demonstrated to decrease cell apoptosis in prostate cancer cells (Zhu, Shao and Peng, 2017) and to regulate apoptosis in hepatocellular cancer (Ji *et al.*, 2019). To assess the

role of hBCATc in apoptosis of TNBC cells siRNA-mediated knockdown of *BCAT1* was used. Knockdown of *BCAT1* was found to increase the percentage of cell in the early apoptotic stage and reduce the percentage of live cells (Figure 4.4), indicating hBCATc expression to allow TNBC cells to evade apoptosis. The underlying mechanism by which hBCATc regulates cell apoptosis has not yet been elucidated. Cancer cells often evade apoptosis by overexpressing or stabilising anti-apoptotic B cell lymphoma 2 (BCL-2) family proteins such as BAX and BAK while inhibiting pro-apoptotic BCL-2 proteins such as BID and Bcl-2-interacting killer (Bik) (Matsuura *et al.*, 2016).

Oxidative stress is induced as tumours initiate and the metabolic activity of cancer cells increases as a result of either an overproduction of reactive oxygen species or insufficient antioxidant activity (Gorrini, Harris and Mak, 2013). ROS has been proposed to support tumour promotion through the activation of signalling pathway that support cell proliferation, survival and metabolic adaptation, however excessive oxidative stress can induce apoptosis (Hole *et al.*, 2013). To counteract excessive ROS accumulation tumour cells increase their antioxidant capacity (Chandel and Tuveson, 2014). The thiol regions of the CXXC motif in hBCATc exhibit sensitivity to changes in the redox environment (Conway *et al.*, 2008) which is perturbed in cancer. Increased levels of glutamate, a product of hBCATc transaminase activity, contribute to GSH biosynthesis, by facilitating the uptake of cystine via the xc-cysteine transporter, which is coupled to the efflux of glutamate (Timmerman *et al.*, 2013). GSH plays an important role in redox homeostasis and tumour cell survival by protecting cells from damage caused by ROS generated during oxidative stress (Zhang, Pavlova and Thompson, 2017). Moreover, expression levels of hBCATc were demonstrated to regulate the expression of Nrf2, which is the primary transcription factor responsible for the

regulation of genes encoding oxidative stress-related proteins (detailed in section 4.4.7). Therefore, overexpression of hBCATc in TNBC can be proposed as a survival mechanism for tumour cells, potentially through the regulation of redox homeostasis.

4.4.3 hBCATc regulates proliferation, migration and invasion through the IGF-1 and insulin signalling pathway

In breast cancer cells, IRS-1 and IRS-2 are the two primary adaptor proteins which regulate IGF-IR and insulin receptor (IR) signalling, in response to IGF-1 and insulin activation (Taniguchi, Emanuelli and Kahn, 2006), whereby IRS-1 mainly promotes cell proliferation while IRS-2 stimulates cell motility (Jackson *et al.*, 2001; Byron *et al.*, 2006). To determine the appropriate concentration of IGF-1 and insulin, which would increase proliferation, MDA-MB-231 cells were incubated with various concentrations of insulin and IGF-1 and proliferation assessed using the TTI assay (Sharma and Kumar, 2018). Concentrations of 100 nM insulin and 100 ng/mL were found to significantly increase proliferation of the TNBC cells (Figure 4.5) as supported by other studies (Gupta and Tikoo, 2013; Davison *et al.*, 2015). Therefore, these conditions were selected for further work involving the IGF-1 and insulin signalling pathways.

As expected, IGF-1 and insulin were found to increase cell migration using Transwell inserts (Figure 4.6). This is in support of previous findings, which have demonstrated that insulin and IGF-1 increased cell migration and markers of migration in TNBC (Morimura and Takahashi, 2011; Rodriguez-Monterrosas *et al.*, 2018). Interestingly, knockdown of *BCAT1* was found to significantly perturb IGF-1 and insulin-mediated increased proliferation and migration (Figure 4.5B and 4.6) indicating hBCATc controls these tumour promoting processes through the IGF-1 and insulin pathway. Leucine deprivation has been shown to improve

insulin sensitivity, under insulin-resistant conditions in hepatoma (HepG2) cells, by the activation of GCN2 and subsequent inhibition of mTOR activation (Xiao *et al.*, 2011). Although lower concentrations of BCAAs increased cell proliferation, higher levels of BCAAs were found to reduce cell proliferation (Figure 4.3). Previous reports have Hagiwara, Nishiyama and Ishizaki (2012) demonstrated BCAAs to directly suppress insulin-induced cell proliferation by induction of IRS1 phosphorylation at the negative regulatory serine residue through mTOR activation (Hagiwara, Nishiyama and Ishizaki, 2012). Therefore, it can be speculated that hBCATc ameliorates the suppressive effect of elevated BCAAs on insulin/IGF-1 mediated cell proliferation and migration.

4.4.4 *BCAT1* knockdown reduces IGF-1 mediated glycolysis in TNBC

Cellular bioenergetics in normal cells are primarily sourced from mitochondrial oxidative phosphorylation (OXPHOS) and aerobic glycolysis to a lesser extent which contribute to cell growth regulation and other cellular functions (Zheng, 2012). Metabolic reprogramming in cancer cells results in enhanced glycolysis and reduced OXPHOS capacity leading to increased lactate production even in the presence of oxygen to support macromolecular synthesis (Warburg, Wind and Negelein, 1927). Attenuation of OXPHOS promotes evasion of apoptosis in tumour cells as the mitochondria is responsible for the activation of multiple pro-apoptotic proteins including Bax and Bak (Yadav *et al.*, 2015). Expression of glucose transporter 1 (GLUT1), which initiates glycolysis, has been reported to be higher in TNBC compared to other subtypes indicating a more glycolytic phenotype for this subtype of breast cancer (Choi, Jung and Koo, 2013). To investigate whether hBCATc is involved in glycolysis in TNBC the Seahorse glycolytic rate assay was utilised. This assay provides precise measurement of the glycolytic rate whilst accounting for extracellular acidification derived from

mitochondrial activity using the mitochondrial inhibitor Rot/AA (Romero *et al.*, 2017).

Inhibition of hBCATc activity has been found to reduce oxygen consumption and glycolysis in activated human macrophages and in activated T cells (Papathanassiu *et al.*, 2017; Ananieva *et al.*, 2014), indicating that hBCATc plays an intrinsic role in glycolysis in immune cells. However, in these experiments knockdown of *BCAT1* did not affect basal glycolytic rate or compensatory glycolysis in TNBC cells (Figure 4.7C and 4.7D) indicating expression of hBCATc does not directly affect metabolic dependency on glycolysis in TNBC cells under normal conditions. Activation of IGF-1R by both insulin and IGF-1 has been reported to increase glycolysis, which was demonstrated by the upregulation of genes involved in glycolysis and the downregulation of genes involved in oxidative phosphorylation in mammary gland tumours (ter Braak *et al.*, 2017). In TNBC, treatment with IGF-1 resulted in a moderate increase of compensatory glycolysis which was ameliorated following *BCAT1* knockdown, suggesting hBCATc may play a role in IGF-1-mediated glycolysis (Figure 4.7).

4.4.5 hBCATc significantly reduces IGF mediated activation of ERK

Knockdown of *BCAT1* led to a significant decrease in the level of IGF-1Rβ, which is responsible for intracellular signal transduction upon receptor activation (Kavran *et al.*, 2015) (Figure 4.9B), indicating hBCATc expression to be required for IGF-1 signalling. Elevated levels of BCAA have been reported to inhibit the activation of IGF-1R (Wubetu *et al.*, 2014), hence indicating increased hBCATc expression, allowing activation of the IGF-1R signalling cascade. This activation triggers two predominant growth signalling pathways, the Ras/ERK and Akt/mTOR pathway.

ERK proteins are central transducers of extracellular signals from hormones, growth factors, cytokines and environmental stress; they regulate many cellular processes throughout the cell and phosphorylate transcription factors, cytoskeletal proteins and other protein kinases and enzymes (Zakrzewska *et al.*, 2013; Han *et al.*, 2018; Soond, 2005). MAPK (ERK 1/2) is activated through a series of phosphorylation reactions, which is transduced through the IGF-1/insulin axis. Initiation of IRS1/2 through IGF-1 and insulin activates Shc, the levels of which were shown to be significantly increased in response to hBCATc overexpression (Figure 4.9C). The Shc substrate binds to the adaptor protein Grb2 and the associated guanine nucleotide exchange protein, SOS, the levels of which were also shown to be increased when hBCATc is overexpressed (Figure 4.9E). Interestingly, expression levels of Shc and SOS were not significantly affected with knockdown of *BCAT1* whilst overexpression of hBCATc significantly increased levels of these two proteins (Figure 4.9C and 4.9E). As discussed in section 3.4.2, metabolic proteins such as hBCATc, can exert extra-metabolic functions; as such these are termed moonlighting proteins (Boukouris, Zervopoulos and Michelakis, 2016). A novel redox chaperone role for hBCATm has previously been reported, through interaction with PDI, a protein-folding protein, which is regulated through S-glutathionylation under oxidative stress (Hindy *et al.*, 2014). Scaffolding proteins play a key role in the co-ordination of the transduction of receptor-mediated signalling pathways, by binding to signalling effectors leading to their sequestration in the cytoplasm and subsequent activation of downstream effectors (Mugabo and Lim, 2018). For example, the IQ motif-containing GTPase-activating proteins (IQGAP) represent a family of scaffolding proteins, of which IQGAP1 has been demonstrated to bind MEK1/2 and ERK1/2 following EGF stimulation to enhance growth factor signalling (Roy,

Li and Sacks, 2005; Zaballos *et al.*, 2019). Here in TNBC, hBCATc may sequester Shc and SOS, acting as a scaffold protein, rather than affecting transcription, to enhance the IGF-1/insulin signalling cascade. Increased levels of SOS allow the exchange of nucleotide guanosine diphosphate (GDP) bound to Ras with nucleotide guanosine triphosphate (GTP) in the cytosol (Pan *et al.*, 2010). GTP-bound Ras allows membrane recruitment and activation of the serine/threonine protein kinase Raf to the plasma membrane followed by phosphorylation and activation of MEK1/2 (Girnita *et al.*, 2014). Normally, this would enhance and activate MAPK. Surprisingly, knockdown of *BCAT1* increased phosphorylation of MAPK, even in the presence of IGF-1 or insulin (Figure 4.10).

Following activation by phosphorylation, MAPK phosphorylates targets in the cytoplasm, and/or translocates to the nucleus, which is mediated by detachment from cytoplasmic anchoring proteins (Burotto *et al.*, 2014). Cytoplasmic targets include proteins responsible for cell movement and metabolism including myosin light chain kinase (MLCK) which facilitates cell motility and ribosomal S6 kinases (RSK) which regulates glycogen synthase kinase 3 (GSK3) involved in cell metabolism (Klemke *et al.*, 1997; Chu *et al.*, 1996). Nuclear translocation of MAPK allows transcriptional regulation of nuclear targets including carbamoyl phosphate synthetase II (CPS II), a regulator of DNA synthesis and p90RSK which is responsible for promoting cell cycle progression (Brunet *et al.*, 1999). Confocal analysis revealed that knockdown of *BCAT1* increased insulin-mediated phospho-MAPK and MAPK nuclear translocation (Figure 4.11 and 4.12), additionally insulin treatment increased hBCATc co-localisation with MAPK (Figure 4.14). Therefore, these results combined indicate that under insulin treatment, hBCATc interacts with MAPK leading to an inhibitory effect of MAPK activation, which is mediated by abrogating MAPK nuclear translocation, which

will therefore reduce activation of downstream nuclear targets responsible for cell proliferation. However, hBCATc overexpression leads to an increase in the levels of Shc and SOS proteins (Figure 4.9), which have also been implicated in the activation of PI3K/Akt activation (Castellano and Downward, 2011). Therefore, hBCATc shows both positive and negative regulation of key proteins involved in the Ras/ERK pathway with the ultimate decrease in insulin-mediated activation of MAPK suggesting that hBCATc-induced regulation of cell proliferation and metastasis is unlikely through this pathway.

4.4.6 hBCATc regulates cross-talk between the Ras/ERK and Akt/mTOR pathways.

Subsequently, it was considered whether hBCATc may be involved in the regulation of the PI3K/Akt/mTOR signalling cascade through activation by IGF-1 or insulin. Indeed, upregulation of hBCATc was found to increase phospho-Akt following insulin treatment (Figure 4.15). Additionally, knockdown of *BCAT1* was found to downregulate IGF-1 and insulin-mediated phosphorylation of mTOR (Figure 4.16). Activation of the mTOR pathway has previously been shown to play a role in cell proliferation with increased synthesis of proteins and lipids (Mirabilli *et al*., 2018). Leucine, a substrate for hBCATc, stimulates protein synthesis through activation of the mTOR pathway as a result of phosphorylation of the eukaryotic initiation factor 4E binding protein and p70 ribosomal S6 kinase 1 (Zhang *et al*., 2017).

As reported above, the levels of Shc and SOS were increased in response to hBCATc overexpression. An increase in these proteins was associated with activation of PI3K/Akt/mTOR with hBCATc overexpression whilst concomitantly attenuating the phosphorylation of MAPK. Whilst it is widely accepted that

activation of IGF-1Rβ, Shc, Grb2 and SOS leads to MAPK activation (Kiyatkin *et al.*, 2008; Pronk *et al.*, 1993), increased levels of SOS activates Ras, which can also activate PI3K, Rac and Rho proteins associated with the regulation of the cytoskeletal and invasiveness of tumour cells (Castellano and Downward, 2011). Shc has been shown to increase Grb2 interaction with PI3K on the p85 regulatory subunit leading to activation of the catalytic subunit of p110 leading to increased PI3k/Akt signalling (Radhakrishnan *et al.*, 2008; Jiménez *et al.*, 2002). Moreover, Grb2 in a complex with the scaffolding protein Grb2-associated binder 1 (GAB) directly binds to p85 and enhances PI3K activation and subsequent Akt signalling (Ong *et al.*, 2002; Kiyatkin *et al.*, 2008). Therefore, in this instance the increased level of the downstream signalling proteins Shc and SOS, mediated by hBCATc overexpression, can be associated with increased activation of the Akt/mTOR axis rather than increased activation of MAPK.

Cross-talk of the RAS/ERK and PI3K/Akt/ pathways in the IGF-1 signalling cascade allows plasticity of tumour cells to utilise different effector molecules to promote tumour survival and progression (extensively reviwed by Mendoza, Er and Blenis, 2011). One mechanism details that MAPK phosphorylation of GAB1 inhibits GAB-1 mediated membrane recruitment of PI3K which leads to the suppression of Akt signalling (Lehr *et al.*, 2004). On the other hand, at high doses of insulin and IGF-1, Akt phosphorylates RAF at Ser259 and thus suppresses the activity of the RAS/MAPK signalling pathway (Arkun, 2016; Moelling *et al.*, 2002). Interestingly, in response to IGF-1 and insulin stimulation hBCATc overexpression enhanced Akt activation, therefore, hBCATc here may play a novel chaperone role allowing metabolic plasticity by sequestering Shc and SOS in the cytoplasm to promote Akt signalling whilst attenuating MAPK activation.

4.4.7 hBCATc regulates expression levels PI3K/Akt signalling effectors

Downstream of the PI3K/Akt pathway, the transcription factor FOXO3a was found to be increased in response to hBCATc overexpression whilst knockdown of *BCAT1* led to reduced expression FOXO3a (Figure 4.17A). FOXO3a is a member of the FOXO subfamily of forkhead transcription factors, which mediate a variety of cellular processes including proliferation, cell cycle progression and apoptosis (Farhan *et al.*, 2017). The role of FOXO3a in breast cancer has been suggested to be ERα+ dependant whereby FOXO3a upregulation in ERα+ MCF-7 cells reduced cell migration, however in TNBC MDA-MB-231 cells FOXO3a was found to promote tumour cell invasion (Sisci *et al.*, 2013; Storz *et al.*, 2009). Therefore, hBCATc positive regulation of this transcription factor in TNBC may contribute to hBCATc-mediated proliferation, migration and invasion, as observed in Figure 4.2. FOXO3a can act on cyclin-dependent kinase inhibitor 1B ($p27^{Kip1}$) directly by disrupting cyclin D/CDK4 and cyclin E/CDK2 complexes to promote cell proliferation (Greer and Brunet, 2005). IGF-1R signalling and subsequent Akt phosphorylation of FOXO3a leads to its translocation from nucleus to cytoplasm, where it associates with 14.3.3 protein (Hao *et al.*, 2018). The 14.3.3 proteins act as a scaffold to integrate signalling proteins with targets involved in biological processes, including cell cycle regulation (Fu, Subramanian and Masters, 2002). Overexpression of 14.3.3 has been associated with breast recurrence indicating a role in therapy resistance, mediated by the downregulation of the pro-apoptotic proteins Bcl-2-associated death promoter (BAD) and Bcl-2-like protein (BIM) (Thistle *et al.*, 2017; Niemantsverdriet *et al.*, 2008).

As previously discussed in section 4.4.2 during tumorigenesis and tumour progression regulation of redox homeostasis is fundamental to ensure cell survival (DeBerardinis and Chandel, 2016). Redox status is predominantly

regulated by the transcription factor Nrf2 and its repressor protein Keap1 in response to redox cellular status (Denicola *et al.*, 2011). PI3K/Akt activation has been demonstrated to be essential for the nuclear translocation of Nrf2, whereby Akt inhibitors, but not MAPK inhibitors, was found to reduce Nrf2 transcriptional activation of antioxidant genes (Nakaso *et al.*, 2003). Akt can also increase the stability of Nrf2 by activating p21 which disrupts the interaction between Keap1 and Nrf2, reducing Nrf2 phosphorylation, preventing its nuclear export and ubiquitination (Chen *et al.*, 2009). Under low concentrations of ROS, Nrf2 is bound to the E3 ubiquitin ligase Keap1 in the cytosol and degraded by the proteasome (Taguchi and Yamamoto, 2017). Under oxidative stress, this interaction is inhibited, by the oxidation of cysteine residues on Keap1, thus allowing Nrf2 translocation to the nucleus to induce the expression of antioxidant genes such as the oxidoreductases, thioredoxin (Trx) and glutaredoxin (Grx) (Ma, 2013). Expression levels of Nrf2 were found to be increased with hBCATc overexpression and decreased with knockdown of *BCAT1* (Figure 4.17B), suggesting levels of hBCATc to positively regulate Nrf2 expression. As hBCATc contains a redox-sensitive CXXC motif, the ability for hBCATc to regulate Nrf2 expression indicates a potential cell protective role in response to changes in the redox state in TNBC.

Additionally, GRP78 was found to be upregulated by hBCATc overexpression (Figure 4.17C). GRP78, is a molecular chaperone protein which is upregulated in response to endoplasmic reticulum (ER) stress as part of the unfolded protein response pathway (Pfaffenbach and Lee, 2011). IGF-1R signalling has been demonstrated to regulate GRP78 expression through the PI3K/AKT/mTOR axis (Pfaffenbach *et al.*, 2012), indicating that hBCATc expression could augment ER homeostasis by sequestering GRP78. Additionally, upregulation of GRP78

suppresses the activity of the pro-apoptotic Bik thus inhibiting apoptosis induction (Fu, Li and Lee, 2007). Therefore, hBCATc may promote evasion of apoptosis as shown in Figure 4.4 through the regulation of GRP78 expression. Hence, regulation of these Akt downstream effector proteins indicates an intrinsic role for hBCATc for tumour survival and progression mediated by cell proliferation, migration, cell cycle control and evasion of apoptosis through the regulation of FOXO3a, Nrf2 and GRP78.

4.4.8 Summary

Using the TNBC cell model, MDA-MB-231, knockdown of *BCAT1* resulted in a significant suppression of IGF-1 and insulin-mediated cell proliferation and migration, indicating that hBCATc regulates its control over proliferation and migration through this pathway. Subsequent analysis revealed that these measures of cell growth were precipitated through activation of the PI3K/Akt/mTOR pathway targeting an increase in FOXO3a, Nrf2 and GRP78, which play fundamental roles in cellular homeostasis and survival. Importantly, hBCATc at the same time reduced phosphorylation of MAPK indicating that regulation of proliferation and migration through hBCATc is primarly through the PI3K/Akt/mTOR pathway rather than through MAPK signalling.

Chapter 5

5.0 Summary and Future Work

Mai Ahmed Shafei

5.1 Summary

Metabolic reprogramming in tumour cells offers targetable vulnerabilities (Long, Li and Zhang, 2016). The expression of key metabolic proteins involved in BCAA metabolism (hBCATc and hBCATm), isocitrate dehydrogenase (IDH1 and IDH2 and serine metabolism (PHGDH) were assessed in a cohort of breast cancer patients (Chapter 4). Differential expression of these metabolic proteins was associated with the different breast cancer subtypes, identifying distinct metabolic dependencies, which may offer novel therapeutic targets.

Expression of hBCATm correlated with smaller tumours and luminal A breast cancer, which associated with a better prognosis compared with other breast cancer subtypes. Using serial sections, hBCATm was found to be significantly associated with IDH1 expression, indicating that these two metabolic pathways are activated concomitantly, potentially through, SREBP1 which regulates both enzymes (Dey *et al.*, 2017; Ricoult *et al.*, 2016). IDH1 is responsible for the oxidative carboxylation of isocitrate to maintain levels of α-ketoglutarate in the cytosol, critical for sustaining the TCA cycle (Kabe *et al.*, 2006), whilst hBCATm utilises α-ketoglutarate in the transamination of BCAAs in the mitochondria to support cell growth (Conway *et al.*, 2016). Therefore, increased expression of IDH1 may replenish the α-ketoglutarate pool in cells with increased hBCATm, which can then be shuttled to the mitochondria (Kabe *et al.*, 2006), to support the TCA cycle. Here, IDH1 and hBCATm metabolism can be proposed to work in conjunction to support tumour growth.

hBCATc expression was found to be significantly associated with the more aggressive HER2+ and TNBC subtypes. Intriguingly, SKBR3 cells, which are a

well-known model for HER2+ subtype, were found to lack hBCATc expression, also reported in related studies (Thewes *et al.*, 2017; Zhang *et al.*, 2013). This suggests that the current breast cancer cell line models of HER2+ subtype do not reflect the pathophysiological outcome observed in the human breast cancer tissue. Expression levels of hBCATc were found to be increased in TNBC, MDA-MB-231 cells. To elucidate the functional role for hBCATc, transient knockdown was optimised with siRNA as well as shRNA mediated knockdown and overexpression of *BCAT1* (Chapter 4). As reported in other relevant studies (Thewes *et al.*, 2017), hBCATc was found to play an intrinsic role in promoting a significant increase in cell proliferation, migration and invasion indicating that activity or its proposed chaperone role enhances tumour progression. Additionally, BCAA levels were found to regulate cell proliferation in a dose dependant manner where lower concentrations increased cell proliferation whilst higher concentrations returned cell proliferation levels to baseline. Increased levels of leucine, a substrate for hBCATc, has been demonstrated to activate mTOR, a serine-threonine kinase that binds with proteins to induce intracellular signalling pathways directly involved in glutaminolysis, nucleotide and protein synthesis, supporting tumour growth (Laplante and Sabatini, 2013; Son *et al.*, 2019; Zhang and Han, 2017).

In breast cancer insulin/IGF-1 signalling has been demonstrated to contribute to tumourigenesis through the activation of growth signalling effectors which is mediated by two chief pathways: Akt/mTOR and RAS/MAPK. For the first time, we show that hBCATc regulates IGF-1 and insulin mediated cell proliferation and migration in TNBC cells. Expression of hBCATc affected the expression of key proteins involved in the insulin/IGF-1 signalling cascade supporting a chaperone

role in this pathway (Figure 5.1). Knockdown of *BCAT1* was found to reduce expression of IGF-1Rβ, which is responsible for intracellular signal transduction upon receptor activation (Kavran *et al.*, 1993). Moreover, downstream IGF-1R signalling effectors Shc and SOS levels were increased with shRNA-mediated *BCAT1* overexpression, however, the expression levels were not significantly affected with knockdown of *BCAT1*. We propose that hBCATc acts as a cytoplasmic scaffold protein, rather than affecting transcription, to enhance the IGF-1/insulin signalling cascade. Increased levels of SOS normally leads to the activation of Ras, Raf and subsequent activation of MAPK (Pan *et al.*, 2010). In this study, knockdown of *BCAT1* was found to increase phosphorylation of MAPK, in the presence of IGF-1 and insulin and nuclear translocation of MAPK. These findings suggest a regulatory role for hBCATc with respect to MAPK activation, mediated by abrogating MAPK nuclear translocation, despite increased expression of the upstream signalling effectors Shc and SOS of the RAS/MAPK signalling cascade.

Interestingly, hBCATc was found to activate Akt in response to insulin stimulation. Shc has been previously reported to increase Grb2 interaction with PI3K leading to activation of PI3K/Akt signalling (Radhakrishnan *et al.*, 2008; Jiménez *et al.*, 2002). Additionally, increased levels of SOS can induce Ras activation of PI3K/Akt (Castellano and Downward, 2011). We propose that hBCATc plays a novel chaperone role by enhancing this mechanism by sequestering the downstream signalling molecules Shc and SOS to promote Akt signalling. Cross-talk of the RAS/ERK and PI3K/Akt pathways in the IGF-1 signalling cascade

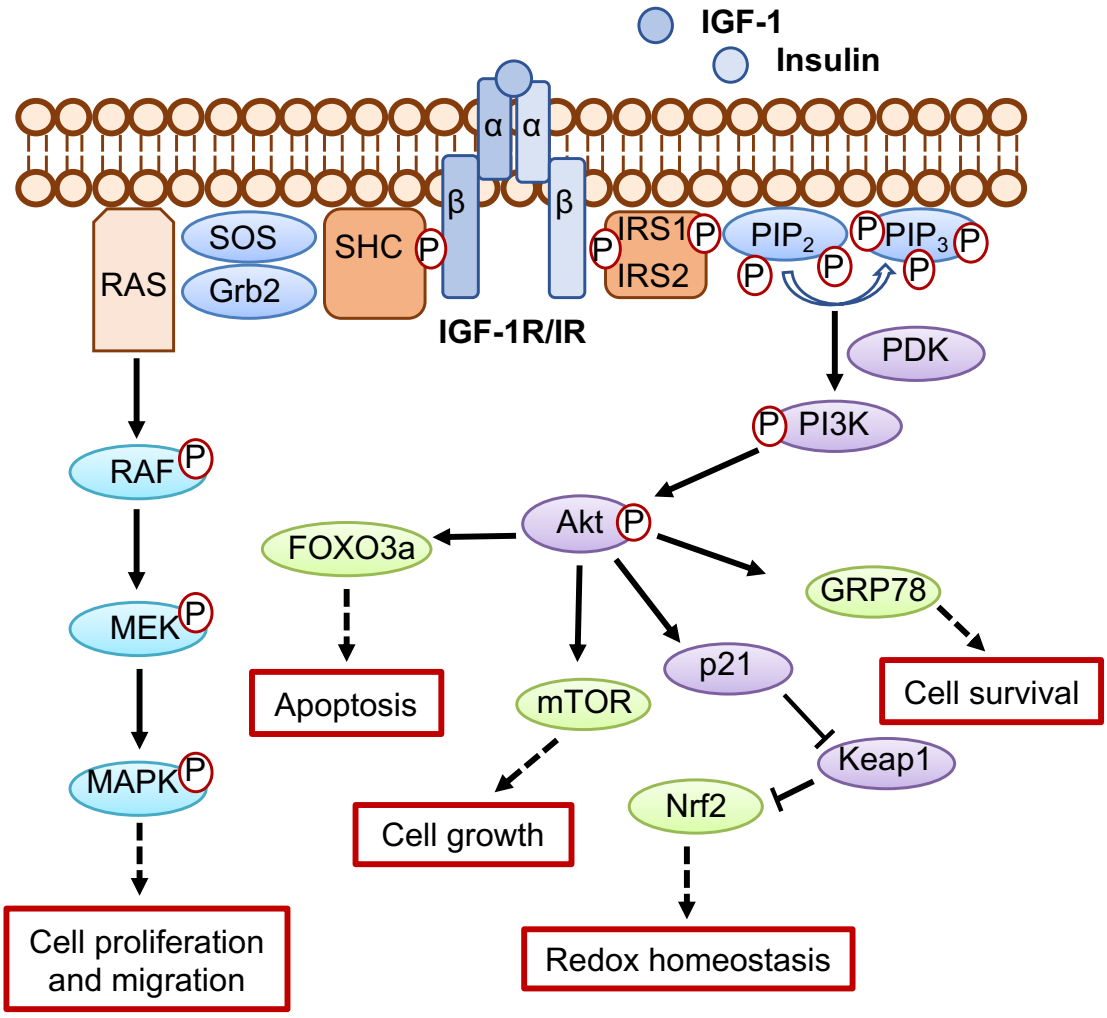

Figure 5.1 The role of hBCATc on the insulin/IGF-1 signalling cascades. Activation of IGF-1R and insulin receptor, mediated by ligand binding of IGF-1 or insulin leads to the subsequent activation of the phosphoinositide-3 kinase (PI3K)/serine/threonine-specific protein kinase (Akt) and the Ras/mitogen-activated protein kinase (MAPK) pathways. Increased expression of hBCATc increased levels of IGF-1Rβ, Src homology 2 domain containing transforming protein (Shc) and son of sevenless (SOS) however MAPK activation was increased with knockdown of *BCAT1* suggesting hBCATc to inhibit MAPK activation. Increased levels of hBCATc increased phosphorylation of Akt and the expression levels of the downstream effector proteins; forkhead box O3 (FOXO3a), mammalian target of rapamycin (mTOR), nuclear factor (erythroid-derived 2)-like-2 (Nrf2) and glucose regulated protein 78 (GRP78) thereby promoting cell proliferation, migration, and evasion of apoptosis in triple negative breast cancer. Grb2, growth factor receptor-bound 2; IRS, insulin receptor substrate; Keap1, kelch-like ECH-associated protein 1; PDK, phosphoinositide-dependent protein kinase; PIP_2, phosphatidylinositol (4,5) bisphosphate; PIP_3, phosphatidylinositol (3,4,5) trisphosphate.

allows plasticity of tumour cells to utilise different effector molecules to promote tumour survival and progression (Mendoza, Er and Blenis, 2011). Downstream of the PI3K/Akt pathway hBCATc was also shown to positively regulate FOXO3a, Nrf2 and GRP78, proteins important to cell proliferation, cell survival and protein folding. In conclusion, data generated in this thesis supports a role for hBCATc as a molecular chaperone through the activation of PI3K/Akt signalling and subsequent Akt downstream effector proteins potentiating tumour survival and progression mediated by cell proliferation, migration, cell cycle control and evasion of apoptosis.

5.2 Future work

Work detailed in this thesis showed novel moonlighting roles for hBCATc, which may contribute to increased proliferation, migration and invasion. In particular, hBCATc was demonstrated to regulate cross-talk between the PI3K/Akt/mTOR and the Ras/ERK pathway under IGF-1 and insulin treatment in TNBC, indicating a novel role for this metabolic protein in the regulation of IGF-1/insulin-mediated proliferation. Moreover, a regulatory role for hBCATc of Nrf2 expression was also identified which may contribute to redox homeostasis and the evasion of apoptosis. Therefore, it is important to further understand the underlying mechanistic role for hBCATc in TNBC as there is the potential for developing a novel targeted therapeutic approach.

- To assess BCAA levels *in vitro* following *BCAT1* knockdown using LCMS to determine hBCATc regulation
- To establish whether hBCATc is involved in the regulation of the redox status of TNBC cells using a direct measure of ROS status
- To assess the expression of proteins involved in the regulation of the redox status such as Grx and Trx following *BCAT1* knockdown and overexpression to determine hBCATc involvement
- To assess the expression of pro-apoptotic proteins such as BAD, BCL and following *BCAT1* knockdown and overexpression to determine the mechanisms by which hBCATc regulates apoptosis
- Develop knockdown shRNA constructs with a rescue system to re-express hBCATc which would further validate findings in this study
- Optimise a method for live-cell observation of hBCATc in order to observe its movements in response to specific stimuli such as insulin and IGF-1

treatment to monitor interaction with downstream signalling effectors such as MAPK and FOXO3a

- Investigate the effect of IGF-1R inhibitors on the expression of hBCATc
- Use of IGF-1R inhibitors and shRNA-mediated overexpression of hBCATc to assess whether hBCATc expression allows the reactivation of the IGF-1/insulin signalling in the absence of ligand activation

6.0 References

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6.0 References

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7.0 Appendix

Mai Ahmed Shafei

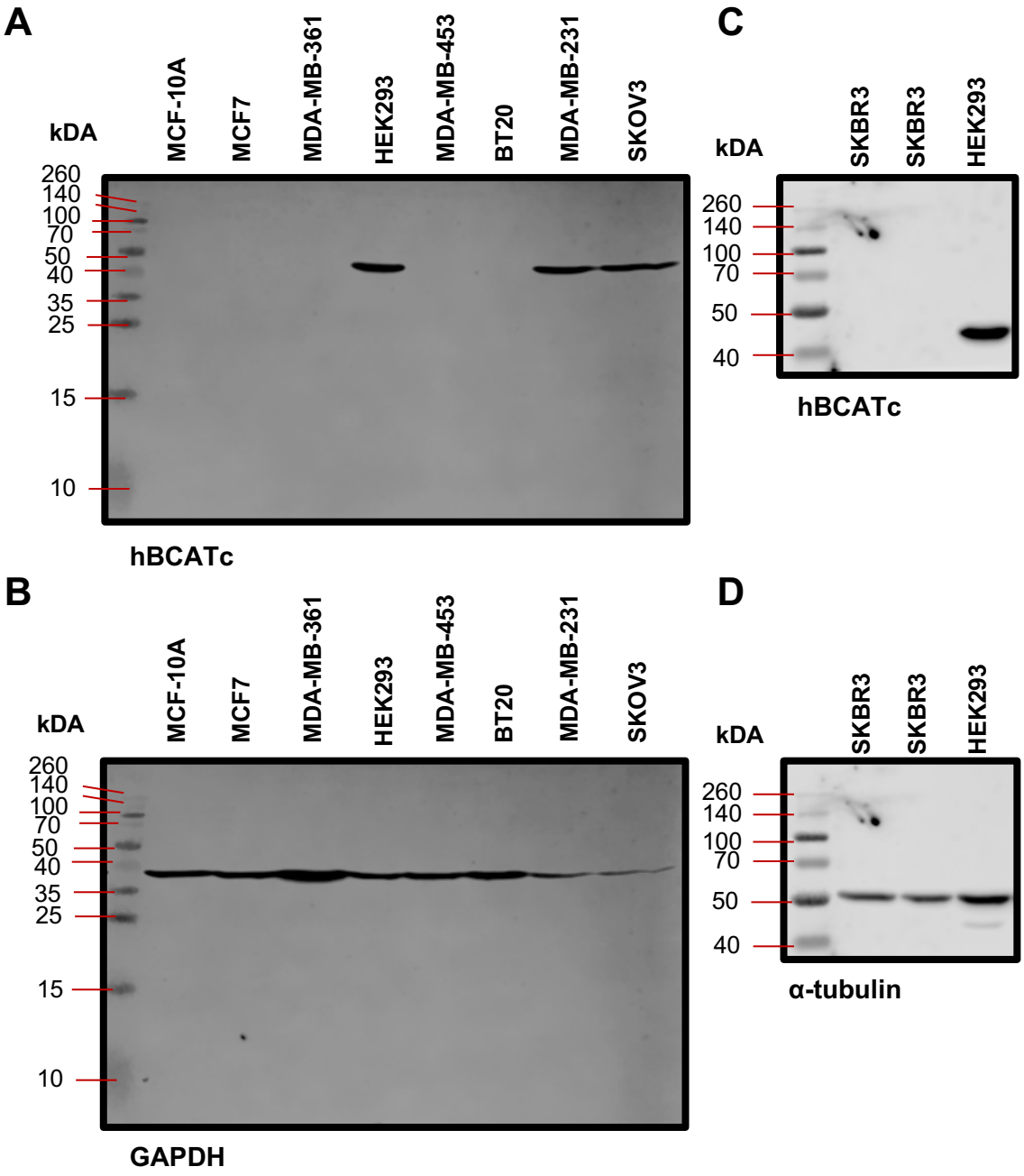

Figure 7.1. Western blot repeats of Figure 3.20A and 3.20C of metabolic protein expression in breast cancer cell lines. 20 µg of cell lysates were separated by SDS-PAGE on a 12% gel and probed for the respective antibodies as described in the materials and methods. (**A**) Full-length immunoblot for hBCATc for panel of breast cancer cell lines (**B**) full-length immunoblot for GAPDH for panel of breast cancer cell lines (**C**) SKBR3 and HEK293 immunoblot for hBCATc with molecular markers (**D**) SKBR3 and HEK293 immunoblot for α-tubulin with molecular markers.

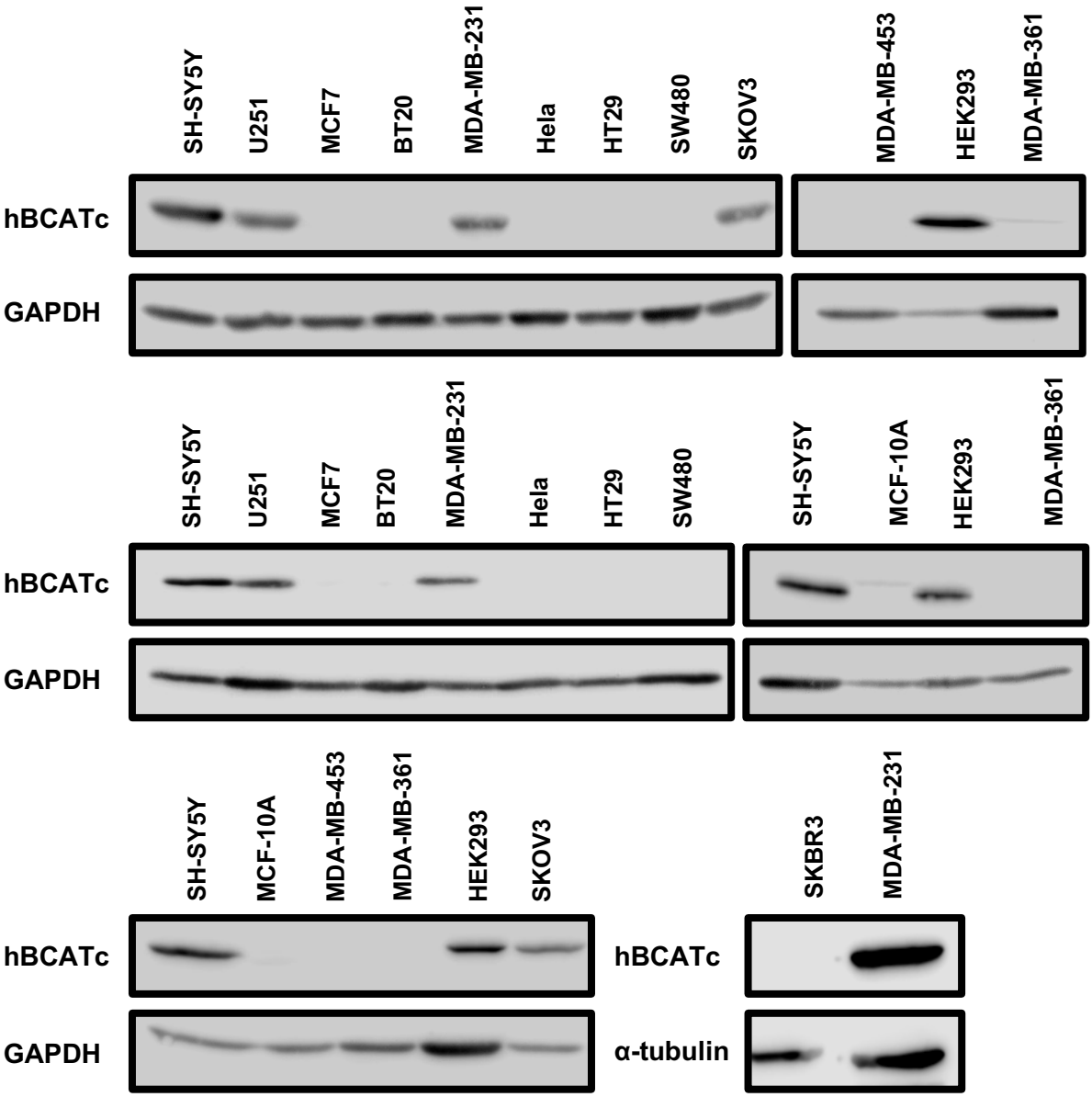

Figure 7.2. Western blot repeats of Figure 3.20A and 3.20C of metabolic protein expression in breast cancer cell lines. 20 µg of cell lysates were separated by SDS-PAGE on a 12% gel and probed for the respective antibodies as described in the materials and methods. Cropped immunoblots for hBCATc and loading control replicates.

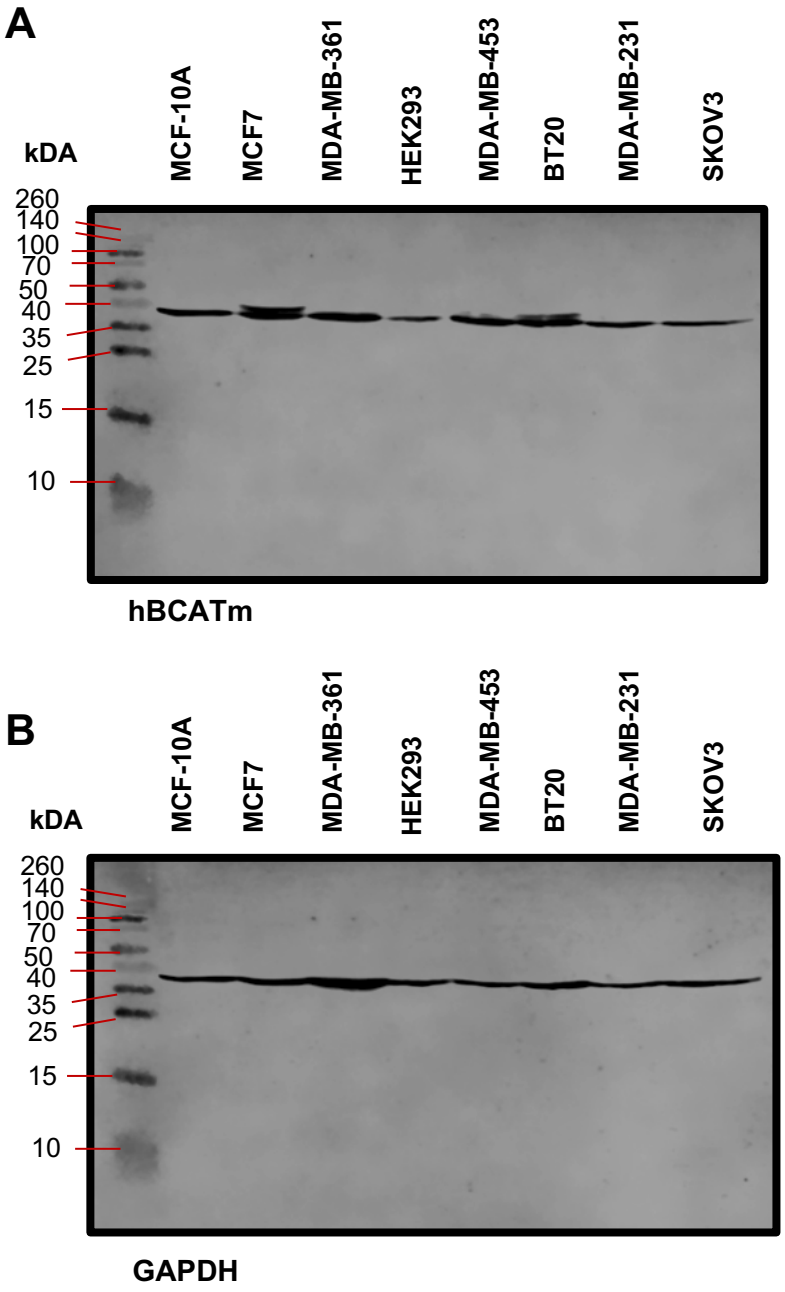

Figure 7.3. Western blot repeats of Figure 3.20A of metabolic protein expression in breast cancer cell lines. 20 µg of cell lysates were separated by SDS-PAGE on a 12% gel and probed for the respective antibodies as described in the materials and methods. (**A**) Full-length immunoblot for hBCATm for panel of breast cancer cell lines (**B**) full-length immunoblot for GAPDH for panel of breast cancer cell lines.

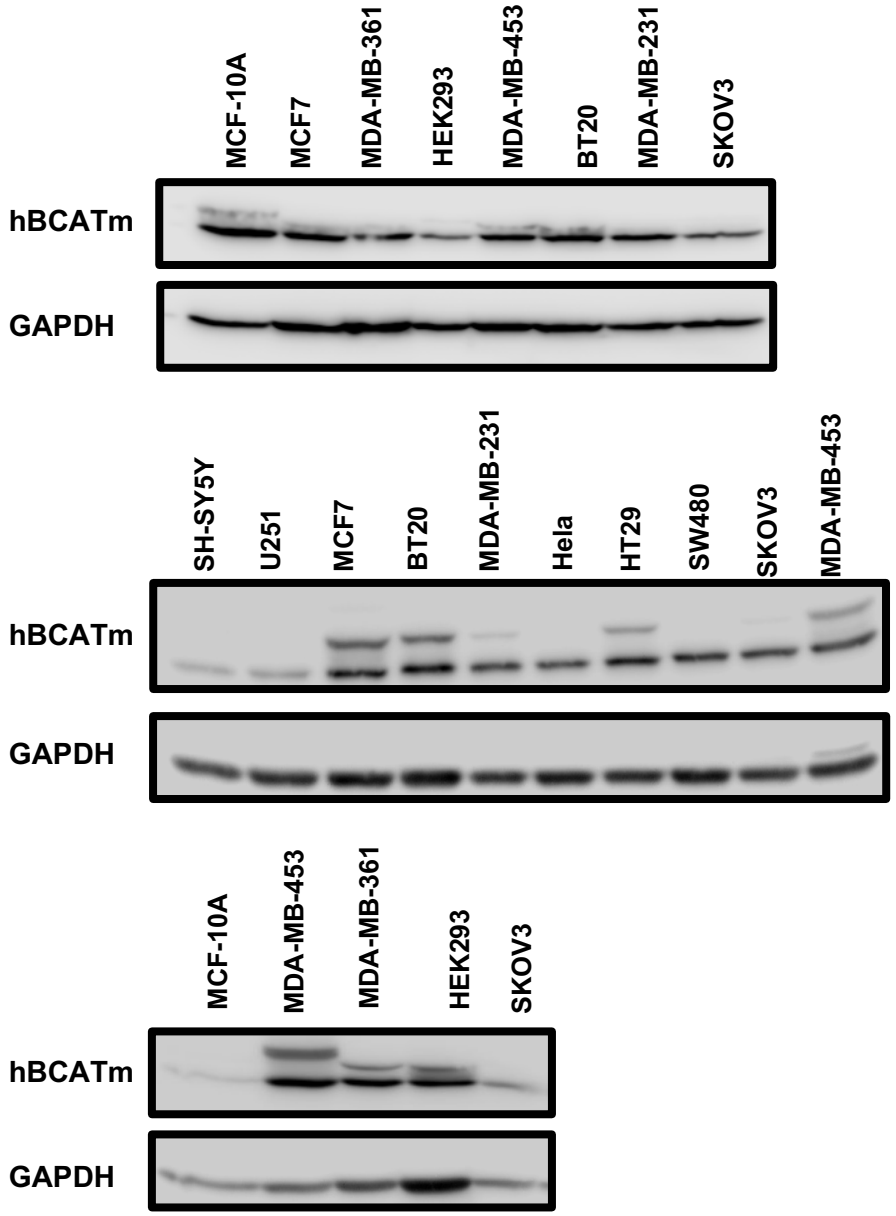

Figure 7.4. Western blot repeats of Figure 3.20A of metabolic protein expression in breast cancer cell lines. 20 µg of cell lysates were separated by SDS-PAGE on a 12% gel and probed for the respective antibodies as described in the materials and methods. Cropped immunoblots for hBCATm and GAPDH replicates.

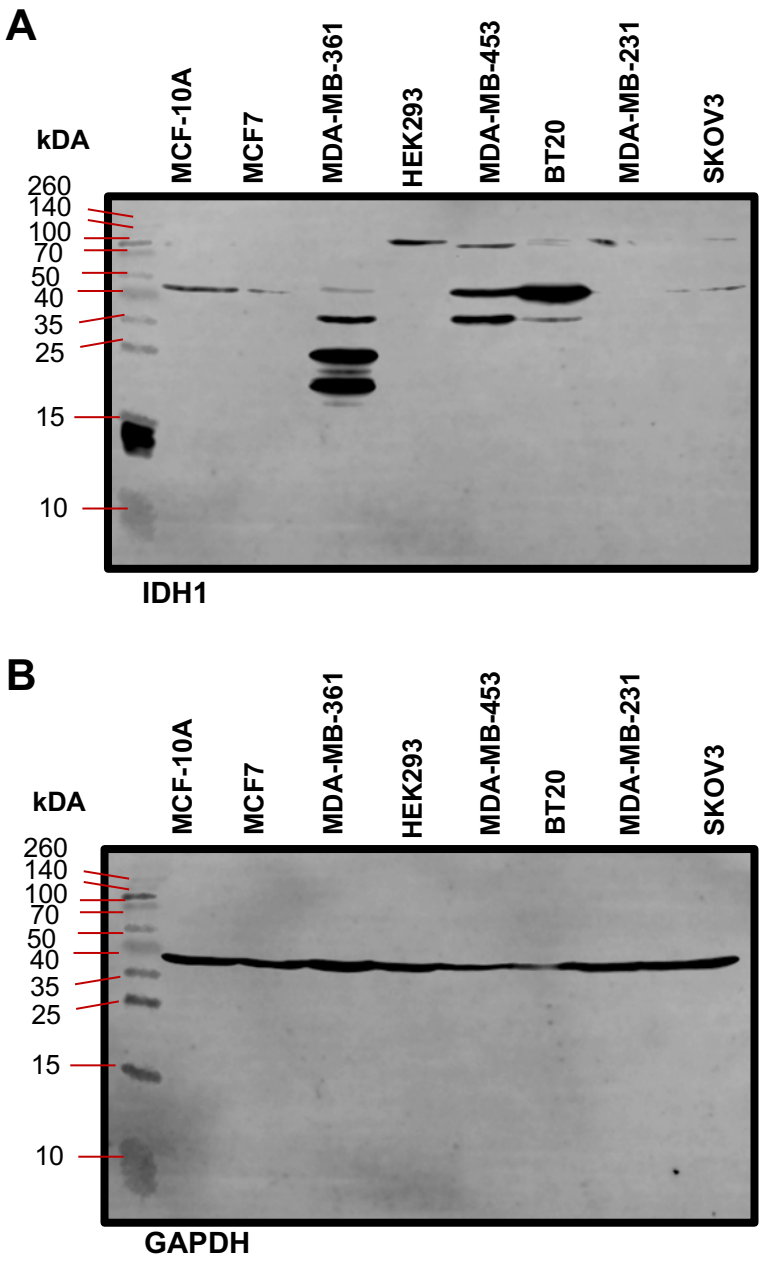

Figure 7.5. Western blot repeats of Figure 3.20A of metabolic protein expression in breast cancer cell lines. 20 µg of cell lysates were separated by SDS-PAGE on a 12% gel and probed for the respective antibodies as described in the materials and methods. (**A**) Full-length immunoblot for IDH1 for panel of breast cancer cell lines (**B**) full-length immunoblot for GAPDH for panel of breast cancer cell lines.

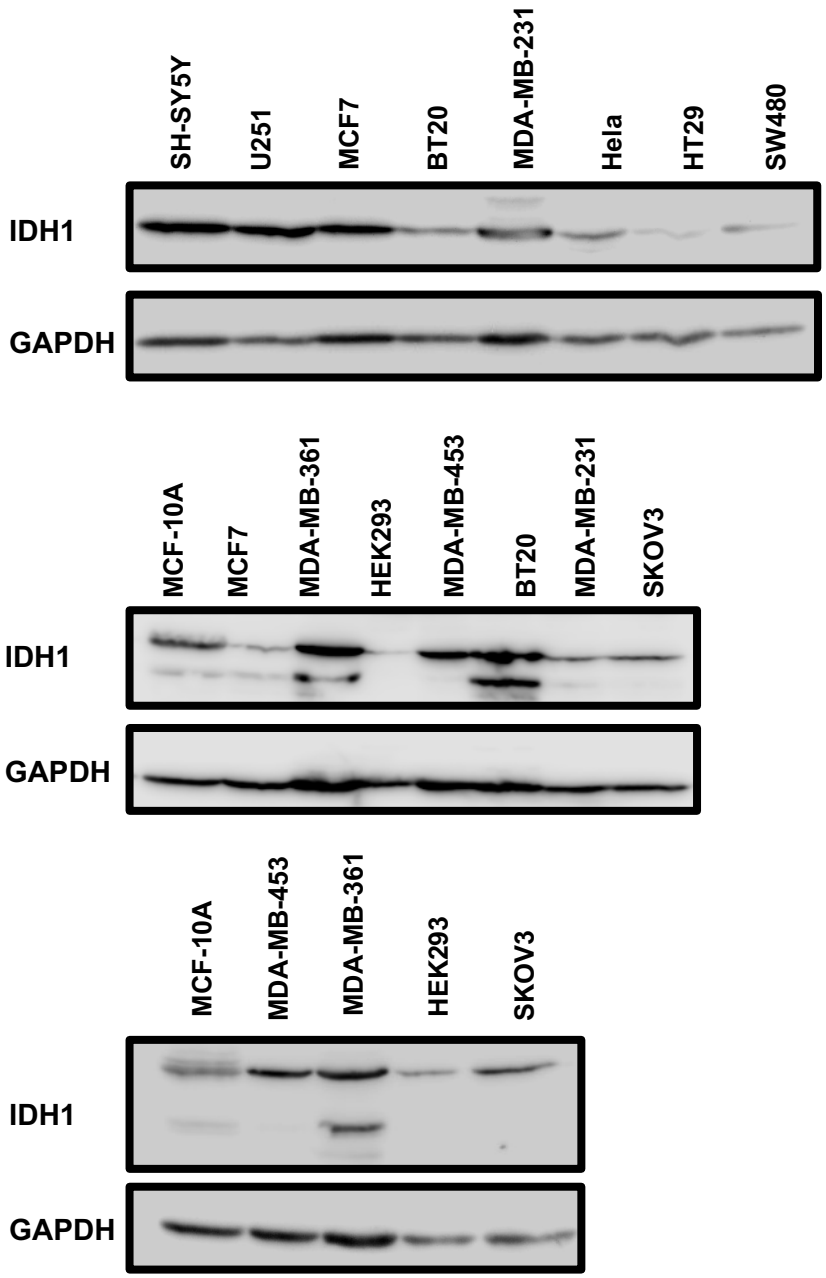

Figure 7.6. Western blot repeats of Figure 3.20A of metabolic protein expression in breast cancer cell lines. 20 µg of cell lysates were separated by SDS-PAGE on a 12% gel and probed for the respective antibodies as described in the materials and methods. Cropped immunoblots for IDH1 and GAPDH replicates.

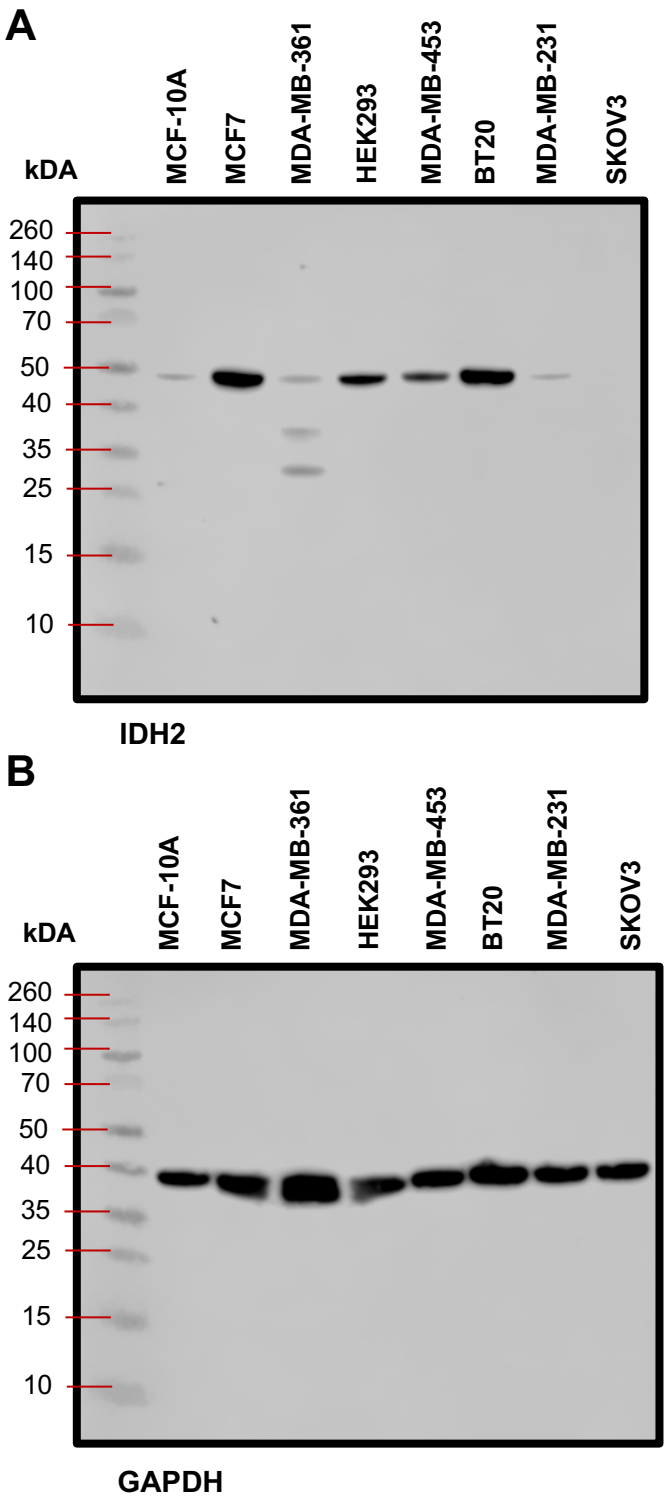

Figure 7.7. Western blot repeats of Figure 3.20A of metabolic protein expression in breast cancer cell lines. 20 µg of cell lysates were separated by SDS-PAGE on a 12% gel and probed for the respective antibodies as described in the materials and methods. (**A**) Full-length immunoblot for IDH2 for panel of breast cancer cell lines (**B**) full-length immunoblot for GAPDH for panel of breast cancer cell lines.

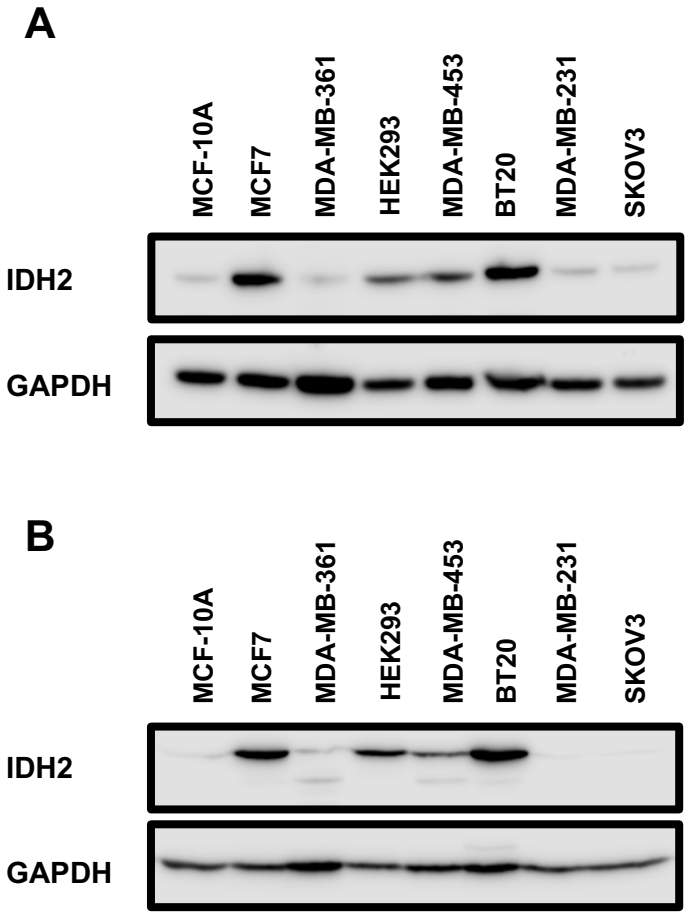

Figure 7.8. Western blot repeats of Figure 3.20A of metabolic protein expression in breast cancer cell lines. 20 µg of cell lysates were separated by SDS-PAGE on a 12% gel and probed for the respective antibodies as described in the materials and methods. Cropped immunoblots for IDH2 and GAPDH replicates.

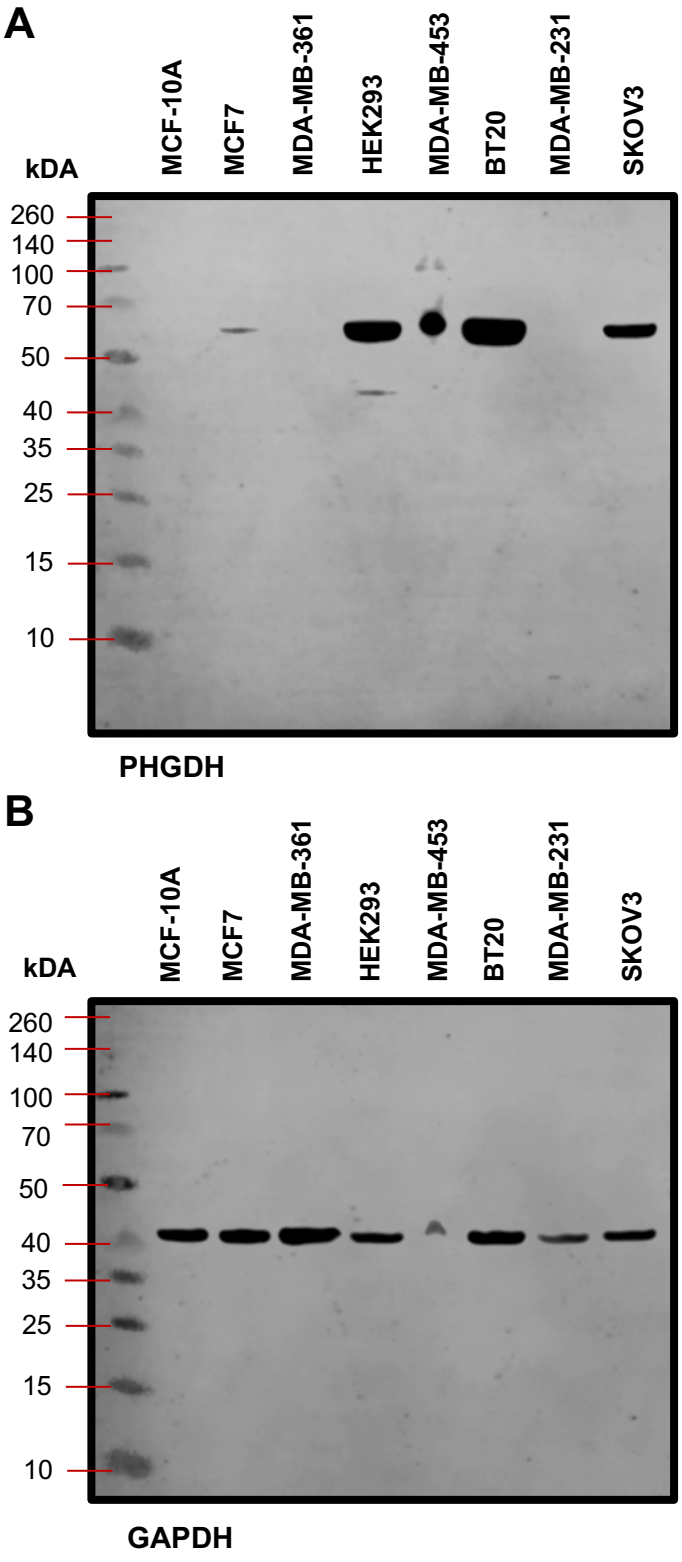

Figure 7.9. Western blot repeats of Figure 3.20A of metabolic protein expression in breast cancer cell lines. 20 µg of cell lysates were separated by SDS-PAGE on a 12% gel and probed for the respective antibodies as described in the materials and methods. (**A**) Full-length immunoblot for PHGDH for panel of breast cancer cell lines (**B**) full-length immunoblot for GAPDH for panel of breast cancer cell lines.

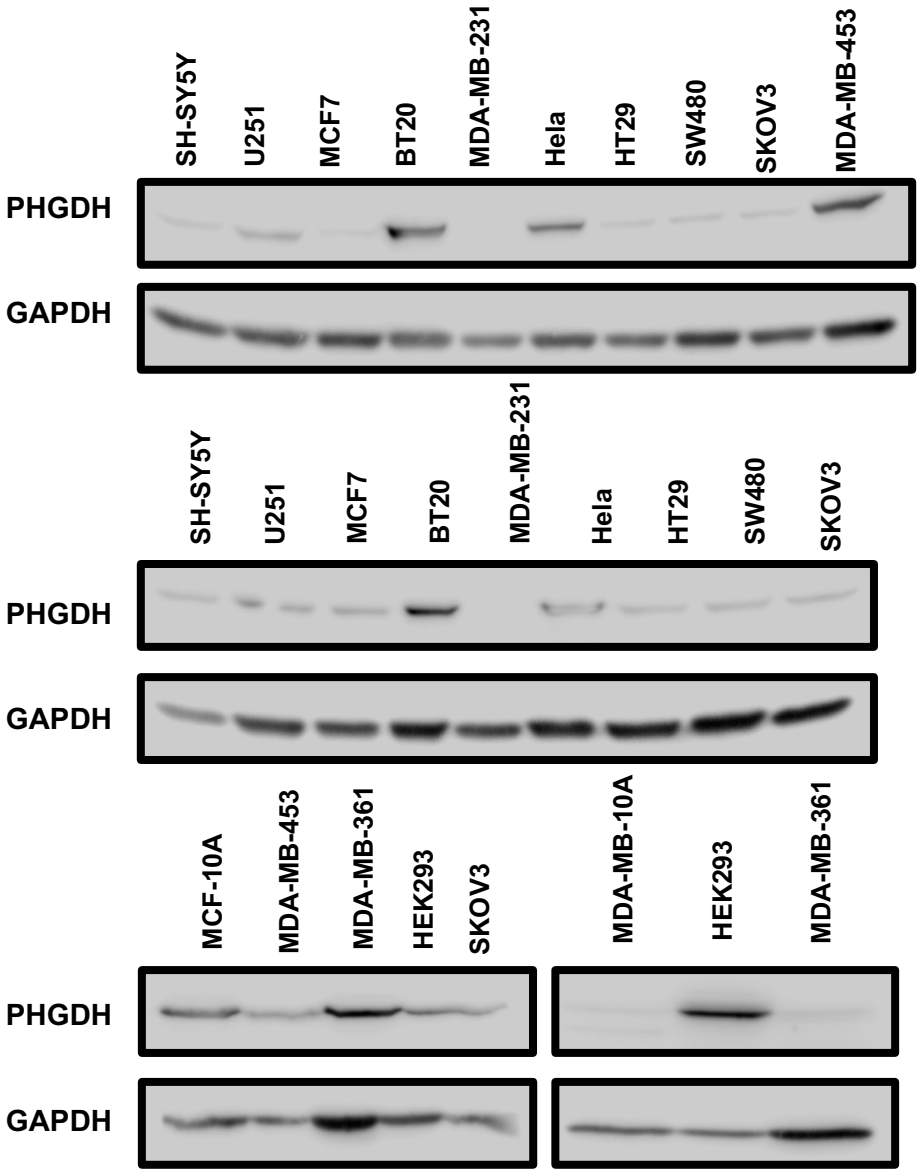

Figure 7.10. Western blot repeats of Figure 3.20A of metabolic protein expression in breast cancer cell lines. 20 µg of cell lysates were separated by SDS-PAGE on a 12% gel and probed for the respective antibodies as described in the materials and methods. Cropped immunoblots for PHGDH and GAPDH replicates.

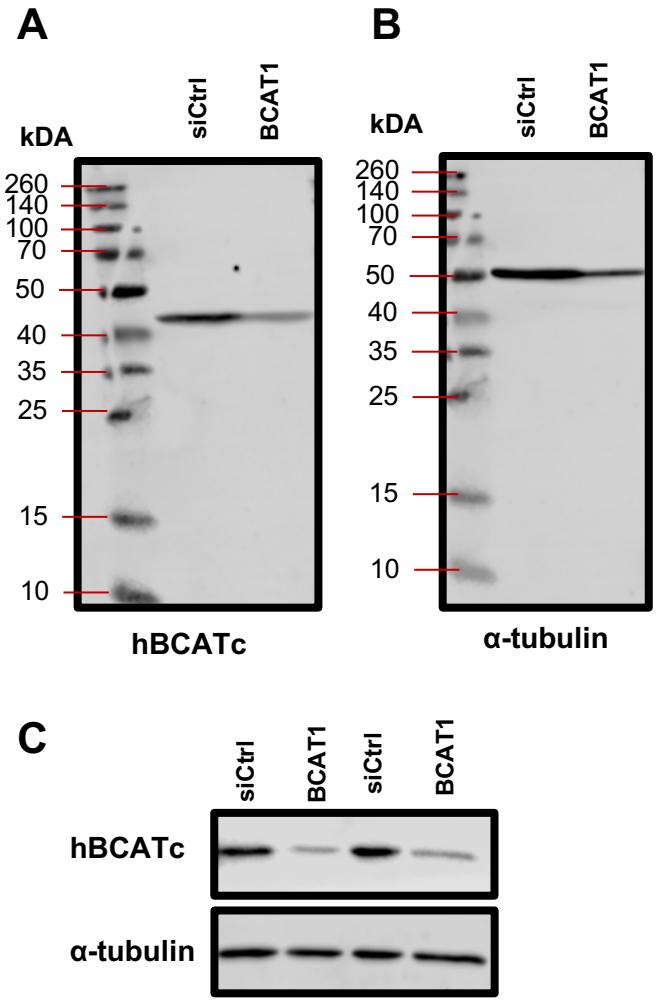

Figure 7.11. Western blot repeats of Figure 4.1A of siRNA knockdown of *BCAT1* in MDA-MB-231 cells. Cells treated with 20 nM *BCAT1* siRNA transfection for 72 hours. (**A**) Full-length immunoblot for hBCATc (**B**) Full-length immunoblot for α-tubulin (**C**) Cropped immunoblots for hBCATc and α-tubulin.

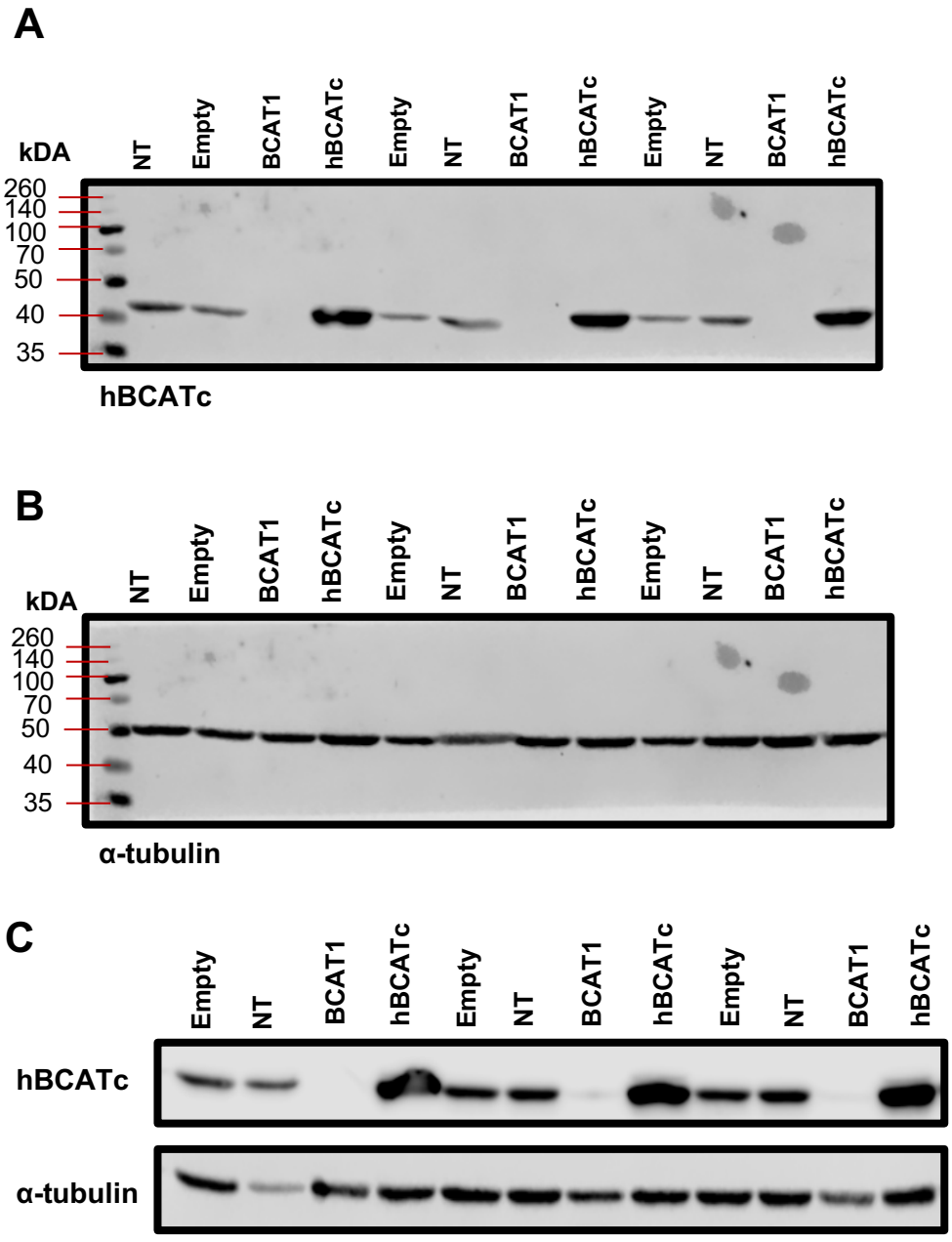

Figure 7.12. Western blot repeats of Figure 4.8B and 4.9A of shRNA knockdown of *BCAT1* and overexpression confirmation in MDA-MB-231 cells. Cells were stably transfected with shRNA for knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty). (**A**) Immunoblot for hBCATc with molecular markers (**B**) immunoblot for α-tubulin with molecular markers (**C**) Cropped immunoblots for hBCATc and α-tubulin.

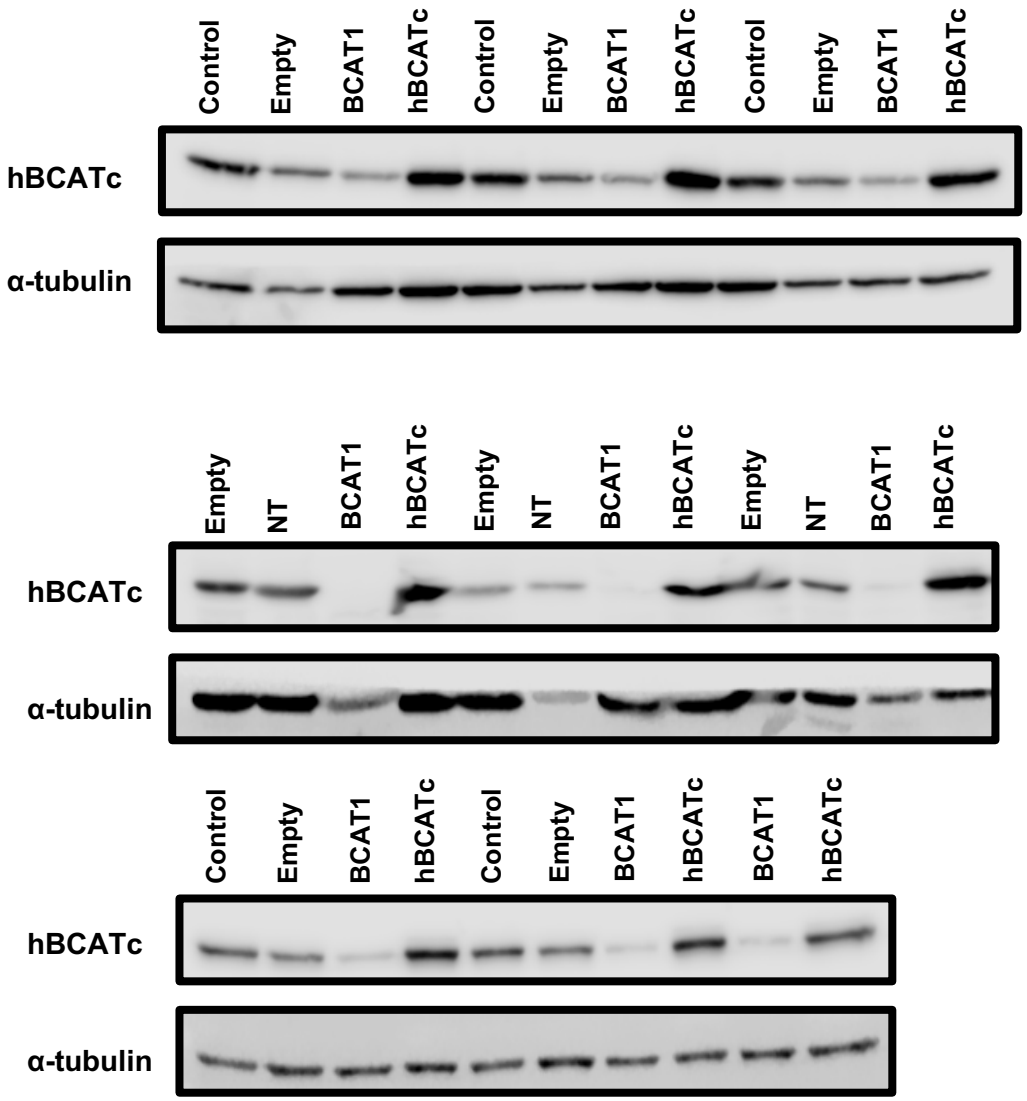

Figure 7.13. Western blot repeats of Figure 4.8B and 4.9A of shRNA knockdown of *BCAT1* and overexpression confirmation in MDA-MB-231 cells. Cells were stably transfected with shRNA for knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty). Cropped immunoblots for hBCATc and α-tubulin.

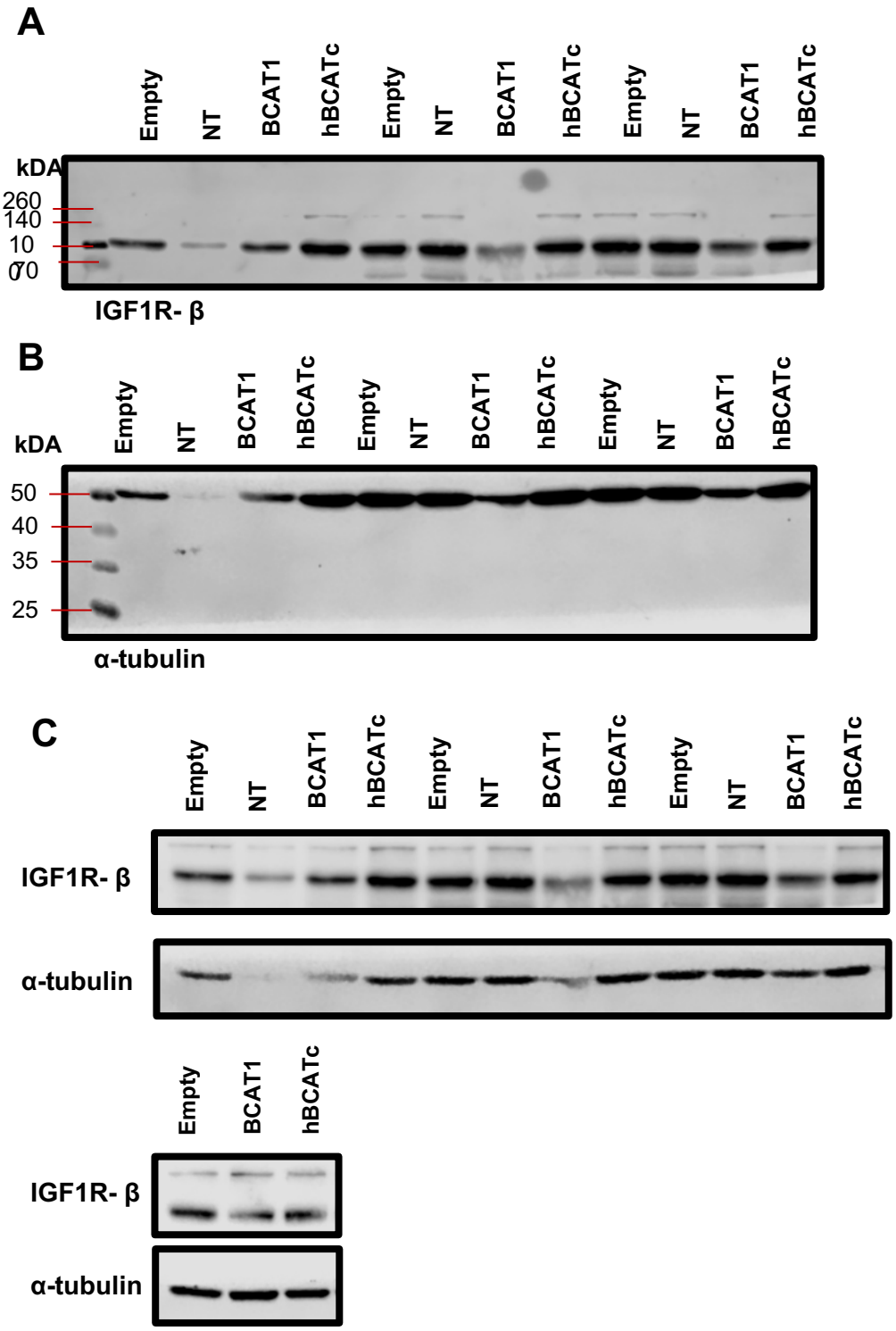

Figure 7.14. Western blot repeats of Figure 4.9B of knockdown and overexpression of *BCAT1* regulates IGF-1/insulin signalling cascade in MDA-MB-231 cells. Cells were stably transfected with shRNA for knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty). (**A**) Immunoblot for IGF1R- β with molecular markers (**B**) immunoblot for α-tubulin with molecular markers (**C**) Cropped immunoblots for IGF1R- β and α-tubulin.

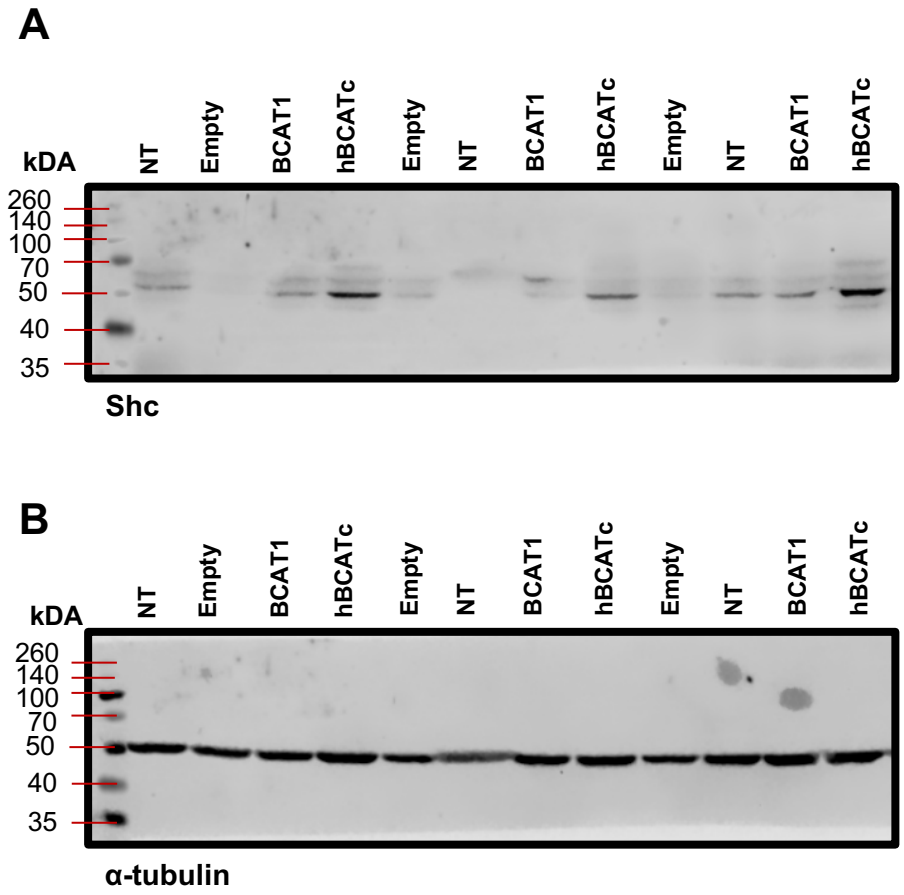

Figure 7.15. Western blot repeats of Figure 4.9C of knockdown and overexpression of *BCAT1* regulates IGF-1/insulin signalling cascade in MDA-MB-231 cells. Cells were stably transfected with shRNA for knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty). (**A**) Immunoblot for Shc with molecular markers (**B**) immunoblot for α-tubulin with molecular markers.

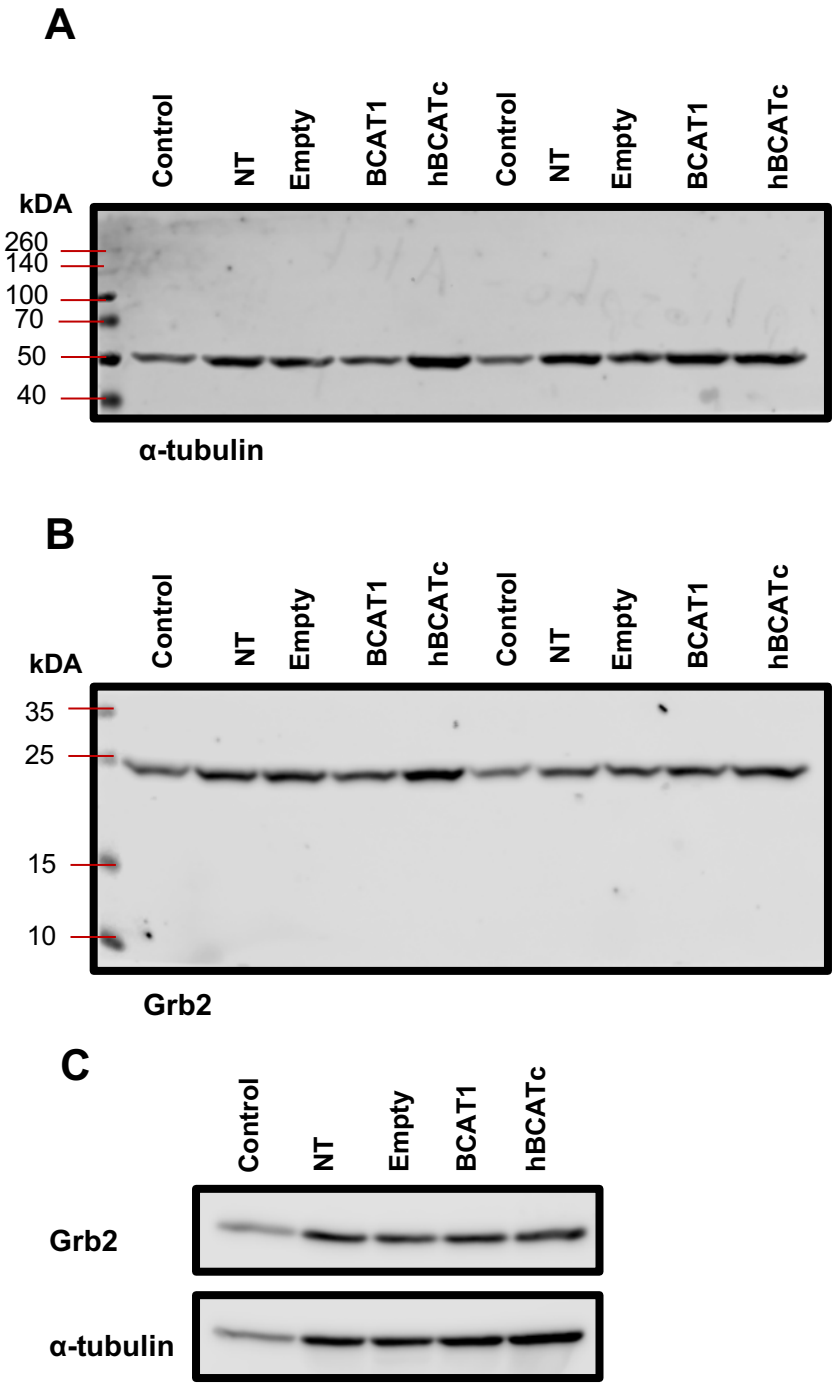

Figure 7.16. Western blot repeats of Figure 4.9D of knockdown and overexpression of *BCAT1* regulates IGF-1/insulin signalling cascade in MDA-MB-231 cells. Cells were stably transfected with shRNA for knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty). (**A**) Immunoblot for α-tubulin with molecular markers (**B**) immunoblot for Grb2 with molecular markers (**C**) Cropped immunoblots for Grb2 and α-tubulin replicates.

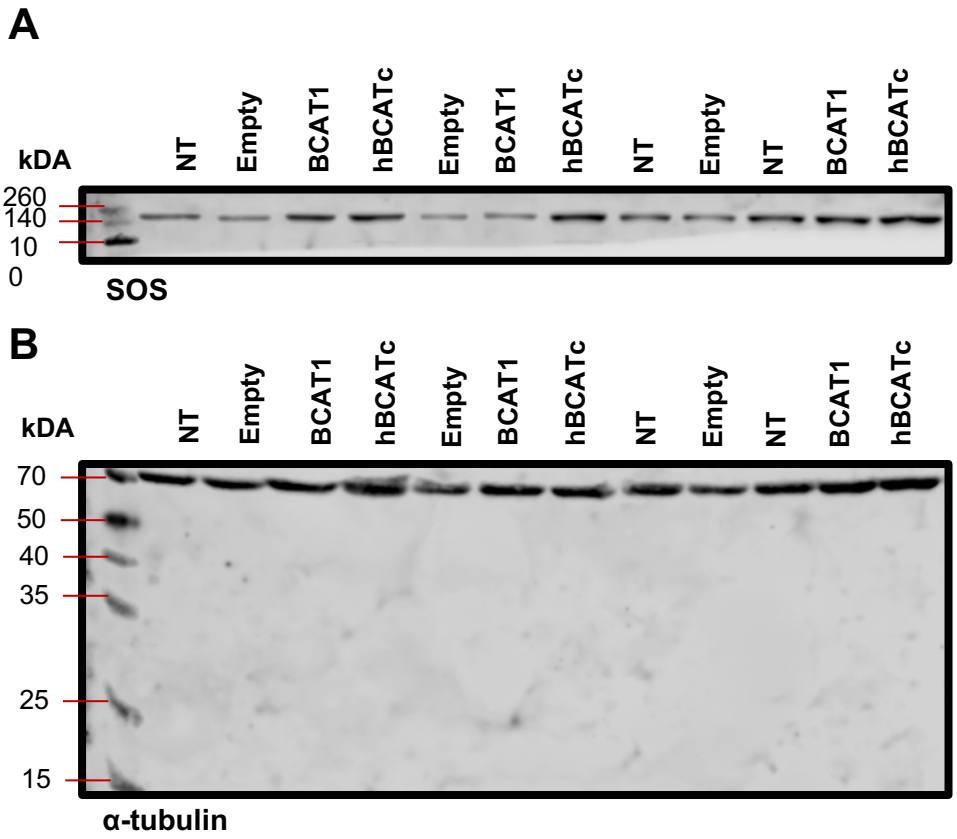

Figure 7.17. Western blot repeats of Figure 4.9D of knockdown and overexpression of *BCAT1* regulates IGF-1/insulin signalling cascade in MDA-MB-231 cells. Cells were stably transfected with shRNA for knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty). (**A**) Immunoblot for SOS with molecular markers (**B**) immunoblot for α-tubulin with molecular markers.

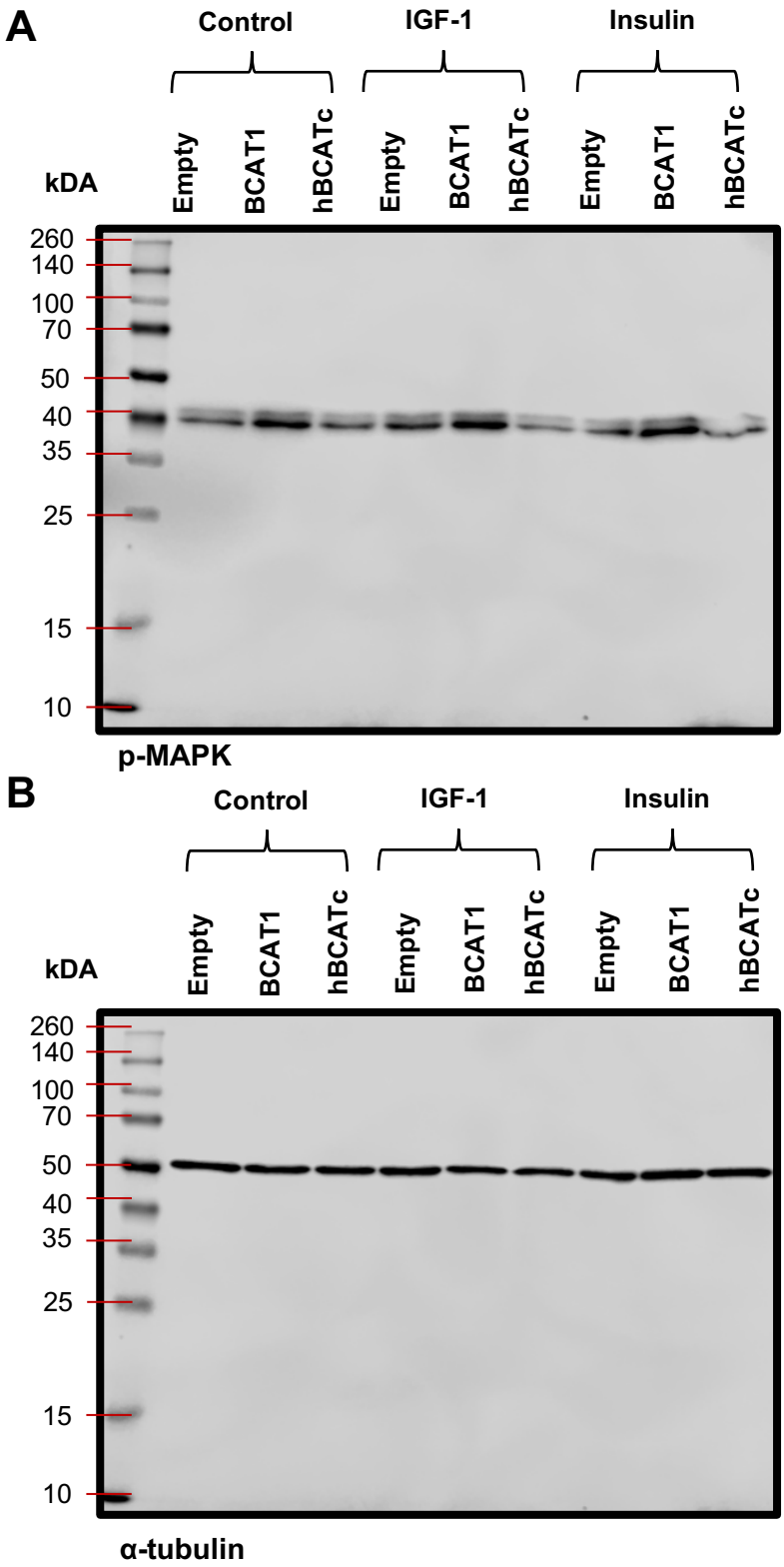

Figure 7.18. Western blot repeats of Figure 4.10A of knockdown and overexpression of *BCAT1* negatively regulates phosphorylation of MAPK in MDA-MB-231 cells. Cells were treated with stable shRNA transfection for *BCAT1* knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty) for 8 days and treated with 100 nM insulin and 100 ng/mL IGF-1 for 24 hours accordingly. (**A**) Full length immunoblot for p-MAPK (**B**) Full-length immunoblot for α-tubulin.

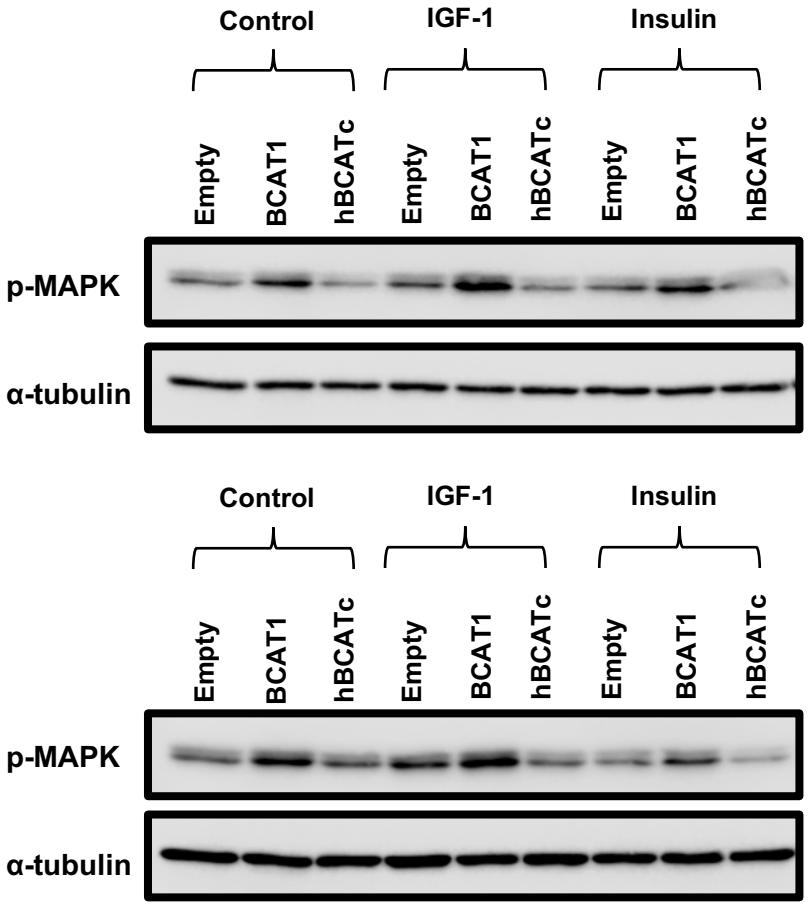

Figure 7.19. Western blot repeats of Figure 4.10A of knockdown and overexpression of *BCAT1* negatively regulates phosphorylation of MAPK in MDA-MB-231 cells. Cells were treated with stable shRNA transfection for *BCAT1* knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty) for 8 days and treated with 100 nM insulin and 100 ng/mL IGF-1 for 24 hours accordingly. Cropped immunoblots for p-MAPK and α-tubulin replicates.

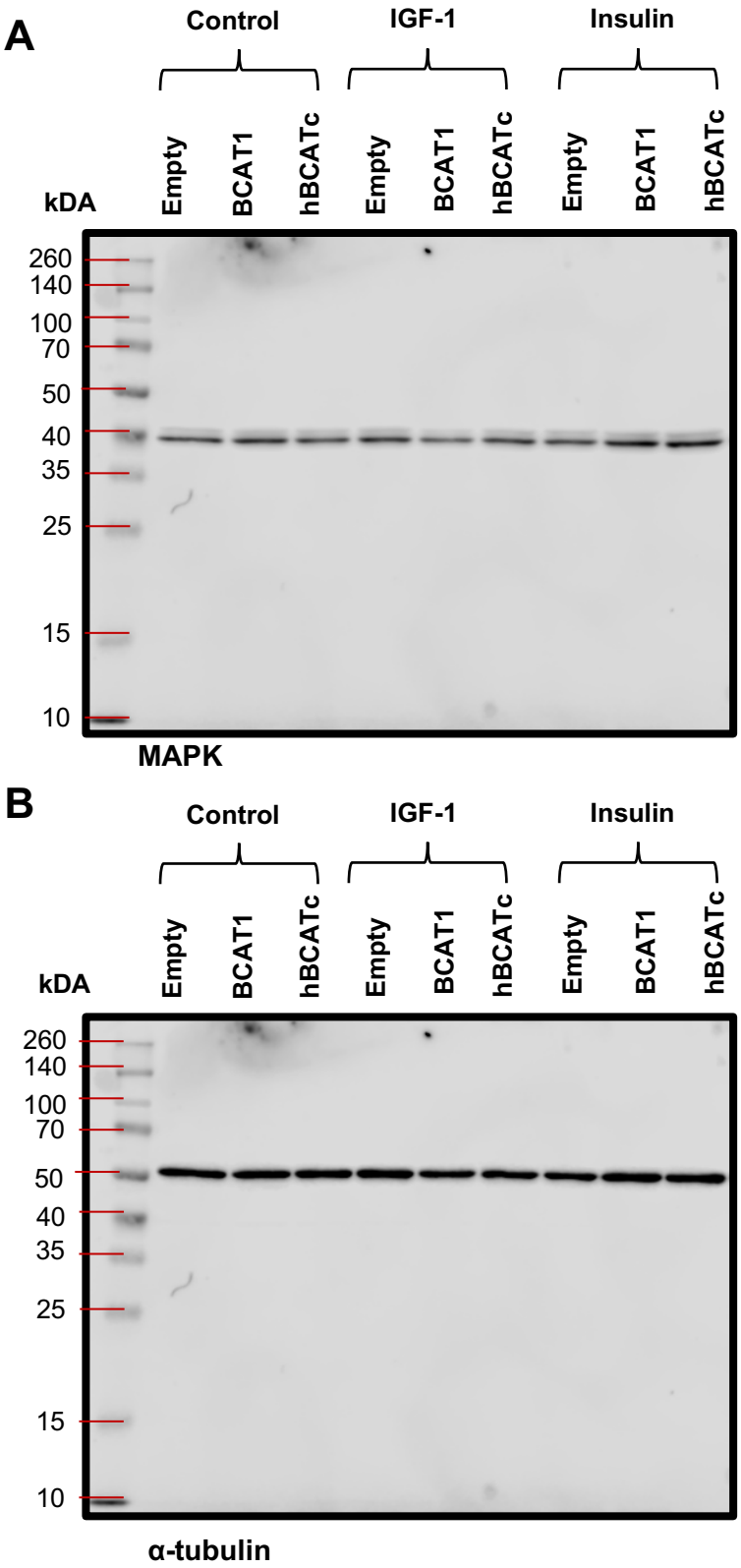

Figure 7.20. Western blot repeats of Figure 4.10B of knockdown and overexpression of *BCAT1* negatively regulates phosphorylation of MAPK in MDA-MB-231 cells. Cells were treated with stable shRNA transfection for *BCAT1* knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty) for 8 days and treated with 100 nM insulin and 100 ng/mL IGF-1 for 24 hours accordingly. (**A**) Full length immunoblot for MAPK (**B**) Full-length immunoblot for α-tubulin.

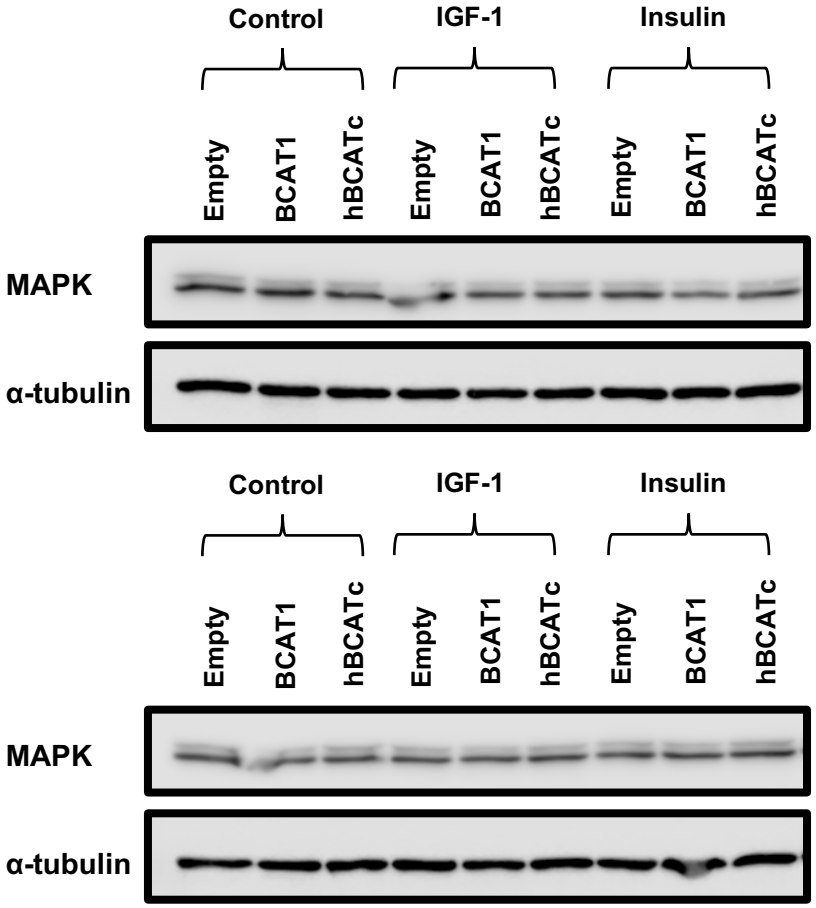

Figure 7.21. Western blot repeats of Figure 4.10B of knockdown and overexpression of *BCAT1* negatively regulates phosphorylation of MAPK in MDA-MB-231 cells. Cells were treated with stable shRNA transfection for *BCAT1* knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty) for 8 days and treated with 100 nM insulin and 100 ng/mL IGF-1 for 24 hours accordingly. Cropped immunoblots for MAPK and α-tubulin replicates.

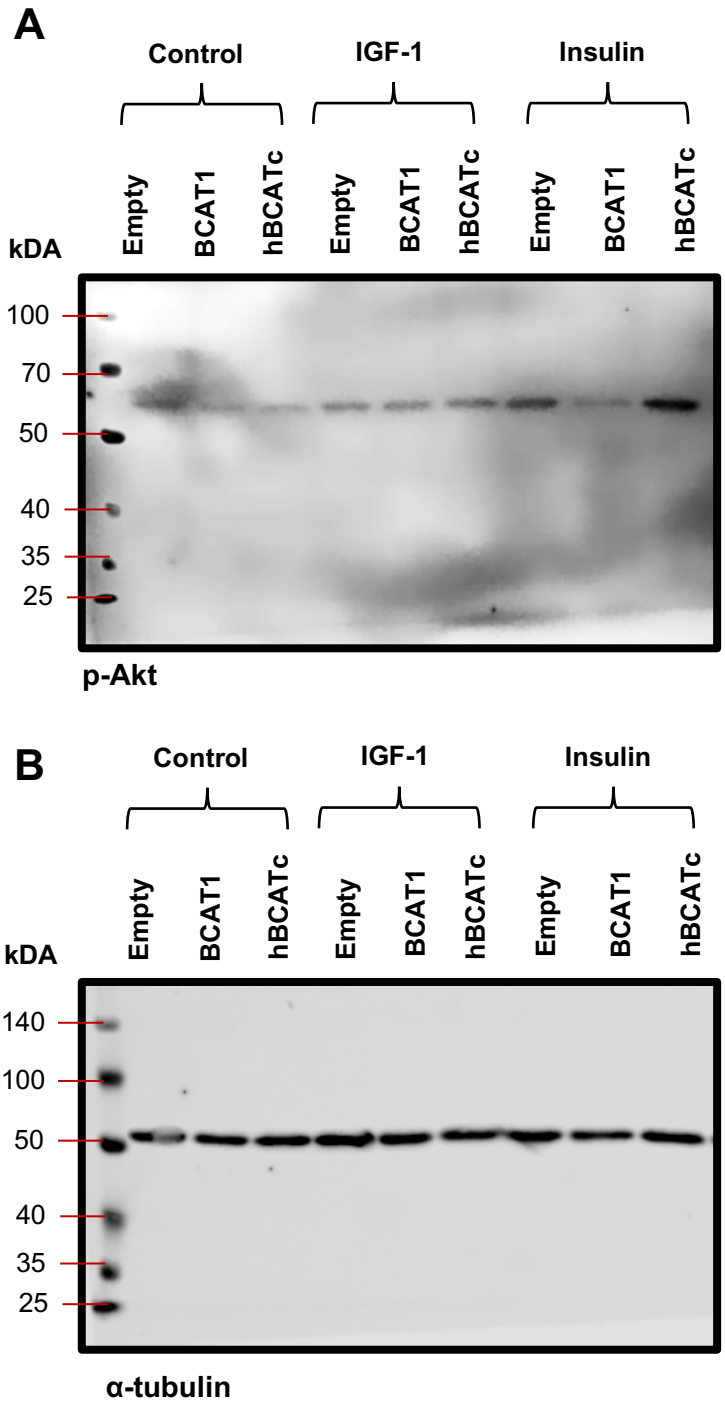

Figure 7.22. Western blot repeats of Figure 4.15A of knockdown and overexpression of *BCAT1* regulates the Akt axis in MDA-MB-231 cells. Cells were treated with stable shRNA transfection for *BCAT1* knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty) for 8 days and treated with 100 nM insulin and 100 ng/mL IGF-1 for 24 hours accordingly. (**A**) immunoblot for p-Akt with molecular markers (**B**) Full-length immunoblot for α-tubulin with molecular markers.

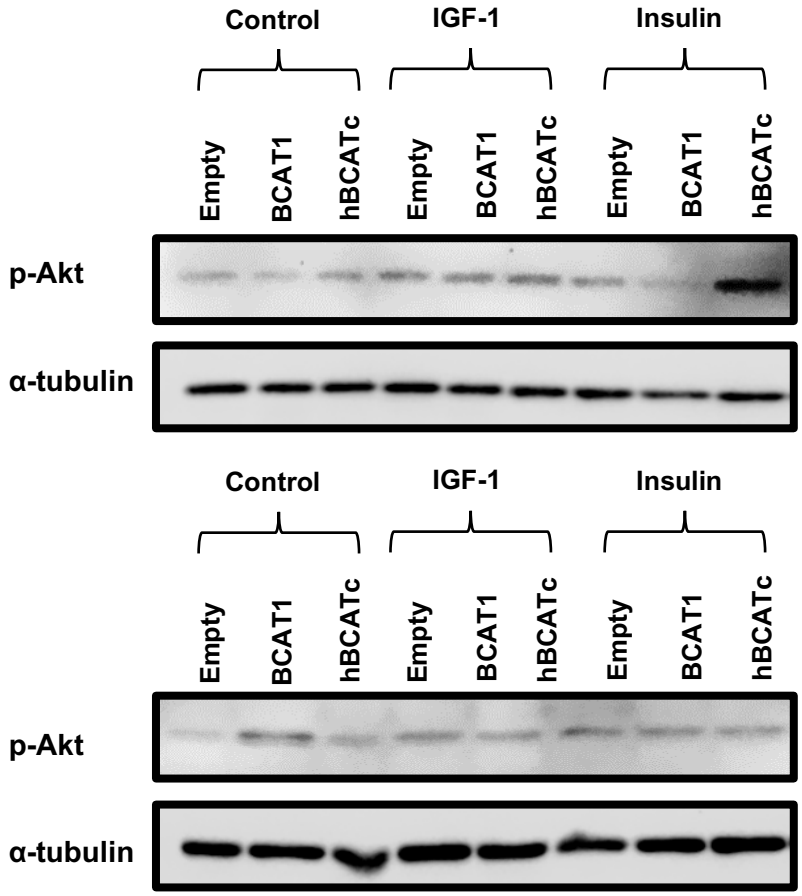

Figure 7.23. Western blot repeats of Figure 4.15A of knockdown and overexpression of *BCAT1* regulates the Akt axis in MDA-MB-231 cells. Cells were treated with stable shRNA transfection for *BCAT1* knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty) for 8 days and treated with 100 nM insulin and 100 ng/mL IGF-1 for 24 hours accordingly. Cropped immunoblots for p-Akt and α-tubulin replicates.

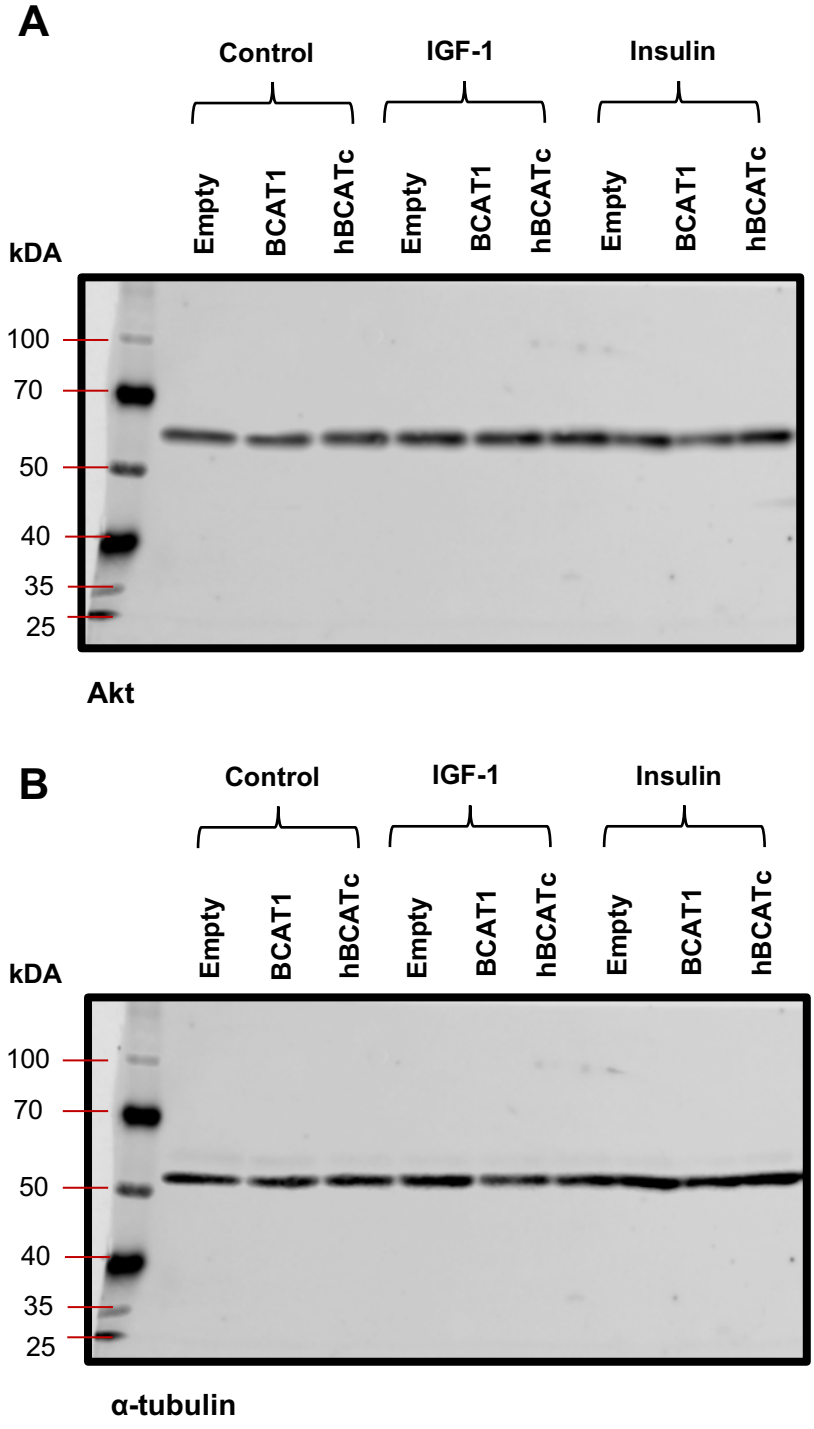

Figure 7.24. Western blot repeats of Figure 4.15B of knockdown and overexpression of *BCAT1* regulates the Akt axis in MDA-MB-231 cells. Cells were treated with stable shRNA transfection for *BCAT1* knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty) for 8 days and treated with 100 nM insulin and 100 ng/mL IGF-1 for 24 hours accordingly. (**A**) immunoblot for Akt with molecular markers (**B**) immunoblot for α-tubulin with molecular markers.

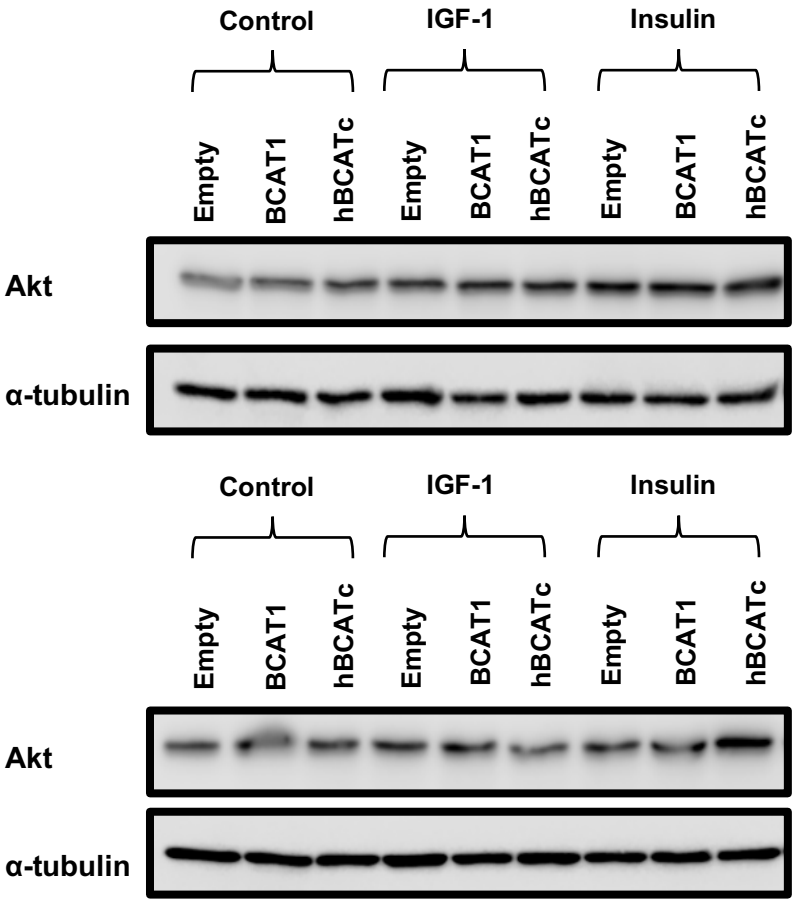

Figure 7.25. Western blot repeats of Figure 4.15B of knockdown and overexpression of *BCAT1* regulates the Akt axis in MDA-MB-231 cells. Cells were treated with stable shRNA transfection for *BCAT1* knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty) for 8 days and treated with 100 nM insulin and 100 ng/mL IGF-1 for 24 hours accordingly. Cropped immunoblots for Akt and α-tubulin replicates

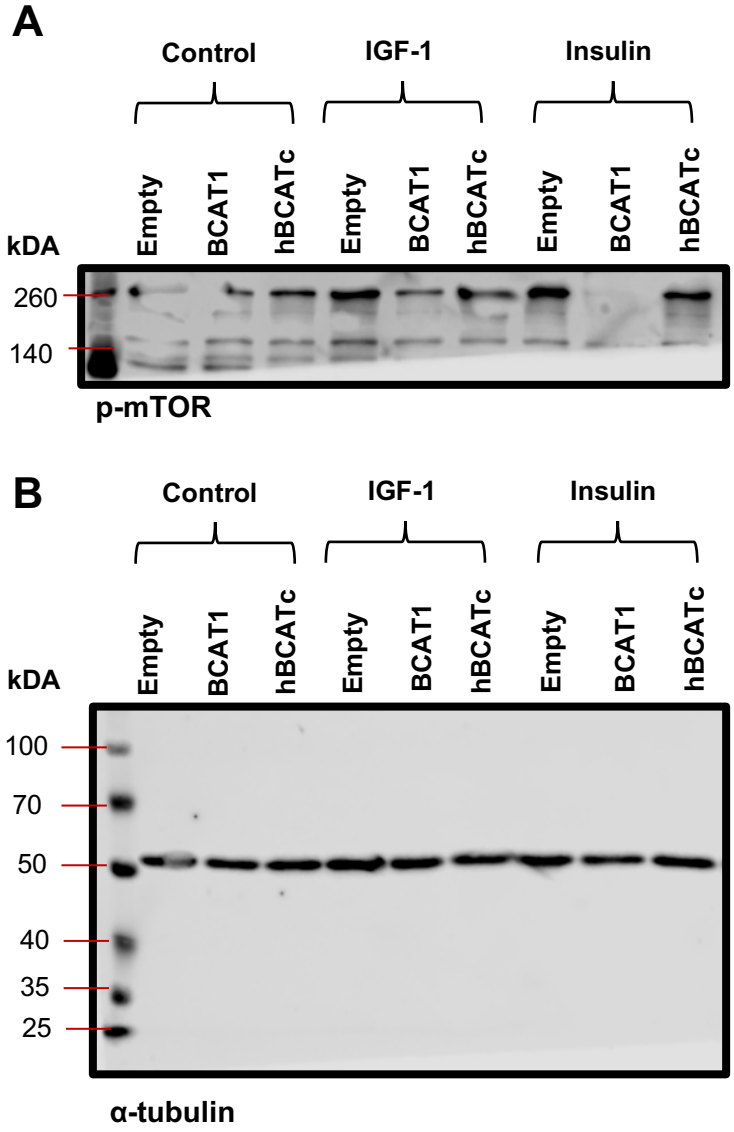

Figure 7.26. Western blot repeats of Figure 4.16A of knockdown and overexpression of *BCAT1* regulates the mTOR axis in MDA-MB-231 cells. Cells were treated with stable shRNA transfection for *BCAT1* knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty) for 8 days and treated with 100 nM insulin and 100 ng/mL IGF-1 for 24 hours accordingly. (**A**) immunoblot for p-mTOR with molecular markers (**B**) immunoblot for α-tubulin with molecular markers.

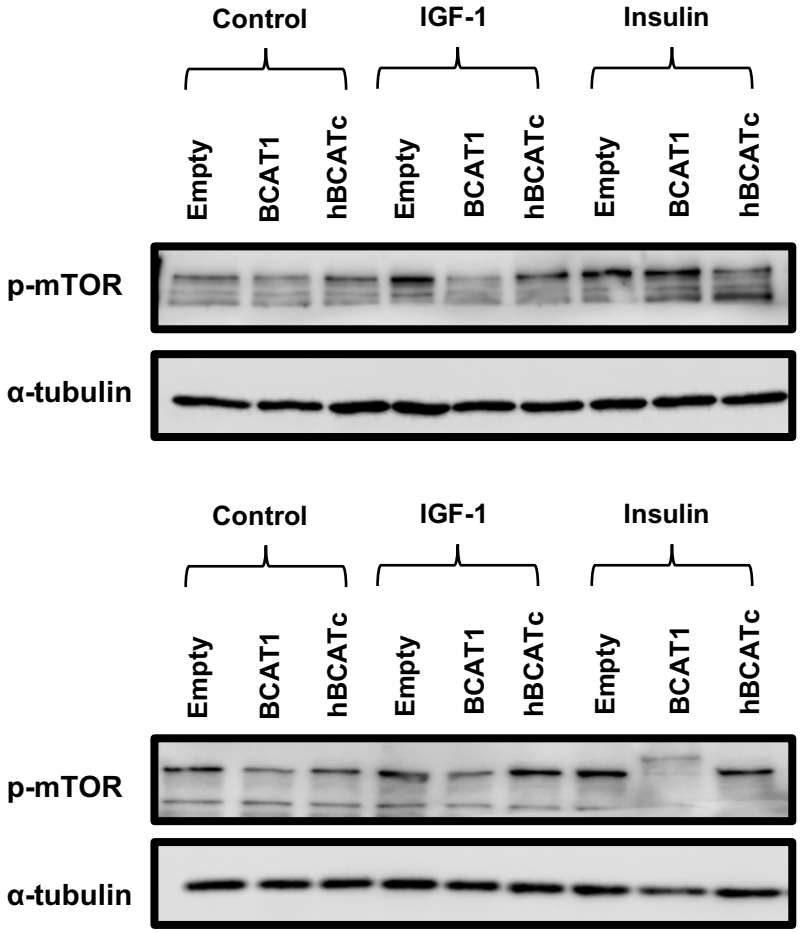

Figure 7.27. Western blot repeats of Figure 4.16A of knockdown and overexpression of *BCAT1* regulates the mTOR axis in MDA-MB-231 cells. Cells were treated with stable shRNA transfection for *BCAT1* knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty) for 8 days and treated with 100 nM insulin and 100 ng/mL IGF-1 for 24 hours accordingly. Cropped immunoblots for p-mTOR and α-tubulin replicates.

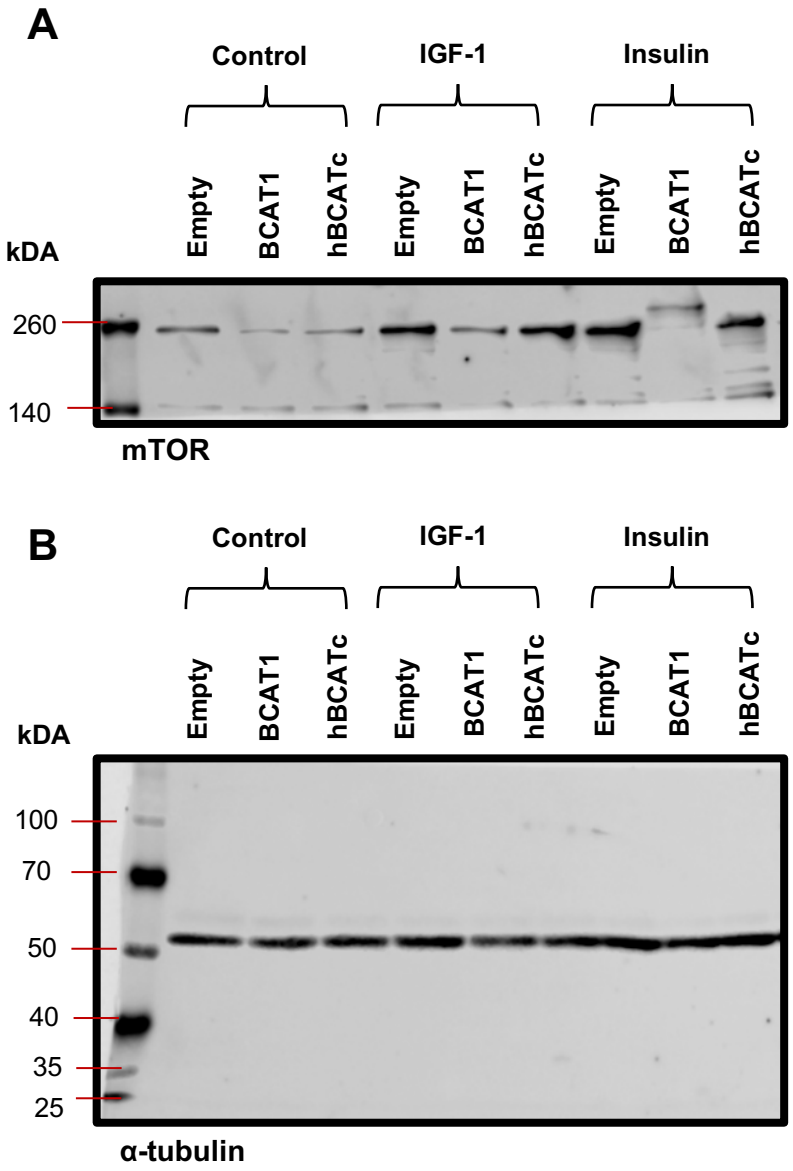

Figure 7.28. Western blot repeats of Figure 4.16B of knockdown and overexpression of *BCAT1* regulates the mTOR axis in MDA-MB-231 cells. Cells were treated with stable shRNA transfection for *BCAT1* knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty) for 8 days and treated with 100 nM insulin and 100 ng/mL IGF-1 for 24 hours accordingly. (**A**) immunoblot for mTOR with molecular markers (**B**) immunoblot for α-tubulin with molecular markers.

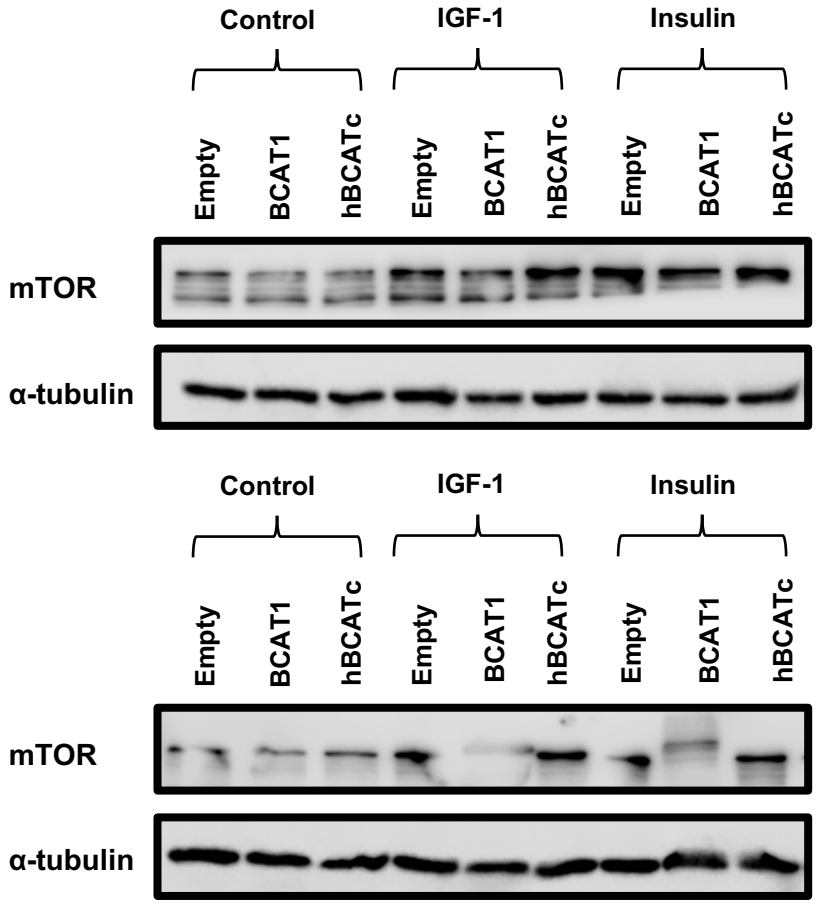

Figure 7.29. Western blot repeats of Figure 4.16B of knockdown and overexpression of *BCAT1* regulates the mTOR axis in MDA-MB-231 cells. Cells were treated with stable shRNA transfection for *BCAT1* knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty) for 8 days and treated with 100 nM insulin and 100 ng/mL IGF-1 for 24 hours accordingly. Cropped immunoblots for mTOR and α-tubulin replicates.

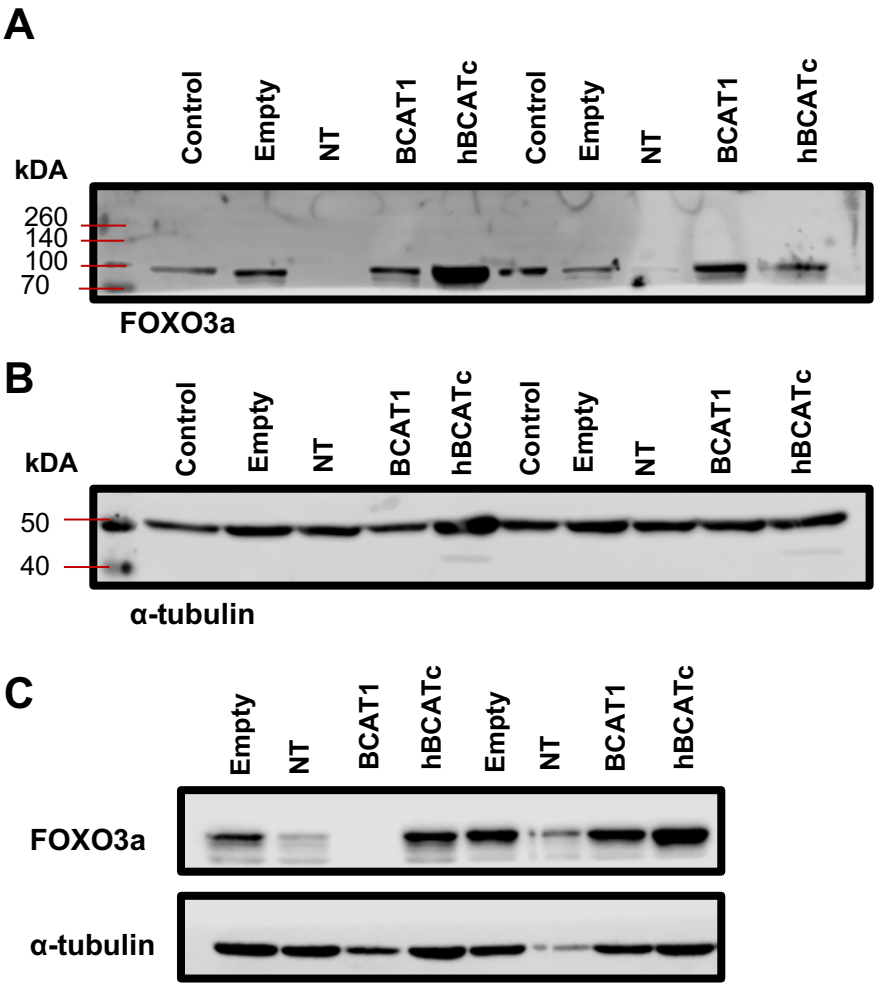

Figure 7.30. Western blot repeats of Figure 4.17A of knockdown and overexpression of *BCAT1* regulates downstream targets of the Akt axis in MDA-MB-231 cells. Cells were stably transfected with shRNA for knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty). (**A**) immunoblot for FOXO3a with molecular markers (**B**) immunoblot for α-tubulin with molecular markers (**C**) cropped immunoblots for FOXO3a and α-tubulin replicates.

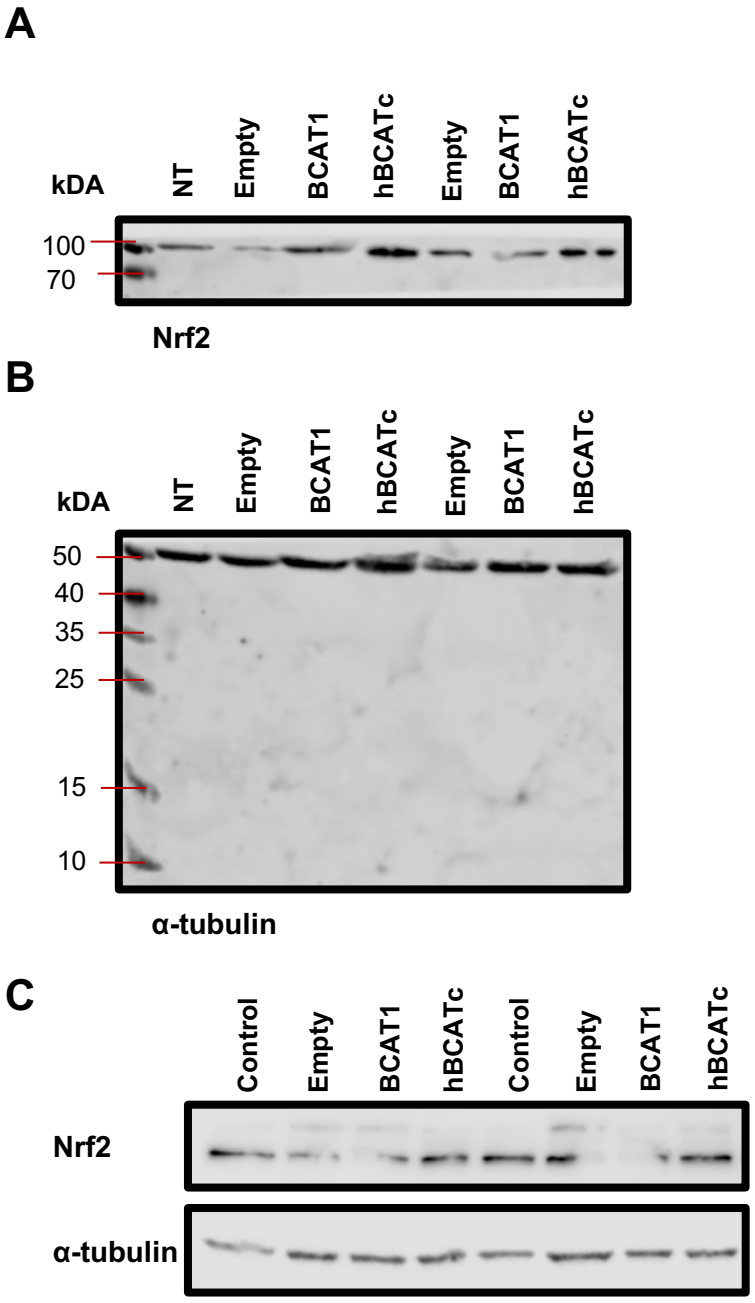

Figure 7.31. Western blot repeats of Figure 4.17B of knockdown and overexpression of *BCAT1* regulates downstream targets of the Akt axis in MDA-MB-231 cells. Cells were stably transfected with shRNA for knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty). (**A**) immunoblot for Nrf2 with molecular markers (**B**) immunoblot for α-tubulin with molecular markers (**C**) cropped immunoblots for Nrf2 and α-tubulin replicates.

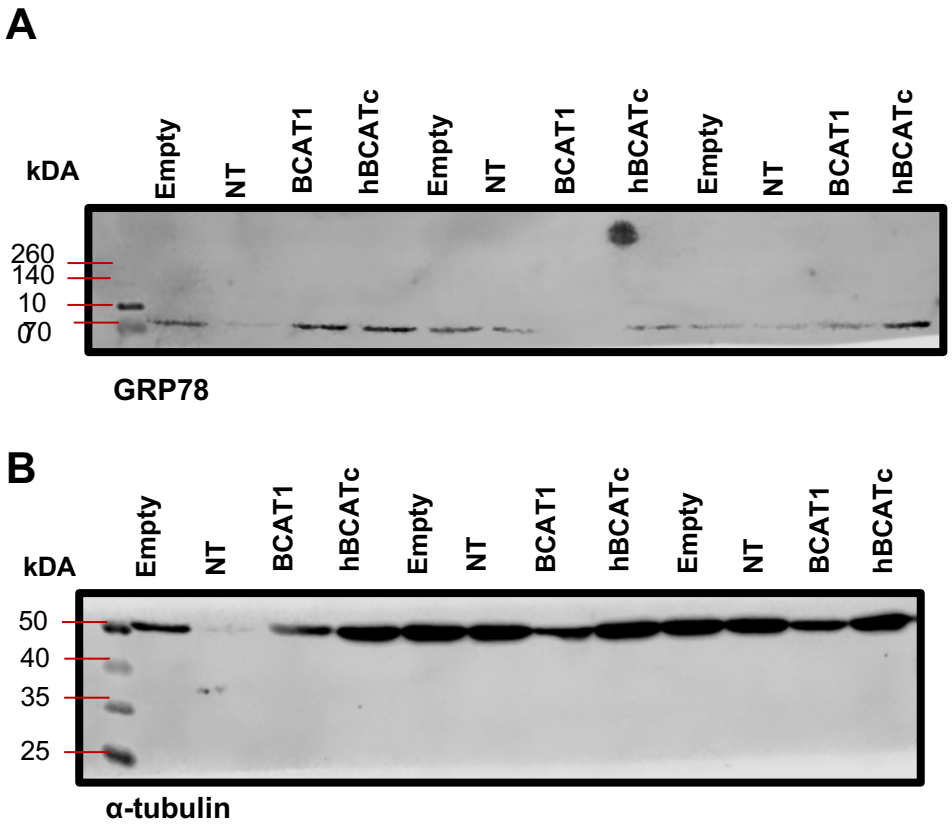

Figure 7.32. Western blot repeats of Figure 4.17C of knockdown and overexpression of *BCAT1* regulates downstream targets of the Akt axis in MDA-MB-231 cells. Cells were stably transfected with shRNA for knockdown (BCAT1) and overexpression (hBCATc) or unmodified pULTRA plasmid with no insertions as a control (Empty). (**A**) immunoblot for GRP78 with molecular markers (**B**) immunoblot for α-tubulin with molecular markers.