The Role of the CBLL1/HAKAI E3 Ubiquitin Ligase component and its association with the RNA methyltransferase complex in Breast Cancer

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Abstract

Breast cancer (BCa), a clinically and pathologically heterogenous cancer, is one of the most commonly diagnosed cancers and one of the leading causes of cancer mortality in women. Women are estimated to have a 1 in 8 chance of being diagnosed with BCa in their lifetimes and constitute 99% of cases, with only 1% of BCa diagnoses affecting 46XY males.

RNA:m^{6}A is the methylation at the N^6 position of certain adenosines in RNA and is one of the most abundant RNA modifications. RNA:m^{6}A is known to be dysregulated in cancer with the potential for some RNA:m^{6}A regulators to be therapeutic targets or biomarkers. These include 'writers' which are methyltransferases and catalyse the addition of the methyl group, 'erasers' which are demethylases and catalyse the removal of methyl group, and 'readers' which are m^{6}A binding proteins. The two methyltransferase components investigated in this study are METTL3 and METTL14 which form a catalytic heterodimer involved in the methyltransferase complex (MTC). This study also investigates the CBLL1 E3 ubiquitin ligase, which was originally identified to play a role in the degradation of E-cadherin and has also been shown to play a role in the RNA:m^{6}A modification and is associated with METTL3 and METTL3 in the MTC.

This study focuses specifically on CBLL1 and investigating its complex relationship with METTL3 and METTL14. The main protein of interest CBLL1 was chosen as it is known to regulate RNA:m^{6}A but its relationship with these two important methyltransferases METTL3 and METTL14 is yet understood. The goal of this project was to test the hypothesis that the CBLL1 E3 ubiquitin ligase regulates METTL3 and METTL14 stoichiometry as previous studies have shown that siRNA-mediated functional depletion of either METTL3 or METTL14, reduces expression of METTL14 and METTL3 respectively. To do this, the main objectives include identifying the effect of simultaneous knockdown of CBLL1 and METTL3 as well as CBLL1 and METTL14 on METTL14 and METTL3 expression was examined through western blotting. Furthermore, another of the objectives includes analysing RNA-seq results to identify the effect of CBLL1 knockdown. This might give insight into the future potential of using CBLL1 as either a biomarker or a therapeutic target.

This study employed BCa cell lines to further understand CBLL1, METTL3 and METTL14 in BCa. These three regulators of RNA:m^{6}A were chosen as it is known that METTL3 and METTL14 form a heterodimer, but it would be interesting to know if and how CBLL1 interacts with these two methyltransferases. The cell lines used were MCF-7 luminal A-like cells, with and without estrogen treatment, and MDA-MB-231 triple negative breast cancer (TNBC) cell lines. The results obtained reveal a complex relationship between CBLL1, METTL3 and METTL14 expression which can be yet further understood. RNA sequencing was employed to examine the effect of siRNA mediated knockdown of CBLL1 which revealed differentially expressed genes and significantly enriched KEGG pathways. Furthermore, expression of the CBLL1, METTL3 and METTL14 MTC components was increased by estrogen in MCF-7 cells.

Collectively these findings advance understanding of the role of CBLL1, the MTC and its association with the RNA:m^{6}A modification and BCa. This study also provides progression into the supporting data for the use of the small molecule inhibitor, STM2457, in the treatment of TNBC.

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Abbreviations and acronyms

A3SS –	alternative 3’ splice site
A5SS –	alternative 5’ splice site
ANOVA -	analysis of variance
APS -	Ammonium persulfate
BCa –	Breast cancer
BRCA -	Breast Cancer Associated gene
BSA -	Bovine serum albumin
CBLL1 -	Cbl proto-oncogene like 1
DEG -	Differentially expressed gene
DNA -	Deoxyribonucleic acid
E2 –	Estradiol
ER -	Estrogen Receptor
FTO -	Fat mass and obesity related
MBC –	metastatic breast cancer
m^6A -	N^6-methyladenosine
MTC -	methyltranferase complex
METTL3 -	Methyltransferase 3
METTL14 -	Methyltransferase 14
NPI -	Nottingham Prognostic Index
padj -	adjusted p-value
PAGE -	Polyacrylamide gel electrophoresis
PARP -	Poly-ADP ribose polymerase
RI –	retained intron
rMATs -	Replicate multivariate analysis of transcript splicing
RNA -	Ribonucleic acid
RNA-seq –	RNA sequencing
SAM -	S-adenosyl-l-methionine

Scr -	Scrambled control
SE –	skipped exon
siRNA -	Short interference RNA
TCGA -	The cancer genome atlas
TEMED -	Tetramethylethylenediamine
TNBC –	triple negative breast cancer
Veh -	Vehicle
WebGestalt -	WEB-based Gene Set Analysis Toolkit
WTAP -	Wilms tumour 1-associating protein

Chapter 1

Introduction

1 Introduction

1.1 Breast cancer

Breast cancer (BCa) is a clinically and pathologically heterogenous cancer (Lüönd et al., 2021, Turner et al., 2021) and one of the most commonly diagnosed cancers with an estimated 310,720 cases to be estimated in the United States in 2024 (Siegel et al., 2024) making it one of the leading causes of morbidity and mortality. BCa is much more common in women, with only 1% of BCa cases affecting males. It is estimated that 1 in 8 women will be diagnosed with BCa in their lifetimes and by 2040 cases and associated mortality are predicted to grow by 40 and 50% respectively (Sung et al., 2021). There are a number of known risk factors for BCa which include the aging, estrogen levels, lifestyle factors, for example a low level of physical activity and hereditary factors (Sun et al., 2017, Sung et al., 2021). Genetic variants affecting the *BRCA1* and *BRCA2* genes are associated with BCa, and ovarian and prostate cancer risk (Sun et al., 2017). Given increasing aging societies in high income countries and increasing BCa incidence in low resource countries in the global south, BCa continues to be a major global clinical challenge. Women that experience later menopause are at a higher risk of BCa since endogenous estrogen is at a higher concentration for a prolonged period of time. Hormone replacement therapy may also increase this BCa risk (Kenemans and Bosman, 2003).

There are several sub-categories of metastasis which include bone metastases include the subgroups and classification into osteolytic, osteosclerotic, or mixed types (Liang et al., 2020a). Out of these classifications osteolytic metastasis is the most common in BCa (Liang et al., 2020a). BCa patients with bone metastasis experience bone pain, fracture, hypercalcemia and also paralysis due to spinal cord compression (Guise, 2013).

1.1.1 Breast anatomy and histological classification

The mammary glands are considered exocrine glands which are composed of skin and subcutaneous tissue, the breast ducts and lobules, and together with the supporting stroma (Jesinger, 2014). In both males and females the two mammary glands cover the pectoral muscle with the female breast usually containing a larger volume of fibro glandular tissue as compared with the smaller male breast of which mostly consists of fat (Jesinger, 2014). The mammary gland develops through three distinct stages of a woman's lifetime: embryonic, pubertal, and reproductive (Macias and Hinck, 2012). During adulthood, the normal mammary gland consists of a number of branching ducts which extend from the nipple and to the lobules (Pellacani et al., 2019).

In the reproductive stage the function of the mammary glands in females is required to generate and deliver milk to young, a unique feature of mammals (Macias and Hinck, 2012). There is evidence to show that involution may be involved in the development of BCa for the reason that the normal conditions of involution present a strong tumour microenvironment as characterisation includes (Schedin, 2006).

Most commonly BCa originates in mammary epithelial cells (Weigelt et al., 2010) and is classified according to many different aspect with the World Health Organization (WHO) using histological presentation as classification (Cserni, 2020). The most common type of BCa is

invasive carcinoma of no special type (NST) which occurs in these epithelial cells (Shea et al., 2020, Sinn and Kreipe, 2013). However, there are a number of special subtypes within invasive breast carcinomas including invasive lobular, tubular, cribriform, metaplastic, apocrine, mucinous, papillary, and micropapillary carcinoma, as well as carcinomas with medullary, neuroendocrine, and salivary gland/skin adnexal type features which effectively emphasises the range of BCa types within patients (Sinn and Kreipe, 2013).

1.1.2 Nuclear hormone receptor functions in normal and malignant mammary cells

The nuclear receptors (NRs) are part of the nuclear receptor family that act as ligand-regulated transcription factors (Yaşar et al., 2017). These are activated by steroid hormones, such as estrogen and progesterone (Sever and Glass, 2013).

Estrogen receptors (ERα and ERβ) are an important aspect in female physiology and play many roles in cellular processes, with ERα particularly important in the initiation and progression of luminal BCa. For example, in females some of these cellular processes are connected to the menstrual cycle, pregnancy, and lactation (Jeffreys et al., 2020). Like all NRs, the structure of ERα comprises of three functional domains: the NH_2-terminal domain (NTD), the DNA-binding domain (DBD), and the COOH-terminal ligand-binding domain (LBD) (Jia et al., 2015). ERα consists of three functional domains which include the N-terminal region with activation function (AF-1), a central DNA binding domain and the C-terminal ligand binding domain also containing an activation function (AF-2) (Gong et al., 2010).

ERα and ERβ are encoded by *ESR1* and *ESR2*, respectively and abnormal signalling by these receptors can promote carcinogenesis and progression (Jia et al., 2015, Jeffreys et al., 2020). *ESR1* is located at chromosome 6q24-27 and *ESR2* is located at 14q22-24 (Menasce et al., 1993). ERα and ERβ are both expressed in normal breast luminal epithelial cells as well as in BCa tumours (Yu et al., 2011). Both ERα and ERβ are able to function as heterodimers however because of the relative distribution being different this would suggest that they act as homodimers (Cowley and Parker, 1999). ERα is mainly expressed in the reproductive tissues, whereas ERβ can be found in a wider range of locations including but not limited to the ovary, central nervous system (CNS), cardiovascular system and lungs (Jia et al., 2015).

In ERα-positive MBC, approximately 20% of cases harbour variants in the ligand-binding domain encoding region of the *ESR1* gene (Toy et al., 2013). These *ESR1* mutations have been shown to be rare in primary BCa, but there is still a need to screen these as this can identify early BCa patients whom are likely to develop estrogen therapy resistance which can therefore lead to relapse (Herzog and Fuqua, 2022).

The progesterone receptor (PR) modulates ERα function in BCa (Li et al., 2022e). In women, the steroid hormone, progesterone, is also involved in the female menstrual cycle, pregnancy and embryogenesis and plays these roles by binding to PR (Li et al., 2022e). Like ER it is a member of the nuclear receptor family and identified as a master regular in tissues associated with female reproduction (Grimm et al., 2016). PR has been shown to undergo several different post-translational modifications including acetylation, ubiquitination, methylation, phosphorylation and sumolysation (Li et al., 2022e) Also similar to ER, the structure of PR

consists of a C-terminal ligand binding domain, a DNA binding domain and an amino terminal domain which can form two different protein isoforms of which are formed both from the same gene *PGR* (Grimm et al., 2016) named PR-A and PR-B (Sampayo et al., 2013).

PR plays key roles in BCa progression with mRNA PR variants with different deleted exons observed in both BCa cell lines and BCa tissues (Springwald et al., 2010). Also in BCa there has been shown a higher level of PR-A as compared with the PR-B isoform (Sampayo et al., 2013) and therefore this may be linked to the development of the cancer.

1.1.3 E-cadherin in non-malignant and malignant mammary cells

E-cadherin is a glycoprotein with a extracellular domain which comprises of five cadherin-motif subdomains, a single-pass transmembrane segment and a short conserved cytoplasmic domain (Berx and Van Roy, 2001). *CDH1*, the E-cadherin gene is positioned on the human chromosome 16q22.1 (Berx and Van Roy, 2001). E-cadherin plays an important role in cell adhesion and loss of e-cadherin expression is common in BCa (Jiang and Mansel, 2000, van Roy and Berx, 2008). The progression of BCa has been shown to be strongly associated with the loss of E-cadherin (Corso et al., 2020), with this loss shown in 1997 to be an early indicator in in situ lobular breast cancer as well as being a precursor of invasive lobular breast cancer (De Leeuw et al., 1997, Hunt et al., 1997). Furthermore, early loss of E-cadherin has been shown to mechanistically induce the activation of Rock-dependent actomyosin as well as leading to hypersensitisation to GFR signals (Bruner and Derksen, 2018), thereby promoting carcinogenesis.

1.1.4 Molecular classification of BCa

BCa can be characterised into biologically and clinically meaningful subgroups based on histological grade and type (Elston and Ellis, 1991) and more recent molecular characterisation based on gene expression signature. To date there are now two methods which can determine the BCa subtype: gene-based assays and immunohistochemistry (IHC) based markers (Gao and Swain, 2018). IHC is a traditional method used to identify the subtype of BCa by utilising markers such as estrogen receptor (ERα), progesterone receptor (PR), human epidermal growth receptor 2 (HER2), and also Ki67 (Gao and Swain, 2018, Perou et al., 2000). BCa can then be divided into a number of subtypes which are determined by the expression of these markers (ER, PR and HER2) and these influence the tumour biology and the effects of different therapeutics. From these the subtypes described include luminal A-like, luminal B-like, HER2 positive and triple negative BCa.

HER2 belongs to the HER family of receptor tyrosine kinases and is important for mammary gland (Ménard et al., 2004). However, the HER2 positive BCa subtype is a result of the overexpression of the HER2 protein (Gradishar et al., 2023). This overexpression of HER2 has been shown to be associated with more aggressive human breast carcinomas (Ménard et al., 2004). Inflammatory BCas have been demonstrated as a subgroup of BCa (Sinn and Kreipe, 2013), and in this subgroup the highest frequency of HER2 overexpression has been shown (Ménard et al., 2004).

The luminal A-like classification is identified using immunohistochemistry (IHC) following the definition of (hormonal receptor) HR positive, HER2 negative as described and Ki-67 at less than 14%, and PR percentage at more than 20% (Prat et al., 2013). The 70-gene and 80-gene signatures are frequently used together to determine which subset of tumours in the luminal A-like classification and both the Neoadjuvant Breast Registry Symphony Trial (NBRST) and a study by Glück et al. have shown that tumours determined in this way have better prognosis as compared with other subtypes (Gao and Swain, 2018, Whitworth et al., 2017, Glück et al., 2013).

Luminal B-like has two subtypes which are HER2-positive and HER2-negative. In comparison to the luminal A-like subtype, Luminal B-like subtype has a poorer prognosis (Castellarnau-Visús et al., 2023). Moreover, when comparing the HER2-positive and HER2-negative within the luminal B-like group, the HER2-positive subgroup has been shown to have a statistically significant higher proportion of bone metastasis (Li et al., 2016). Currently there is limited information available for the management and subsequent decisions of Luminal B-like subtype within clinics (Castellarnau-Visús et al., 2023)

Triple negative (TNBC) is the subtype of tumours that do not express ER, PR, or HER2 (Lee et al., 2020). TNBC is generally highly aggressive and is associated with high invasiveness, high metastatic potential and increased rate of relapse contributing to poor prognosis (Yin et al., 2020). Within this tumour type there are specific subtypes categorised into four groups named basal-like 1 (BL1), basal-like 2 (BL2), mesenchymal (M), and luminal androgen receptor (LAR) (Lehmann et al., 2016). However, recently slight variations has been made on these groups leading to four subtypes mesenchymal-like (MES), luminal androgen receptor (LAR), immunomodulatory (IM), and basal-like and immune-suppressed (BLIS) subtypes (Yang et al., 2023b, Lee et al., 2020). The LAR group was characterized by the upregulation of oxidized phosphatidylethanolamines and glutathione metabolism. There is an urgent need for new therapeutic approaches given the current lack of targeted therapies for TNBC.

1.1.5 Diagnosis, current therapies, and treatment resistance in BCa

Early BCa diagnosis remains the best approach to deliver better outcomes (Yi-Sheng Sun, 2017). Since 1988 BCa screening is supported in the UK through the NHS (NICE, 2018). When women are between the age of 50-70 years old they are invited for routine mammography screening in the UK which improves early diagnosis (NICE, 2020). However screening a healthy population can have undesired effects such as false-positive mammography results which have been shown are common (Hubbard et al., 2011) and can have a number of physiological consequences (Bond et al., 2013). Despite the success of screening programs, the number of BCa diagnoses continues to increase. For this reason, there has been considerable interest in the development of improved histopathological, biomarker and genomic-based prognostic approaches.

The Nottingham Prognostic Index (NPI) was developed using BCa cases diagnosed and treated at Nottingham City Hospital (Haybittle et al., 1982) and has been widely used in clinical practice internationally (Blamey et al., 2007). Haybittle and colleagues identified that the three major factors to indicate prognosis were tumour size, lymph node stage and histological

grade (Haybittle et al., 1982). The NPI score is calculated as follows: NPI score = Lymph node stage (1-3) + Histological grade (1-3) + 0.2 x tumour size (cm) (Lee and Ellis, 2008).

Microarray-based gene expression profiling became available at the start of the 2000s which have contributed to the understanding of the heterogeneity of BCa and that it includes a range of distinct molecular subtypes (Colombo et al., 2011, Perou et al., 2000). This can therefore be taken into the context of clinical trials which was previously considered BCa as a single group of tumours (Colombo et al., 2011, Milioli et al., 2016). In turn the advent of next-generation sequencing has transformed understanding of the molecular heterogeneity of BCa and the pathways involved in driving disease and are now entering clinical use. Furthermore, as an example of gene prognostic and a predictive assay, Oncotype® DX Breast Recurrence Score is used worldwide (Syed, 2020). This 21-gene recurrence score assay can be used for BCa tumour samples and works by generating a recurrence score from the expression levels of a number of cancer-related genes (Syed, 2020). Recently this assay has been shown to have the potential to utilise it as an important tool for the guidance of treatment decisions in neoadjuvant setting (Taylor et al., 2023).

Collectively histopathologic and genomics tools are used to inform BCa treatment decisions. The recommended treatment is and should be different depending on the subtype of BCa diagnosed. For luminal A-like subtype the current would include endocrine treatment. Endocrine treatment/ therapy works by blocking the effect of the estrogen receptor or by inhibiting the production of this estrogen (Reinbolt et al., 2015). There are a number of types of endocrine treatments which include selective estrogen receptor modulators (SERM), selective estrogen receptor down-regulators (SERD), aromatase inhibitors (AI), luteinizing hormone releasing hormone agonists (LHRH agonists) and the tyrosine kinase inhibitors (TKIs) (Reinbolt et al., 2015).

Recently novel anti-HER2 agents have been developed for the HER2 positive BCa subtype which include trastuzumab, neratinib, tucatinib, margetuximab and the antibody drug conjugate trastuzumab-deruxtecan (Gradishar et al., 2023, Rugo et al., 2023, Nguyen et al., 2021, Perez et al., 2022). Margetuximab is a new HER2 receptor antagonist which has been used in combination with chemotherapy when treating metastatic HER2-positive BCa patients (Gradishar et al., 2023).

There is currently research to develop improved treatments for TNBC. For example, pembrolizumab is an anti–programmed death 1 (PD-1) monoclonal antibody which has been shown to have anti-tumour activity (Schmid et al., 2020). When early triple-negative BCa patients were given pembrolizumab plus neoadjuvant chemotherapy a significantly higher pathological complete response (Schmid et al., 2020). Pembrolizumab has also been shown to have promising results in HER2-positive patients specifically the results of a phase Ib trial shows that a combination of pembrolizumab plus T-DM1 is positive in metastatic BCa patients (Waks et al., 2022).

Poly (ADP-ribose) polymerase (PARP) inhibitors result in cell death (Wang et al., 2022a) and is a cancer therapy treatment which targets the poly(ADP-ribose) polymerase (Lord and Ashworth, 2017). In tumours with germline mutations such as BRCA1 and BRCA2 have been

shown to be sensitive to these PARP inhibitors for the reason that they have a DNA repair defect (Lord and Ashworth, 2017). However, with advanced disease or cancer types, resistance develops to these PARP inhibitors therefore there is a need to manage this (Lord and Ashworth, 2017). These PARP inhibitors have been shown to be used in BCa types which carry the loss-of-function mutations in the HR pathway genes (Wang et al., 2022a).

Even though there are a number of treatments for specific subtypes of BCa, the emergence of treatment resistance is common and emphasises the need to identify novel targeted therapies.

1.2 N^6-methyladenosine (RNA:m^6A)

The focus of this dissertation is covalent RNA modifications and their potential mechanistic and clinical relevance to BCa initiation, progression and response to therapy. The three most common RNA methylation modifications include m^6A, m^5C and m^1A which are important regulators of gene expression (Sheehan et al., 2023, Yu et al., 2022, Meyer et al., 2012, Wang et al., 2023d). The N^6 position methylation of certain adenosines, N^6-methyladenosine (m^6A), has been shown to be the most abundant RNA modification (Sun et al., 2019). RNA:m^6A sites are typically enriched near stop codons and 3' UTRs, near the consensus motif "RRACH" (R = A/G; H = A/C/U) (Meyer et al., 2012, Chen et al., 2023c). 5-methylcytosine (m^5C) methylation is the modification at the fifth N of cytosine position when a methyl group is added (Yu et al., 2022, Aslam et al., 2023). N^1-methyladenosine is the modification at the first nitrogen of adenosine (Li et al., 2022b). Furthermore another of the post transcriptional RNA modifications is N^6-2'-O-methyladenosine (m^6Am) which has been identified to be present on mRNA and snRNA (Oerum et al., 2021). The focus of this project is the m^6A modification as there is there in increasing interest especially in the context of cancer.

There is now considerable interest in RNA:m^6A as it has been shown to play crucial roles in gene regulation though splicing, RNA stability and translational regulation (Zhu et al., 2023, Huang et al., 2018, Chen et al., 2023d). This modification is also evolutionary conserved in eukaryotes, prokaryotes and viruses (Ying Yang, 2018, Fray and Simpson, 2015) specifically with RNA:m^6A playing critical roles in yeast and plants (Liang et al., 2020b, Jenjaroenpun et al., 2021). RNA:m^6A has been investigated in a number of plant species which include *Arabidopsis thaliana* (Zhong et al., 2008), *Solanum lycopersicum* (tomato plant) (Zhou et al., 2019) and *Oryza sativa* (rice) (Li et al., 2014).

In *A. thaliana*, RNA:m^6A was first discovered in its poly(A) RNA using 2D thin-layer chromatography (Zhong et al., 2008). Just as in mammals RNA:m^6A sites are enriched around the stop codon and within 3'UTRs in *A. thaliana* (Reichel et al., 2019). In plants the organs where RNA:m^6A-modified transcripts are most commonly located are the leaves, flowers, and roots, therefore suggesting that RNA:m^6A plays an important role in cellular differentiation (Reichel et al., 2019).

As shown in **Figure 1** the modification involves 'writers' which catalyse the addition of the methyl group (methyltransferases), 'readers' which are RNA:m^6A binding proteins, is regulated and 'erasers' which remove the methyl group (demethylases) (Ying Yang, 2018). RNA:m^6A binding proteins mainly involve the YT521-B homology (YTH) domain-containing

proteins which consists of YTHDF and YTHDC subtypes specifically YTHDF1-3 and YTHDC1-2 (Liao et al., 2022). In mammalian cells, these proteins bind directly to m^{6}A sites. YTHDC1 preferentially binds to the GG(m^{6}A)C sequence therefore indicating YTHDC1 preference to a guanosine nucleotide over an adenosine nucleotide when at the position prior to the m^{6}A nucleotide (Xu et al., 2015). YTHDC2 has been shown to enhance or 'fast track' translation and does this by facilitating interactions between m^{6}A-containing mRNAs and the ribosomes (Kretschmer et al., 2018).

Also included in the RNA:m^{6}A binding proteins are heterogenous nuclear ribonucleoproteins (HNRNPs) HNRNPA2B1, HNRNPC and HNRNPG (Geuens et al., 2016). This hnRNPs family comprises of approximately 30 proteins (Liu and Shi, 2021). HNRNPA2B1 has been shown to bind to m^{6}A-containing sites and shows similar alternative splicing effects as compared to the methyltransferase METTL3 (Alarcón et al., 2015).

To date there are two identified demethylases: fat mass and obesity-associated protein (FTO) and α-ketoglutarate–dependent dioxygenase alkB homolog 5 (ALKBH5) both of which are part of the ALKBH family (Zhao et al., 2014, Zheng et al., 2013, Marcinkowski et al., 2020). FTO was first associated with obesity in humans and then was later discovered to demethylate m^{6}A (Azzam et al., 2022). When *FTO* gene was knocked out in mice the results reported a lower fat mass and body weight (Fischer et al., 2009). In regard to m^{6}A, the demethylation requires the oxidative function of FTO for the reason that FTO catalyses the oxidative demethylation of m^{6}A in an iron (II)- and α-KG-dependent manner (Jia et al., 2011). ALKBH5 localises in the nuclear speckles and the expression of this demethylase is controlled by the hypoxia inducible factor 1 (HIF-1α) transcription factor when under hypoxic conditions (Zheng et al., 2013, Marcinkowski et al., 2020). ALKBH5 also effects mRNA export and RNA metabolism specifically examples such as pre-mRNA processing, mRNA decay and translation and has been found to only catalyse the removal of RNA:m^{6}A on single stranded RNAs (Zheng et al., 2013, Qu et al., 2022).

There are a number of proteins involved in the RNA:m^{6}A methyltransferase complex, including Wilms tumour 1-associating protein (WTAP), RNA-binding motif protein 15 (RBM15), Vir-like m^{6}A methyltransferase-associated (VIRMA), and, most importantly in the context of this dissertation, the CBLL1/HAKAI ubiquitin-ligase, methyltransferase-like 14 (METTL14) and methyltransferase-like 3 (METTL3) (Liu et al., 2014, Knuckles et al., 2018). WTAP recruits several adapter proteins to the methyltransferase complex which include VIRMA, RBM15, RBM15B and ZC3H13 (Lothion-Roy et al., 2022).

Further additional identified RNA:m^{6}A methyltransferases include METTL16, zinc finger CCHC domain-containing protein 4 (ZCCHC4), METTL5, and METTL4 (van Tran et al., 2019). METTL16 has been shown to control S-adenosylmethionine (SAM) homeostasis (Doxtader et al., 2018). METTL5 has been shown that it is necessary for the m^{6}A formation in the *18S* ribosomal RNA as well as forming a complex with TRMT112 (Ignatova et al., 2020, van Tran et al., 2019). The METTL3:METTL14 complex is however the core of this larger complex of methyltransferases as reviewed in Oerum et al. (Oerum et al., 2021) and therefore emphasising the importance of METTL3 to the RNA:m^{6}A modification.

Recent studies have shown that the dysregulation RNA:m^{6}A plays essential roles in disease progression these including many types of human cancers, neuronal disorders (Jiang et al., 2021, Angelova et al., 2018).

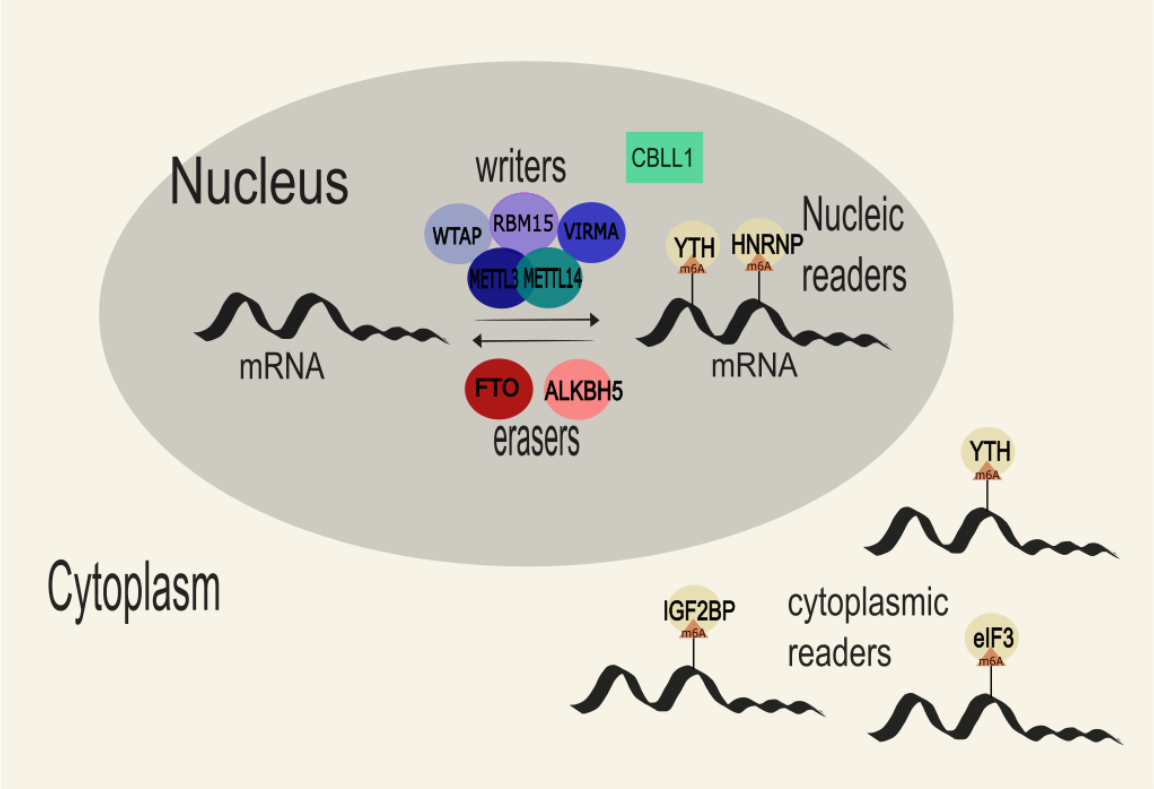


Figure 1. Components of the RNA:m^{6}A modification.

The RNA:m^{6}A modification is read by m^{6}A binding proteins (readers) and regulated by methyltransferases (writers) and demethylases (erasers). Here readers are shown as both nucleic and cytoplasmic, with the YTH family found in both.

1.2.1 RNA:m^{6}A in Cancer

RNA:m^{6}A is known to be dysregulated in cancer with research showing that some of the regulators of RNA:m^{6}A to be potential therapeutic targets and biomarkers. The methyltransferases, demethylases and RNA:m^{6}A binding proteins are to harbour both pro-oncogenic and tumour suppressor functions. RNA:m^{6}A has also been shown to be associated with immune system involved in cancer but the roles of this m^{6}A modification in macrophages are still unknown (Yin et al., 2021). This project focuses on RNA:m^{6}A in BCa as several of the regulators of RNA:m^{6}A have been shown to play roles in BCa progression. Therefore, this would be an interesting area to investigate for future potential therapeutics.

YTHDF2 has been shown to have as association with breast cancer, specifically in TNBC (Wu et al., 2023) as when YTHDF2-dependant mRNA degradation is disrupted this leads to the triggering of apoptosis (Einstein et al., 2021). YTHDF2 is needed to sustain MYC-driven cell

growth and that it is essential for the survival of TNBC cells (Einstein et al., 2021). Also, in breast cancer cells it has been shown that YTHDF2 is the m^6A binding protein (reader) and METTL3 the methyltransferase (writer) for the mRNA LATS1 which promoted tumorigenesis and glycolysis in BCa (Xu et al., 2023b).

The FTO and ALKBH5 demethylases have also been found to be associated with cancer. ALKBH5 has been shown to regulate gene expression in a number of human cancers (Qu et al., 2022). When under hypoxic conditions the hypoxia-inducible factors HIF-1α and HIF-2α activate *ALKBH5* transcription in BCa which has been demonstrated to mediate NANOG expression (Zhang et al., 2016). Therefore, by inhibiting ALKBH5 then the expression of NANOG decreases. In triple negative BCa ALKBH5 regulates the mRNA stability of FOXO1, said to be the master regulator, which reduces cellular reactive oxygen species (ROS) in doxorubicin (DOX) resistant triple negative BCa cells (Liu et al., 2024d). As well as being associated with obesity, FTO is found to be overexpressed in a number of human cancers (Azzam et al., 2022). FTO is upregulated in BCa and the overexpression of this demethylase leads to the increase in energy metabolism which works via the PI3K/AKT signalling pathway (Azzam et al., 2022, Niu et al., 2019). In acute myeloid leukaemia (AML) the expression of FTO enhanced cellular viability and proliferation while inhibiting apoptosis (Li et al., 2017).

In the HNRNP family hnRNP A1 is generally considered an oncogene (Siculella et al., 2023). In MDA-MB-231 and MCF-7 BCa cell lines, Zhang and colleagues showed knockdown of members of the HNRNP family, *HNRNPF*, *HNRNPH1* and *HNRNPK* increase of expression of the gene *Mcl-1$_S$* which in turn promotes the apoptosis of these BCa cells therefore delaying progression (Zhang et al., 2023). Also focusing on readers, YTHDC1 has been shown to be associated with BCa. Research shows that when there is increased expression of *YTHDC1* there is a positive correlation to the poor prognosis of these BCa patients (Tan et al., 2022a). YTHDF1 has also been shown to have an association with BCa and thereby the growth of BCa cells (Sun et al., 2022).

1.3 CBLL1

1.3.1 The biological function

Cbl proto-oncogene like 1 (CBLL1) was discovered in 2002 and was originally identified as an E3 ubiquitin ligase which plays a role in the degradation of E-cadherin (Gong et al., 2010, Fujita et al., 2002) and does this by targeting the tyrosine-phosphorylated E-cadherin (Mukherjee et al., 2012). In epithelial cells, CBLL1 has been shown to interact with the Src kinase phosphorylated E-cadherin (Lu et al., 2018).

CBLL1 is located on chromosome 7 and its structure consists of phosphotyrosine-binding, RING finger, and proline-rich domains following similarities with c-Cbl (Gong et al., 2010, Hui et al., 2019). Even though CBLL1 has been shown to regulate E-cadherin, many E3-ligases have multiple ubiquitination substrates suggesting that it may have other roles (Figueroa et al., 2009). CBLL1 dimerisation allows for the formation of the phosphotyrosine-binding pocket which is able to recognise these phosphorylated tyrosine's and surround the amino acids of substrates such as E-cadherin (Mukherjee et al., 2012). In terms of the E-cadherin complex,

the CBLL1 ubiquitin ligase functions as a posttranslational regulator and therefore induces the ubiquitination and subsequent degradation of E-cadherin (Roca-Lema et al., 2022, Bawankar et al., 2021). However, CBLL1's precise function in terms of RNA:m^{6}A still remains unclear.

More importantly in the context of this study, CBLL1 has also been shown to be involved in BCa (Figueroa et al., 2009) and as a component of the METTL3:METTL14 m^{6}A methyltransferase complex. Knockdown of CBLL1, shown in Hela cells, has resulted in lower levels of RNA:m^{6}A (Yue et al., 2018). Both METTL3 and METTL14 have been shown to be two stable sub-complexes which are part of the RNA:m^{6}A writer complexes (Knuckles et al., 2018). CBLL1 has been shown to be a core component for the stabilisation of METTL3 and METTL14 (Song et al., 2022). It remains unclear of how CBLL1 interacts or potentially regulates METTL3 and METTL14.

CBLL1 is shown to be a component of the RNA:m^{6}A methyltransferase complex in vertebrates and plants specifically shown in *Drosophila melanogaster* and *A. thaliana* models (Zhang et al., 2022, Bawankar et al., 2021) In *D. melanogaster*, CBLL1 has been shown to have functions associated with the sex determination pathway (Bawankar et al., 2021) where RNA:m^{6}A is also known to play a critical role in early embryonic development (Kaido et al., 2009). In *A. thaliana* the CBLL1 homolog is named HAKAI, has been shown to have no lethal effects on the plant when knocked out whereas knockouts of MTA (METTL3 human homolog), MTB (METTL14 human homolog), FIP37 (WTAP human homolog) and VIR did not progress past the embryonic globular stage, therefore further suggesting that CBLL1 has complex functions (Růžička et al., 2017). To date, there has been no published development or completion of a CBLL1 knockout mouse model.

1.3.2 CBLL1 and cancer

Ubiquitination plays key roles in cellular processes specifically affecting the function of the intracellular proteins those including master regulatory proteins (Pickart, 2001), therefore indicating that the dysregulation of CBLL1 would be associated with a number of types of cancers (Figueroa et al., 2009). However, recent studies have described other players involved in regulating the E-cadherin complex. For example, the Slit-Robo signalling has been shown to recruit CBLL1 and therefore ubiquitinates E-cadherin (Zhou et al., 2011). This signalling has been shown to demonstrate that when Slit2 is expressed in tumours which is able to communicate with Robo1 in the endothelial cells therefore showing that there is a crosstalk pathway which mediates tumour-induced angiogenesis with the recruitment of CBLL1 (Wang et al., 2003, Zhou et al., 2011).

On the other hand, Rack1 acts as a substrate and inhibits the phosphorylation of E-cadherin by Src and the ubiquitination of E-cadherin by CBLL1 thus promoting cell to cell adhesion (Swaminathan and Cartwright, 2012, Aparicio et al., 2012). Therefore, as the disruption of the cell to cell adhesion allows cancer cells to invade and metastasize, Rack1 acts as a tumour suppressor (Swaminathan and Cartwright, 2012).

As well as being a part of the E-cadherin complex, CBLL1 has been shown to be associated with BCa by co-regulating the estrogen receptor (ERα) (Gong et al., 2010). More specifically

CBLL1 binds to the DNA-binding domain of ERα thereby inhibiting its transcriptional activity (Gong et al., 2010).

There are a number of other diseases and cancers where CBLL1 has been shown to play a role. CBLL1 has been shown to be upregulated in non-small cell lung cancer as compared to adjacent non-tumour tissues (Hui et al., 2019). When CBLL1 is silenced in the colon cancer tumour spheres, a 3D model of colon cancer stem cells, a clear reduction in the tumour sphere size was observed (Alfonsín et al., 2024). This silencing also downregulates the stem cell biomarkers LGR5 and c-MYC at both mRNA and protein levels (Alfonsín et al., 2024).

1.4 METTL3

1.4.1 Biological function

METTL3 comprises 580 amino acids and consists of a zinc finger domain (ZFD) and a methyltransferase domain (Zeng et al., 2020). METTL3 has been shown to have be the catalytic subunit and forms a heterodimer with METTL14 (Vu et al., 2017) which is said to be the core component of the methyltransferase complex (Yue et al., 2018). Moreover, in the terms of the m^6A modification METTL3 has been shown to methylate miRNAs, lincRNAs and circRNAs (Han et al., 2019). METTL3 has a S-adenosylmethionine (SAM) binding site and is therefore able to crosstalk with SAM signalling (Wang et al., 2016a). Cytoplasmic METTL3 is able to recognise the m^6A sites that are on the 3' UTR which therefore promotes translation (Liu et al., 2020). It does this by interacting with the eukaryotic translation initiation factor 3 subunit h (eIF3h) which results in the facilitation of the translation loop formation (Choe et al., 2018).

Yan et al. has identified that METTL3 has a novel cleaved form named METTL3a which is the residues 239–580 of METTL3 and that this cleaved form is needed for the METTL3–WTAP interaction (Yan et al., 2023). SAM plays a crucial role in the methylation process by catalysing the transfer of the methyl group through the use of SAM-dependent methyltransferases (MTases) such as METTL3 (Struck et al., 2012).

METTL3 has been shown to play roles in a number of cellular processes which include the cell cycle, proliferation, apoptosis, migration and invasion as well as differentiation (Li et al., 2022a) with these processes having an involvement in a number of diseases including cancer (Liu et al., 2020). It has also been shown to regulate alternative splicing of genes related to the cell cycle of which are dependent on RNA:m^6A (Kim et al., 2023). This understanding of the mechanism involved in alternative splicing is important for the treatment of diseases such as cancer (Kim et al., 2023).

METTL3 has been shown to be essential for the normal signalling of progesterone during embryo implantation and this METTL3 dependent RNA:m^6A methylation also facilitates female fertility (Zheng et al., 2023c, Wan et al., 2023), therefore these both indicate that m^6A plays an important role in reproduction. As well as being progesterone METTL3 also modulates a number of important pathways including JAK/STAT (Yao et al., 2019), PI3K/AKT (Tian et al., 2019), Wnt/β-catenin (Miao et al., 2019) and MAPK/NF-κB (Liu et al., 2020, Feng et al., 2018). Both progesterone and a number of these pathways have shown an association with cancer.

1.4.2 Association of METTL3 with cancer

METTL3 dysregulation can contribute to cancer and both pro-oncogenic and tumour suppressor functions have been identified (Zeng et al., 2020). METTL3 has shown to promote a number of different cancer types which include lung cancer (Lin et al., 2016), bladder cancer (Cheng et al., 2019), BCa (Cai et al., 2018) and also myeloid leukaemia progression (Barbieri et al., 2017).

This study focuses on the role of METTL3 in BCa which has been shown to be upregulated in these BCa cells and tissue (Wang et al., 2020b, Cheng et al., 2019). Cai et al. identified that HBXIP, which is an oncoprotein found in BCa, upregulated METTL3 and did this by inhibiting the expression of let-7g, a tumour suppressor (Cai et al., 2018). When promoting BCa cell proliferation METTL3 has been shown to be regulated by the p21 expression (Cheng et al., 2021).

1.5 METTL14

1.5.1 Biological function

METTL14 is currently believed to function an adapter protein for METTL3 activity and enhances the methyltransferase activity by recognising the RNA substrates and methyl localisation (Guan et al., 2022). It is structurally similar to METTL3 but differs by lacking the SAM binding site and therefore does not have catalytic function (Wang et al., 2016b, Guan et al., 2022). Together METTL14 and METTL3 form a stable catalytic heterodimer which is the core complex of the methyltransferase complex (Liu et al., 2014). The histone H3 trimethylation at lysine 36 (H3K36me3) has been shown to co-transcriptionally guide RNA:m^{6}A through interacting with METTL14 and therefore in turn the methyltransferase complex involvement (Huang et al., 2019).

1.5.2 Association of METTL14 with cancer

METTL14 has been shown to be a tumour suppressor gene in a number of different types of cancers (Guan et al., 2022). In bladder cancer, METTL14 was shown to be at lower levels and correlated with the clinical stages of bladder cancer and associated with worse clinical outcome (Sun et al., 2020). In colorectal cancer METTL14 expression was also significantly reduced and thereby the association with poor overall survival (Chen et al., 2020). Also found in colorectal cancer was that METTL14 supressed proliferation and invasion by down-regulating the lncRNA named X inactivate-specific transcript (*XIST*) (Yang et al., 2020). In BCa METTL14 may accelerate migration and invasion of BCa cells and does so in an m^{6}A-dependent manner (Yi et al., 2022a, Yi et al., 2020).

A cancer associated METTL14 with the heterozygous R298P mutation and has been shown to promote cancer cell proliferation (Miyake et al., 2023). However, if the R298P mutation is homozygous it is shown to reduce cancer cell proliferation (Miyake et al., 2023). Mechanistically this mutation has been shown to promote RNA:m^{6}A modification at aberrant motifs whereas at canonical sites it reduces methylation efficiency (Miyake et al., 2023). Furthermore, RNA:m^{6}A modfication is enriched at these non-canonical cleavage sites in the

3’UTR and had a tendency to be located at non-canonical alternative polyadenylation (APA) sites (Zhang et al., 2020).

1.6 The potential of therapeutic targeting RNA:m^{6}A in cancer

There is a need for novel therapeutics to treat a range of cancers including BCa. Recent interest has supported RNA:m^{6}A as a therapeutic target in many cancers which has most prominently involved the methyltransferase METTL3 and the demethylase FTO.

The recent development of small molecule inhibitors of METTL3/METTL14 including STM2457 and STC-15 has begun an era of epitranscriptomic therapies (Yankova et al., 2021, Patke et al., 2024). These small molecule inhibitors were developed by STORM Therapeutics and have been shown to inhibit enzymatic activity and function of METTL3 by binding to the SAM-binding site located on METTL3 (Sanderson et al., 2019). STC-15 is currently in phase I clinical trials (clinicaltrials.gov accession#: NCT05584111). Also, shown in myeloid leukaemia the pharmacological inhibition of METTL3 using STM2457 has demonstrated that the cells when treated showed a reduction in cancer cell growth (Yankova et al., 2021). Recently, STM2457 has been shown to reduce tumour progression oral squamous cell carcinoma both *in vitro* and *in vivo* in mice models (Liu et al., 2024b).

Another drug to potentially exploit METTL3 as a therapeutic target is metformin which works by targeting p21 which has been shown to interact with METTL3 to promote BCa proliferation and therefore using Metformin can inhibit cell proliferation (Cheng et al., 2021). This could indicate the use of metformin as a potential indirect RNA:m^{6}A therapeutic (De and Kuppusamy, 2020). Berberine, an isoquinoline alkaloid has been shown that it also targets METTL3 but only partially by regulating METTL3-mediated m6A modification of FGF7 mRNA (Fu et al., 2024).

There is a number of inhibitors that target the demethylase FTO which include rhein, meclofenamic acid and other repurposed therapeutics to inhibit FTO (Aik et al., 2013, Huang et al., 2015). Rhein is a reversible and competitive FTO inhibitor and is a main constituent of rhubarb of which is found in medicinal herbs (Aik et al., 2013, Wang et al., 2020a). This inhibitor, discovered by Chen et al., has shown to have several pharmacological properties such as anticancer, anti-inflammatory and ant-angiogenic (Zhang et al., 2024, Chen et al., 2012). Rhein works by competitively binding to the active site on FTO and therefore inhibits its ability to demethylate the m^{6}A modification (Chen et al., 2012). It also been shown to induce apoptosis in a number of cancer cell types (Wang et al., 2020a) including in acute myeloid leukaemia where Rhein was shown to inhibit FTO and subsequently inhibit the AKT/mTOR pathway (Zhang et al., 2024).

Meclofenamic acid, a non-steroidal anti-inflammatory drug, has been shown to selectively inhibit FTO demethylation over ALKBH5 *in vitro* (Huang et al., 2015). This works by indirectly targeting the m^{6}A-containing the single stranded DNA or single stranded RNA in a dose-dependent manner through inhibiting FTO demethylation (Huang et al., 2015).

1.7 The aims of this study

In recent unpublished work completed by two former PhD students (Drs. Anna Harris and Jennifer Lothion-Roy), the clinical significance and function CBLL1 and METTL3 and METTL14 were independently examined, primarily in luminal-type BCa. These studies had also identified mutual regulation of METTL3 and METTL14, through an as yet unknown mechanism. Thus, understanding of the clinical significance of the MTC in TNBC remains incomplete and the potential role for CBLL1 in the regulation of METTL3 and METTL14 expression remains unknown. For these reasons this study works to investigate the expression of METTL3, METTL14 and CBLL1 in TNBC cell lines and determine the functional effects of individual and combinatorial knockdown of METTL3, METTL14 and CBLL1 in BCa cells. To access the therapeutic potential of targeting METTL3, the effect of the STM2457 METTL3 inhibitor on TNBC proliferation and gene expression was assessed. By using the currently available inhibitors targeting the regulators of the RNA:m^6A modification could give insight into the future of novel inhibitors. Such as, there might be potential to target CBLL1 as a therapeutic target in the future and use this therapeutic as standalone or in combination with STM2457. Furthermore, the aim of this would be to look at how the effect might have different impacts on different Bca cancer subtypes particularly TNBC.

Chapter 2

Materials and methods

2 Materials and Methods

2.1 Cell culture

BCa cell lines MCF-7, MDA-MB-231 and MDA-MB-436 were utilised in this study. These cell lines were kindly provided by Dr. Cinzia Allegrucci and Dr. Andrew Green and their identity confirmed by STR profiling (Eurofins Genomics).

2.1.1 An overview of the cell lines used in this study

MCF-7, MDA-MB-231 and MDA-MB-436 are widely used in BCa cell line studies.

MCF-7 was established at the Michigan Cancer Foundation in 1973 from a pleural effusion derived from a breast carcinoma (Soule et al., 1973). MCF-7 is ER and PR positive as well as HER2 negative therefore positioning it under the subgroup luminal A-like (Holliday and Speirs, 2011). This cell line has been shown to express E-cadherin (Eslami Amirabadi et al., 2019).

Both MDA-MB-231 and MDA-MB-436 are triple negative BCa cell lines which lack ER, PR and HER2 expression (Holliday and Speirs, 2011). The MDA-MB-436 cell line originates from a Caucasian female (Chavez et al., 2010). MDA-MB-231 has been shown to display representative epithelial to mesenchymal transition (EMT) which research has shown to be associated with BCa metastasis (Huang et al., 2020). MDA-MB-231 lacks E-cadherin expression (Eslami Amirabadi et al., 2019, Garcia et al., 2021).

2.1.2 Conditions

MCF-7 and MDA-MB-231 were maintained at 37°C and 5% CO_2 and cultured in RPMI-1640 medium and supplemented with 100 µg/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate all purchased from Gibco® by Life Technologies™ as well as 10% heat inactivated fetal bovine serum (FBS) purchased from Sigma-Aldrich. MDA-MB-436 was cultured in the same conditions and supplementation, but instead DMEM media with HEPES was used. For the estrogen experiments, β-estradiol (E2) was added at a concentration of 10 nM (Tocris Biosciences, UK) and the cell lines were cultured in phenol-red free RPMI-1640 media also supplemented with 100 µg/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate all purchased from Gibco® by Life Technologies™, however 10% charcoal and dextran treated "steroid depleted" FBS was prepared in the laboratory using the Privaslky protocol. These cell lines were frozen in RPMI-1640 with the addition of 10% DMSO.

2.1.3 siRNA-mediated depletion

MCF-7 and MDA-MB-231 were plated in 6 well plates in RPMI-1640 medium as described 24 hours before the first siRNA transfection. As METTL3 protein is known to have an extended half-life, experiments utilising anti-*METTL3* siRNA used a double knockdown approach which was performed with the first transfection 24 hours after plating and the second transfection after 48 hours from the first transfection. Prior to the second transfection, plated MCF-7 cells were replaced with stripped phenol red-free RPMI-1640 containing charcoal stripped FBS with the addition of either 10nM E2 or DMSO vehicle control. Protein was harvested after an additional 72 hours.

All transfections followed manufacturer's instructions, using DharmaFect 1 Transfection Reagent from GE Dharmacon™, USA. The siRNAs used in this study were: for METTL3 (SMARTpool: ON-TARGETplus METTL3 siRNA L-005170-02-0010; GE Dharmacon™, USA), METTL14 (SMARTpool: ON-TARGETplus METTL14 siRNA L-014169-02-0010; GE Dharmacon™, USA and/or CBLL1 (SMARTpool ON-TARGETplus siRNA targeting human CBLL1 (L-007069-00-0005; GE Dharmacon™, USA). For the negative control, ON-TARGETplus non-targeting scrambled control siRNA (D-001810-10-05; GE Dharmacon™, USA) was utilised.

Overall, the transfection experiments were conducted for collectively 120 hours with n=3 technical repeats performed for each experiment, with a second biologically independent replicate completed on another occasion.

The method of siRNA-mediated deletion was chosen as it was sufficient to answer the hypothesis under question. An advantage to using siRNA-mediated depletion is that it is time efficient for the user. This option was chosen over CRISPR-Cas9, or other gene-editing tools used for knockouts, as m^6A is known to be fundamental to cells. However, it may be interesting to investigate a knockout of the genes described in this study.

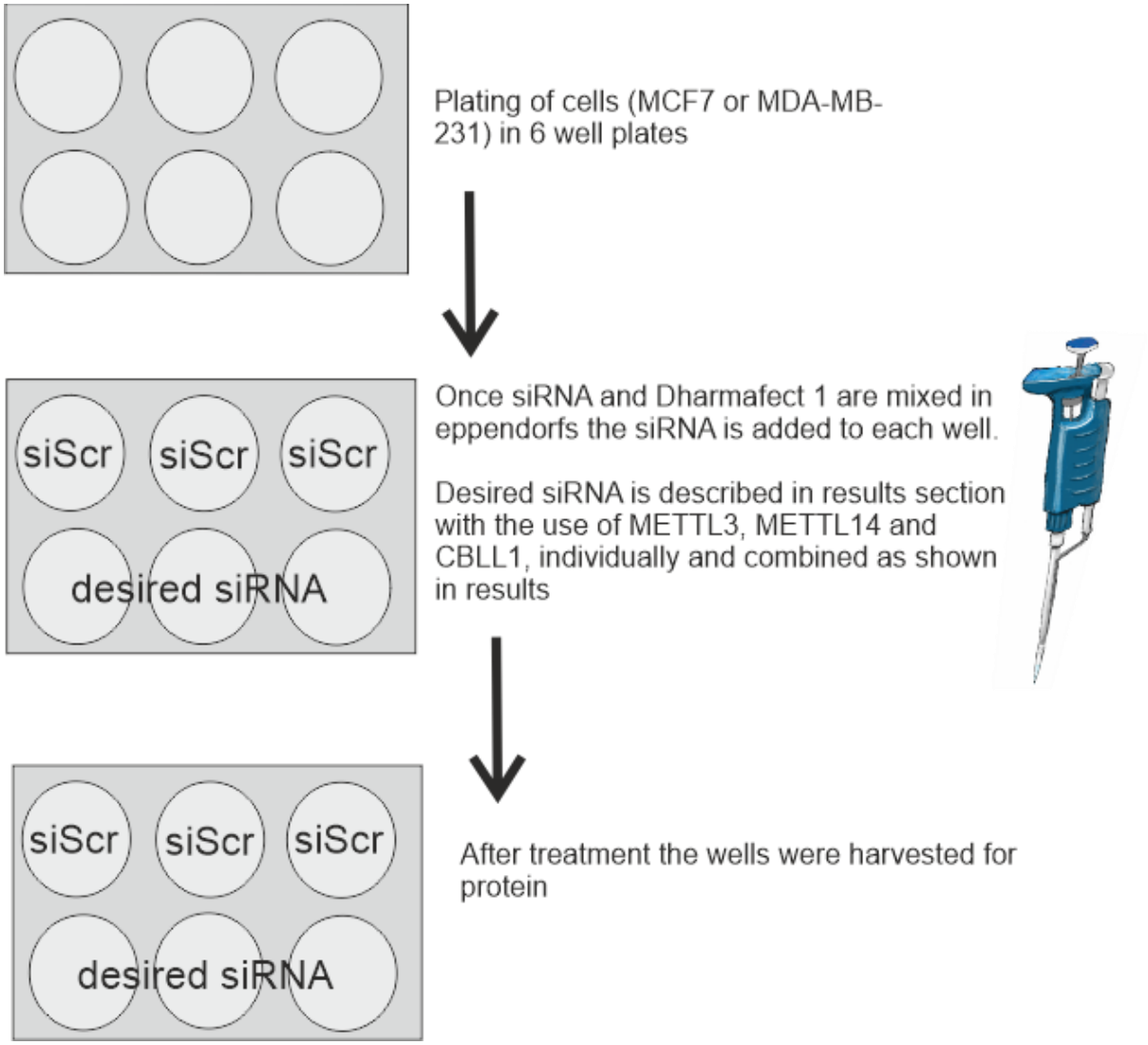


Figure 2. Flowchart to summarise the siRNA-mediated depletion of the experiments described in results.

The flowchart describes the process in which protein is harvested followed siRNA mediated depletion of the siRNAs used in this study.

2.1.4 Proliferation assay

The effect of STM2457 on MDA-MB-436 proliferation was assessed using the CyQuant Assay (Promega). For MDA-MB-436, 500 cells were plated per well in 96-well plates. After 24 hours the cells were treated with STM2457 (0µM, 10µM, 20µM, 30µM and 40µM concentration) with n=9 for each condition. Cells were treated at 0 and 72 hours when media was also changed to remove dead cells. CyQUANT™ Direct Cell Proliferation Assay (Promega, C35011) following manufacturer's instructions was used but replacing the background suppressor with PBS, pipetting 200µl of this mixture into the wells. After an hour incubation at 37°C, the plate was read using the Varioskan LUX Multimode Microplate Reader (ThermoFisher).

2.1.5 DNA extraction

The identity of MDA-MB-436 was confirmed by STR profiling (Eurofins Genomics). Genomic DNA was isolated using the DNeasy® Blood and Tissue Kit from Qiagen. Cultured MDA-MB-436 cells (a maximum number of 5 x 10^6) were centrifuged for 5 minutes at 300 x *g* and then resuspended in 200µl of PBS. 20µl of Proteinase K and 200µl of Buffer AL were added and then mixed by vortexing. This was then incubated in a water bath at 56°C for 10 minutes. After incubation 200µl of 100% ethanol was added and vortexed thoroughly to yield a heterogeneous solution. The mixture was then pipetted into the DNeasy mini spin column which is in a 2ml collection tube. This was centrifuged at ≥6000 x *g* for 1 minute with the flowthrough and collection tube discarded after. Once the DNeasy mini spin column is placed in a new 2ml collection tube, 500µl of Buffer AW1 was added and then this was centrifuged for 3 minutes at 20,000 x *g* with again the flowthrough and collection tube discarded after. Finally, the DNeasy mini spin column was placed in a 1.5ml microcentrifuge tube and 50µl of ddH_2O was pipetted directly onto the DNeasy membrane. This was then incubated at room temperature for 1 minute and then centrifuged for 1 minute at ≥6000 x *g* to elute. This sample was then sent for genotyping.

2.2 Western Blots

2.2.1 Protein sample preparation

Protein samples were prepared using the Laemmli approach. Briefly 20µl Laemmli final sample buffer (FSB)-diluted protein samples were combined with 5X Laemmli loading buffer (250mM Tris HCl pH 6.8, 10% SDS, 30% Glycerol, 5% β-Mercaptoethanol, and 0.02 % Bromophenol Blue) and 10mM DL-dithiothreitol (DTT). The cell lysates were then prepared by boiling at 95°C for 5 minutes and carefully sonicated.

2.2.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The 1mm 10% SDS-PAGE gels used were created as shown in Table 1. These gels were then placed in the tank and then filled with 1X electrophoresis buffer. Each 20µl of the protein was then loaded next to 8µL of Spectra™ Multicolor Broad Range Protein Ladder posited in the first well of each gel. The gel was then run at 180 volts for approximately 1 hour using the PowerPac™ Basic (Bio-Rad Laboratories, USA).

2.2.3 Semi-dry Transfer

Following the semi-dry transfer method, the polyvinylidene difluoride (PVDF) membrane (Immobilon-P membrane, 0.45µm; Merck, USA) was soaked in methanol for 2-3 seconds for activation. This PVDF membrane, the Whatman® paper pre-cut to match the size of the PVDF membrane with two sets of four sheets for each gel, and the now completed SDS-PAGE gel were all then soaked in transfer buffer (created as shown in Table 2) for 10 minutes. The PVDF membrane, Whattman paper and SDS-PAGE gel were then assembled in a sandwich. The blotting sandwich order consists of, starting from the bottom upwards, four sheets of soaked Whatman® paper, followed by the PVDF membrane, topped with the SDS-PAGE gel and finished with four more sheets of soaked Whatman® paper prepared on the cassette. Before closing the cassette any air bubbles in the blotting sandwich were removed using a roller to

ensure the desired transfer. The cassette was then placed in the Trans-Blot Turbo Transfer System (BioRad) and run at 25 volts for 30 minutes.

2.2.4 Antibody probing and chemiluminescence imaging of the PVDF membrane

Following the semi-try protein transfer, the PVDF membrane was blocked in 5 % milk powder in Tris-buffered saline with Tween 20 (5% milk/TBST) for 1 hour at room temperature. After this the membrane was then probed with the desired primary antibody and incubated at 4^{o}C in the cold room overnight. In the following day the PVDF membrane was washed 4 times each for 5mins in TBST. 5ml of 5% milk/TBST with the addition of anti-rabbit or anti-mouse HRP conjugated secondary antibodies was then probed for 1 hour at room temperature. Then following 3 washes for 5 mins each and another wash for 45mins the membrane was then imaged by adding mixed enhanced chemiluminescence (ECL) solution reagents A and B. The images were taken using a BioRad chemiluminescent detection system.

2.2.5 Quantification of the western blot

Using Image Studio Lite version 5.2 (LI-CoR Biosciences) the western blot signal was quantified with the calculated signal relative to the β-ACTIN and the ratio of the vehicle to the siRNA scrambled control.

Table 1. Reagents for producing gels.

Reagents	Resolving gel (10% for a pair of gels)	Stacking gel (for a pair of gels)
ddH_2O	4ml	3ml
1.5 M Tris HCL, pH 8.8	2.5ml	N/A
1.25 M Tris HCL, pH 6.8	N/A	1.3ml
10 % SDS	100µl	50µl
30% acrylamide and 0.8 % bisacrylamide	3.3ml	660µl
TEMED	5µl	5µl
10 % APS	50µl	25µl

Table 2. Buffer recipes

Buffer Name	Reagents
10x electrophoresis buffer	1.92 M Glycine + 0.25 M Tris in 1L
1 x electrophoresis buffer	100ml 10x electrophoresis buffer, 10ml 10% SDS and 890ml ddH2O
1 x transfer buffer	100ml 10x electrophoresis buffer, 100ml 100% methanol 800ml ddH2O

TBST	100ml of 10x TBS with 900ml water and 1ml Tween
PBST	100ml of 10x PBS with 900ml water and 1ml Tween
Mild stripping buffer	15g glycine, 1 g SDS, 10 ml Tween20, adjusted pH to 2.2 and then volume brought up to 1 L with dH20

2.3 Statistical analysis

The statistical analyses were completed using GraphPad Prism with one-way ANOVA, with Geisser-Greenhouse correction and uncorrected Fisher's LSD, with individual variances computed for each comparison. All the graphs were created using GraphPad Prism and p values were shown as * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.001$, ****$p < 0.0001$, ns = not significant.

2.4 RNA sequencing and bioinformatics of publicly available data

Bioinformatic analysis was performed by Professor Nigel Mongan which consisted of reads being trimmed using the TrimGalore (Martin, 2011) wrapper to remove reads with a Phred quality score of <30 and the contaminating adapter sequences. Following this the retained reads were aligned using STAR (Dobin et al., 2013) to the Ensembl annotated human reference genome (GRCh38.83). Gene expression was then quantified using FeatureCounts (Liao et al., 2014) with the differential gene expression calculated using DESeq2 (Love et al., 2014). All RNA sequencing data related to this project were deposited in NCBI-GEO under the following accession numbers: GSE271326, GSE271330, GSE234571, GSE234322, GSE195487, GSE195532).

Significantly differentially expressed genes identified by RNA sequencing analysis were defined as padj <0.05 and log2FC >±0.58496250072 (equivalent to ±1.5 fold change). Further analysis was then performed using WebGestalt, Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/) and VolcaNoseR (https://huygens.science.uva.nl/VolcaNoseR/) tools. WebGestalt was using to analysis the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways of the significantly differentially expressed genes. Venny 2.1 was used to produce Venn diagrams of this RNA sequencing data. VolcaNoseR was also utilised to produce volcano plots of the significantly differentially expressed genes from the RNA sequencing analysis. Further validation methods to confirm the significance of gene expression changes would be interesting in future experiments.

Replicate multivariate analysis of transcript splicing (rMATs) was used to identify differential alternative splicing genes and events (version 3.2.5 was used) (Shen et al., 2014). Genes and events (transcripts) were considered significantly differentially spliced with percentage spliced in ≥5% (dPSI ≥5%) and false discovery rate < 0.05 (FDR < 0.05). KEGG pathway analysis, as described for RNA-seq, was also used to analysis alternative splicing events.

The TCGA-BRCA RNA-seq dataset was dichotomized into quartiles based on normalised CBLL1 expression. Patients with low (<Q1) versus high (Q4) *CBLL1* expression were analysed using DESeq2 package.

To complete the analysis of publicly available data within this study, several online tools were used. cBioPortal was utilised to identify and analysis *CBLL1*, *METTL3*, and *METTL14* gene alterations and mutations within the Breast Invasive Carcinoma (TCGA-BRCA, PanCancer Atlas dataset) (n=996) cohort. Furthermore, the Broad Institute and Novartis Cancer Cell Line Encyclopedia from (2019 update with 961 samples) was used to investigate the expression of *CBLL1*, *METTL3*, and *METTL14* in different patients and cancer cell lines. The genomic profiles selected were mutations, structural variant and putative copy number alterations from GISTIC. Patient/Case set, samples with mutation and CNA data was also selected. Kaplan-Meier plots were performed using the Kaplan-Meier plotter online tool (Győrffy, 2021). The settings were on default options which are selected on the tool OS (n=2976) as the auto select best cutoff (percentile), no restrictions on analysis to subtypes or cohorts and the follow-up threshold was 'all'.

Chapter 3

Results

3 Results

3.1 Clinical significance of *CBLL1*, *METTL3* and *METTL14* expression

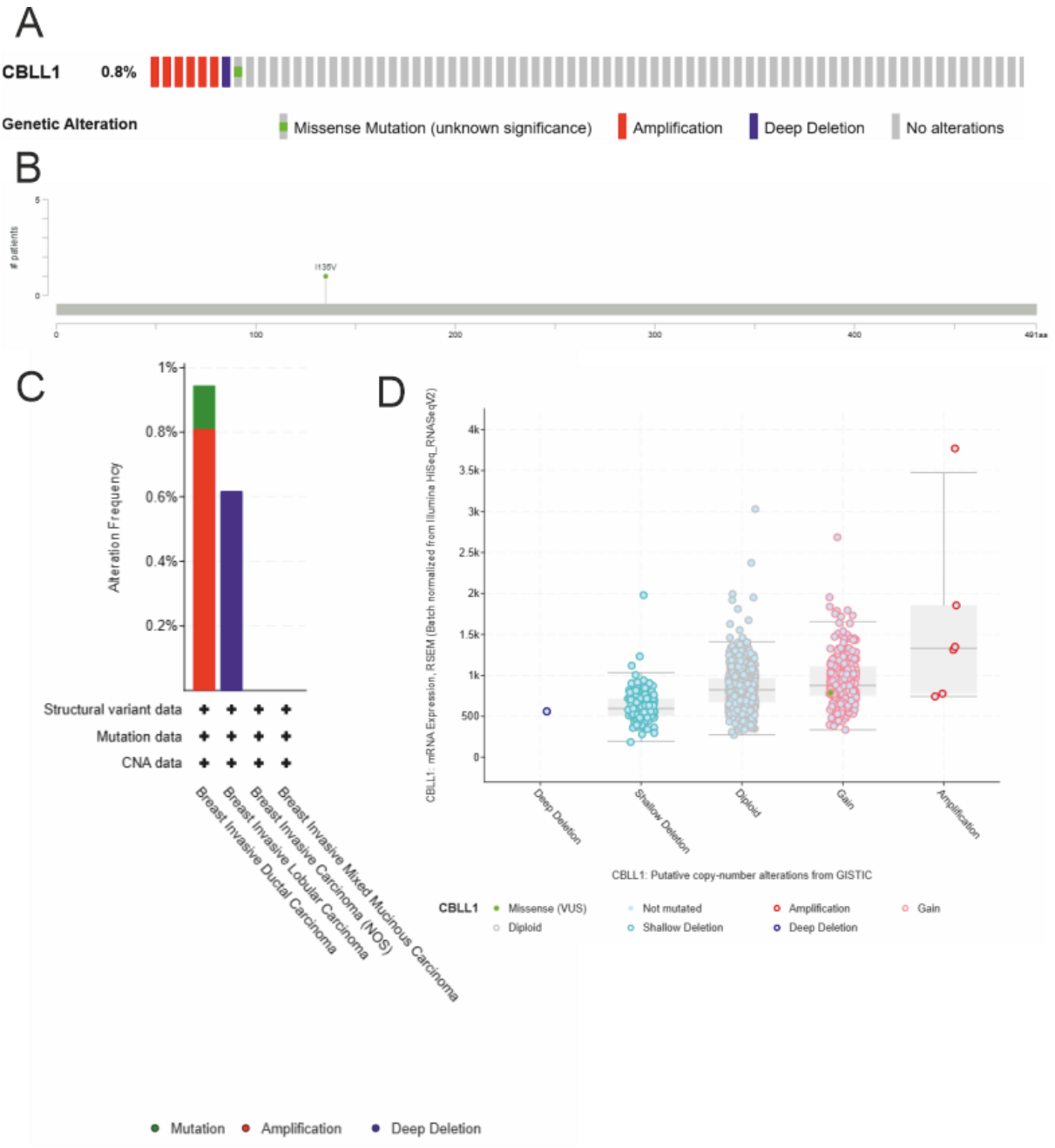
3.1.1 Genetic alterations and mutations of *CBLL1*, *METTL3* and *METTL14* in breast cancer

To investigate if *CBLL1*, *METTL3* and *METTL14* mRNA expression, genetic alterations and mutations were associated with invasive BCa, cBioPortal was used to analyse genetic variants, copy number and expression changes in the TCGA PanCancer Atlas Breast Invasive Carcinoma (BRCA) cohort comprising n=996 patients' specimens.

Putative copy number alterations from GISTIC resulting in the most *CBLL1* mRNA expression in the cohort were diploid, but *CBLL1* mutations were not detected. This analysis identified a single deep deletion affecting one patient sample (**Figure 3**). In this cohort, *CBLL1* also showed shallow deletions, gain and amplification (**Figure 3**). From analysis there is only a singular mutation found in *CBLL1*, I135V missense mutation, in the cohort of 996 patients, (**Figure 3**).

The putative copy number alternations from GISTIC for *METTL3* mRNA expression (**Figure 4**) showed two deep deletions, and seven amplifications. Furthermore, shallow deletions, diploid and gain of copies were observed (**Figure 4**). There were three observed *METTL3* mutations of which are all in the MT-A70 domain with two of these being missense mutations and the other being a frame shift deletion (**Figure 4**)

The putative copy number alternations from GISTIC for *METTL14* mRNA expression (**Figure 5**) showed no deep deletions, but shallow deletion, diploid, gain of copies and amplification were observed. For the *METTL14* mRNA expression, 5 amplification alterations were observed. In *METTL14* five mutations resulted from the analysis with two of these being in the MT-A70 domain (**Figure 5**).

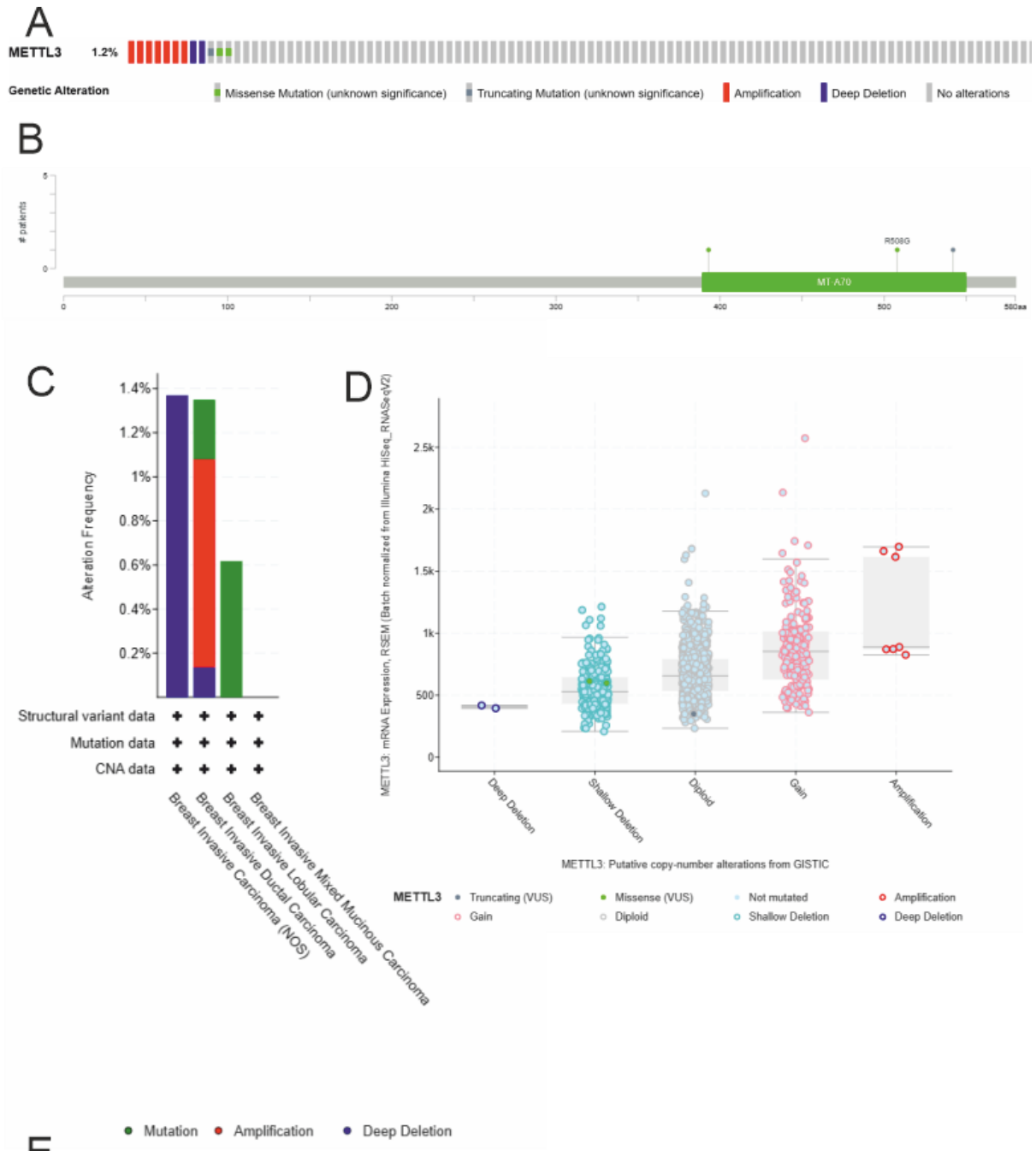


E

Name of gene	Sample ID	Detailed cancer type	Protein change	Mutation type	Copy number	Variant Allele frequency in tumour sample	Total number of nonsynonymous mutations in sample
CBLL1	TCGAAO-A128-01	Breast Invasive Ductal Carcinoma	I135V	Missense	Low level Gain	0.16 (10 variant reads of 64 total)	826

Figure 3. *CBLL1* genetic alternations and mutations analysis using cBioPortal.

cBioPortal analysis of *CBLL1* in the TCGA PanCancer Atlas Breast Invasive Carcinoma (n=996). (**A**) shows genetic alterations (**B**, **C**, **D**, **E**) and mutations.



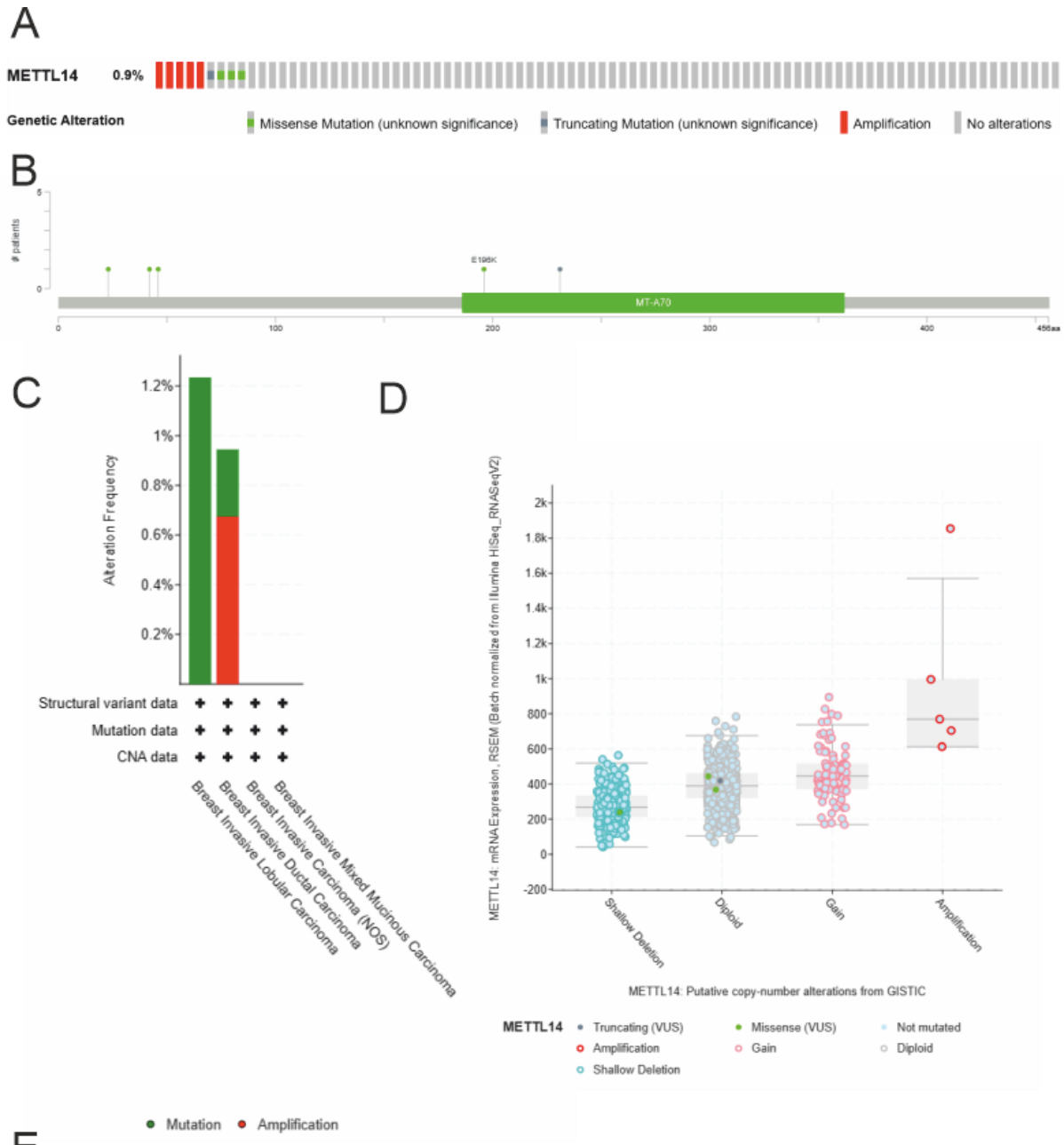
E

● Mutation ● Amplification ● Deep Deletion

Name of gene	Sample ID	Detailed cancer type	Protein change	Mutation type	Copy number	Variant Allele frequency in tumour sample	Total number of nonsynonymous mutations in sample
METTL3	TCGA-AR-A251-01	Breast Invasive Ductal Carcinoma	M393V	Missense	Shallow deletion	0.32 (12 variant reads out of 37 total)	159
	TCGA-B6-A1KF-01	Breast Invasive Ductal Carcinoma	R508G	Missense	Shallow deletion	0.79 (86 variant reads out of 109 total)	49
	TCGA-D8-A27V-01	Breast Invasive Lobular Carcinoma	N543Tfs*15	Frame shift deletion	Diploid	0.10 (14 variant reads out of 144 total)	3080

Figure 4. *METTL3* genetic alternations and mutations analysis using cBioPortal

cBioPortal analysis of *METTL3* in the TCGA PanCancer Atlas Breast Invasive Carcinoma (n=996). (**A**) shows genetic alterations (**B**, **C**, **D**, **E**) and mutations.



E

Name of gene	Sample ID	Detailed cancer type	Protein change	Mutation type	Copy number	Variant Allele frequency in tumour sample	Total number of nonsynonymous mutations in sample
METTL14	TCGA-AR-A0U1-01	Breast Invasive Ductal Carcinoma	L23F	Missense	Shallow deletion	0.18 (38 variant reads out of 207 total)	78
	TCGA-D8-A27G-01	Breast Invasive Lobular Carcinoma	R42K	Missense	Diploid	0.20 (11 variant reads out of 54)	813
	TCGA-D8-A27G-01	Breast Invasive Lobular Carcinoma	E46K	Missense	Diploid	0.18 (9 variant reads out of 49)	813
	TCGA-AC-A23H-01	Breast Invasive Ductal Carcinoma	E196K	Missense	Diploid	0.27 (26 variant reads out of 96)	4259
	TCGA-D8-A27V-01	Breast Invasive Lobular Carcinoma	L233Sfs*43	Frame shift deletion	Diploid	0.11 (18 variant reads out of 169)	3080

Figure 5. *METTL14* genetic alternations and mutations analysis using cBioPortal

cBioPortal analysis of *METTL14* in the TCGA PanCancer Atlas Breast Invasive Carcinoma (n=996). (**A**) shows genetic alterations (**B**, **C**, **D**, **E**) and mutations.

3.1.2 Clinical significance of *CBLL1, METTL3 and METTL14* expression at transcriptomic levels

Using the online tool Breast Cancer Gene-Expression Miner v5.0 (Jézéquel et al., 2021) the correlation between *METTL3*, *METTL14* and *CBLL1* mRNA expression and clinical parameters in both the TCGA (n = 743) and SCAN-B (n=3,678) cohorts together within the RNA-seq data was analysed. For the age analysis, 51 years of age is chosen as this is the median age women experience menopause in western industrialised countries as supported by Laven et al. (Laven et al., 2016).

This analysis for *METTL3* mRNA expression has been shown for ER, PR, and HER2 with the additional comparison between ER and PR. The Dunnett-Tukey-Kramer test was carried out by Breast Cancer Gene-Expression Miner v5.0 and showed that the comparison between ER+/PR+ versus ER-/PR- was significant ($p<0.0001$). The comparison of ER+/PR+ versus ER+/PR- was also significant ($p<0.05$). The subtypes were also compared using the Dunnett-Tukey-Kramer test for *METTL3* (**Figure *6*L**).

Analysis of *METTL14* mRNA expression was shown in the bee swarm plots in **Figure 7**. Comparison of ER and PR gave all significant results when analysed with the Dunnett-Tukey-Kramer test except for ER-/PR+ verses ER-/PR- with a p value of >0.10. Furthermore, comparison of the subtypes (PAM50) was carried out utilising the Dunnett-Tukey-Kramer test (**Figure *7*L**)

CBLL1 mRNA analysis was also carried out and shown in bee swarm plots in **Figure 8**. When comparing ER+/PR+ as compared to ER-/PR- was significant ($p<0.0001$), ER+/PR+ versus ER+/PR- was significant ($p<0.01$) and the following other comparisons were not significant. As with *METTL14* the comparison of the subtypes (PAM50) was analysed using the Dunnett-Tukey-Kramer test for *CBLL1* (**Figure *8*L**).

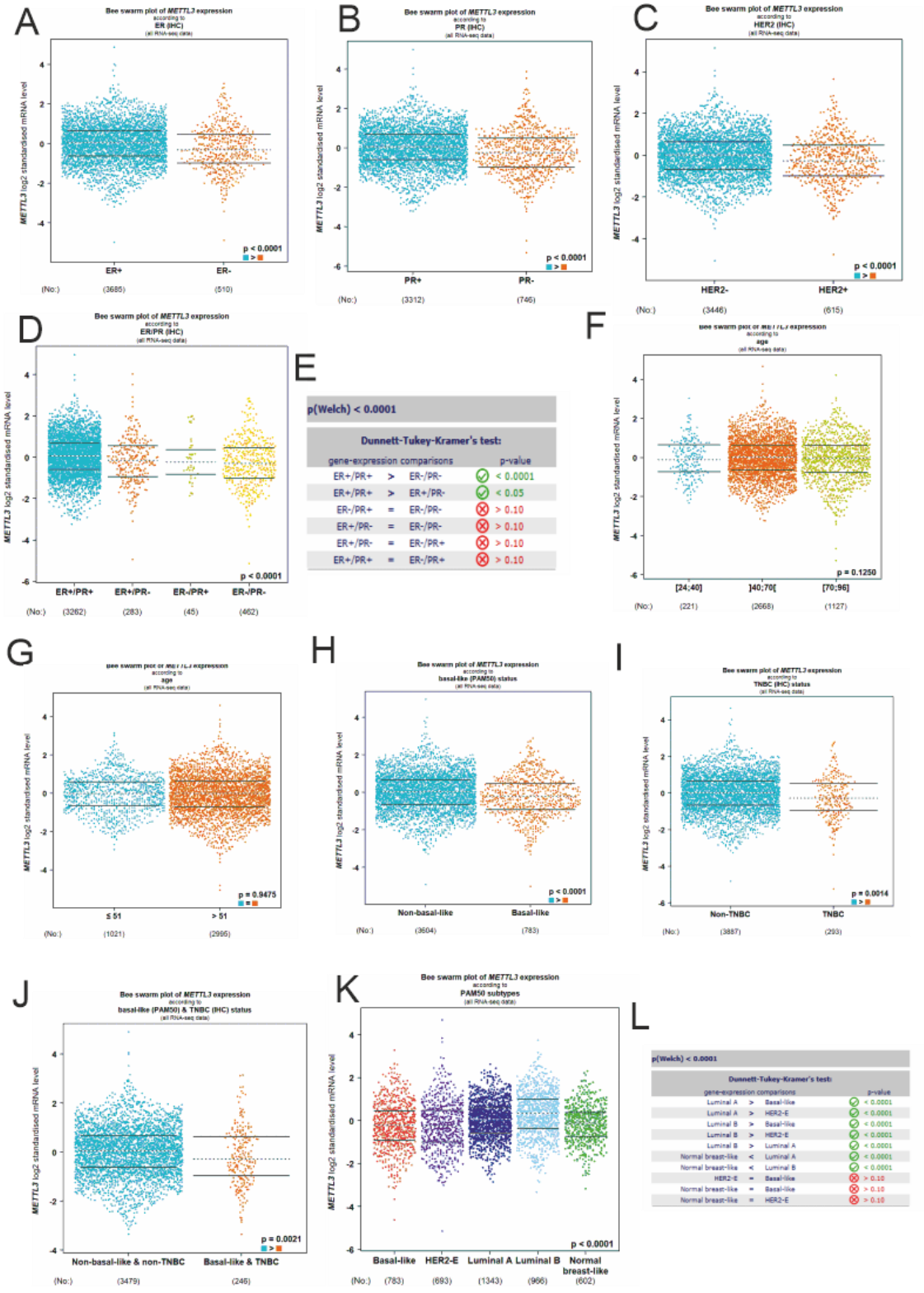


Figure 6. Clinical significance of *METTL3* expression at transcriptomic level under several different parameters

The association between *METTL3* mRNA expression levels and (**A**) ER, (**B**) PR, (**C**) HER2, (**D**) comparison between ER and PR (**E**) and significance, (**F**) range of age groups (**G**) above and below 51 years of age (**H**) basal like, (**I**) TNBC, (**J**) basal like and TNBC, (**K**) PAM50 subtypes (**L**) and significance.

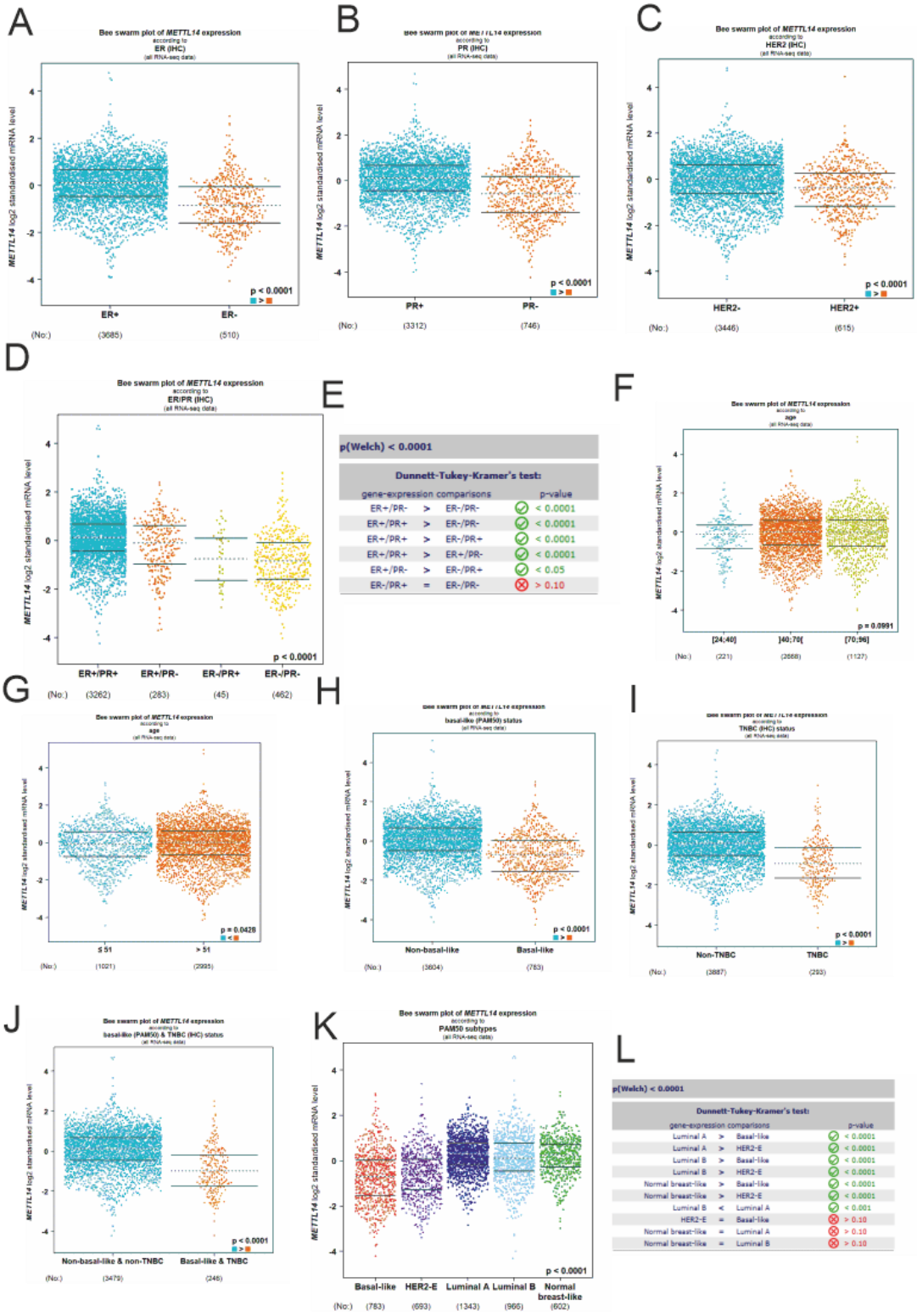


Figure 7. Clinical significance of *METTL14* expression at transcriptomic level under several different parameters

The association between *METTL14* mRNA expression levels and (**A**) ER, (**B**) PR, (**C**) HER2, (**D**) comparison between ER and PR (**E**) and significance, (**F**) range of age groups (**G**) above and below 51 years of age (**H**) basal like, (**I**) TNBC, (**J**) basal like and TNBC, (**K**) PAM50 subtypes (**L**) and significance.

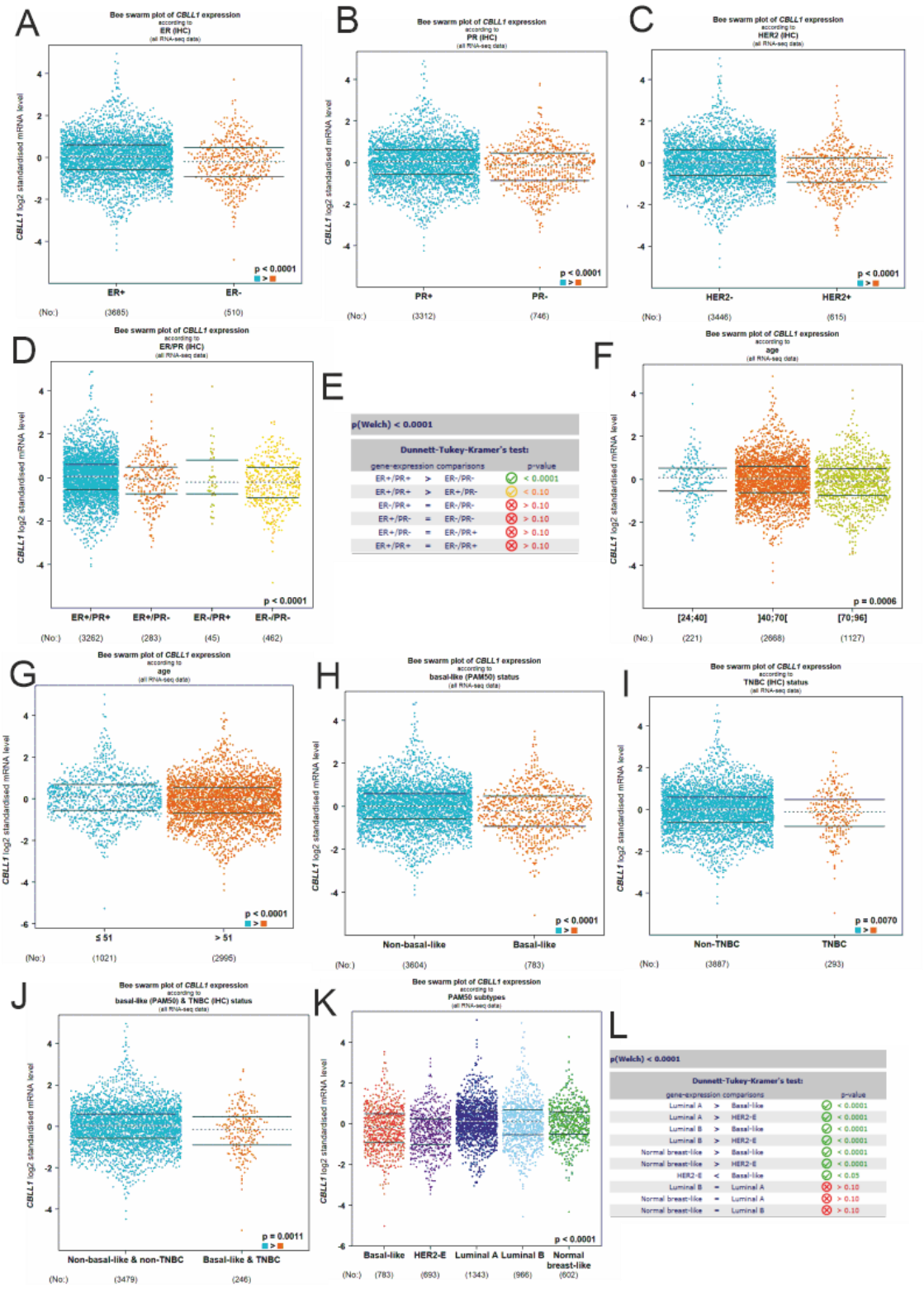


Figure 8. Clinical significance of *CBLL1* expression at transcriptomic level under several different parameters.

The association between *CBLL1* mRNA expression levels and (**A**) ER, (**B**) PR, (**C**) HER2, (**D**) comparison between ER and PR (**E**) and significance, (**F**) range of age groups (**G**) above and below 51 years of age (**H**) basal like, (**I**) TNBC, (**J**) basal like and TNBC, (**K**) PAM50 subtypes (**L**) and significance.

3.1.3 Kaplan-Meier survival estimates for the expression of *CBLL1*, *METTL3* and *METTL14*

The Kaplan-Meier plots were created using the Kaplan-Meier plotter online tool (Győrffy, 2021). This online tool uses publicly available BCa RNA-seq (n=2976). The survival estimates for the expression of CBLL1, METTL3 and METTL14 are shown in to analyse the association of these with patient outcomes (**Figure 9**). Additionally, ERBB2 (HER2) has been analysed as this may give an interesting comparison of an already known marker.

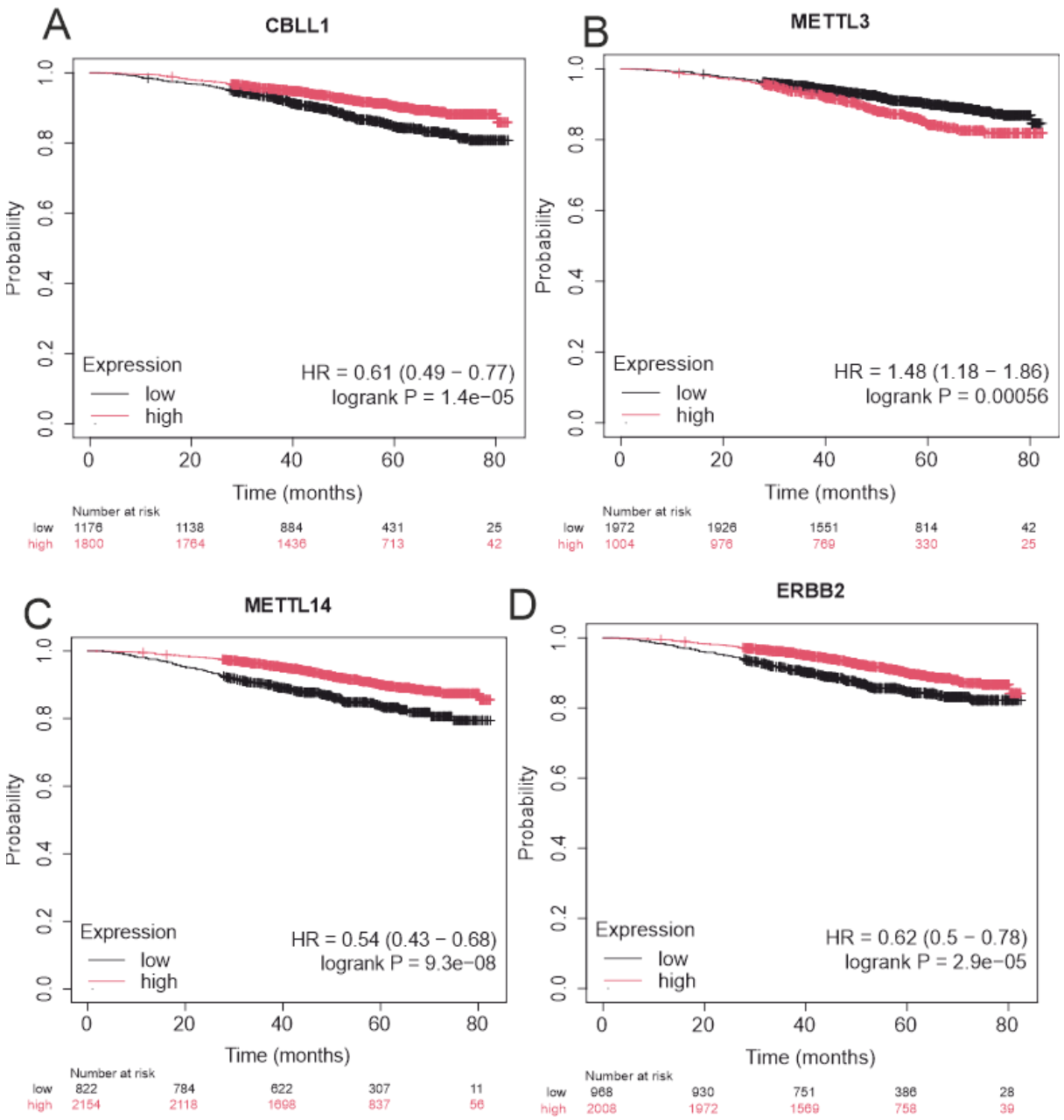


Figure 9. Kaplan-Meier survival estimates for *CBLL1, METTL3 and METTL14.* Altered expression of *CBLL1, METTL3* and *METLL14* were significantly associated with overall survival outcome.

The Kaplan-Meier plots show overall survival (OS) from RNA-seq data for four genes (A) CBLL1, (B) METTL3, (C) METTL14 and (D) ERBB2 (HER2) for comparative data. These were created using the online tool (Győrffy, 2021).

3.2 The functional role of CBLL1 in breast cancer cell line MCF7

3.2.1 siRNA-mediated depletion of CBLL1 following estrogen treatment

To investigate the role CBLL1 has in both expression of E-cadherin and components of the MTC and estrogen signalling, a siRNA-mediated depletion of CBLL1 was completed in the MCF-7 luminal A-like breast cancer cell line. This cell line was chosen as it expresses ERα and therefore can be used to investigate the role of CBLL1 following estrogen treatment. The results obtained (**Figure 10**) show successful siRNA-mediated depletion of the CBLL1 protein.

In non-gene targeting scramble (siScr) control, estrogen treatment induced the significant upregulation of *CBLL1, METTL3* and *METTL14* protein (**Figure 10**). However, following siRNA-mediated knockdown of CBLL1, METTL3 and METTL14 protein expression does not significantly change, when compared with the siCBLL1 vehicle and siCBLL1 estrogen treated indicating knockdown is selective for CBLL1. Also, there is no significant change in METTL3 and METTL14 expression when comparing the scramble vehicle to the siCBLL1 vehicle. On the contrary, knockdown of CBLL1 in the presence of estrogen, results in downregulation of METTL3 and METTL14 and CBLL1 protein expression.

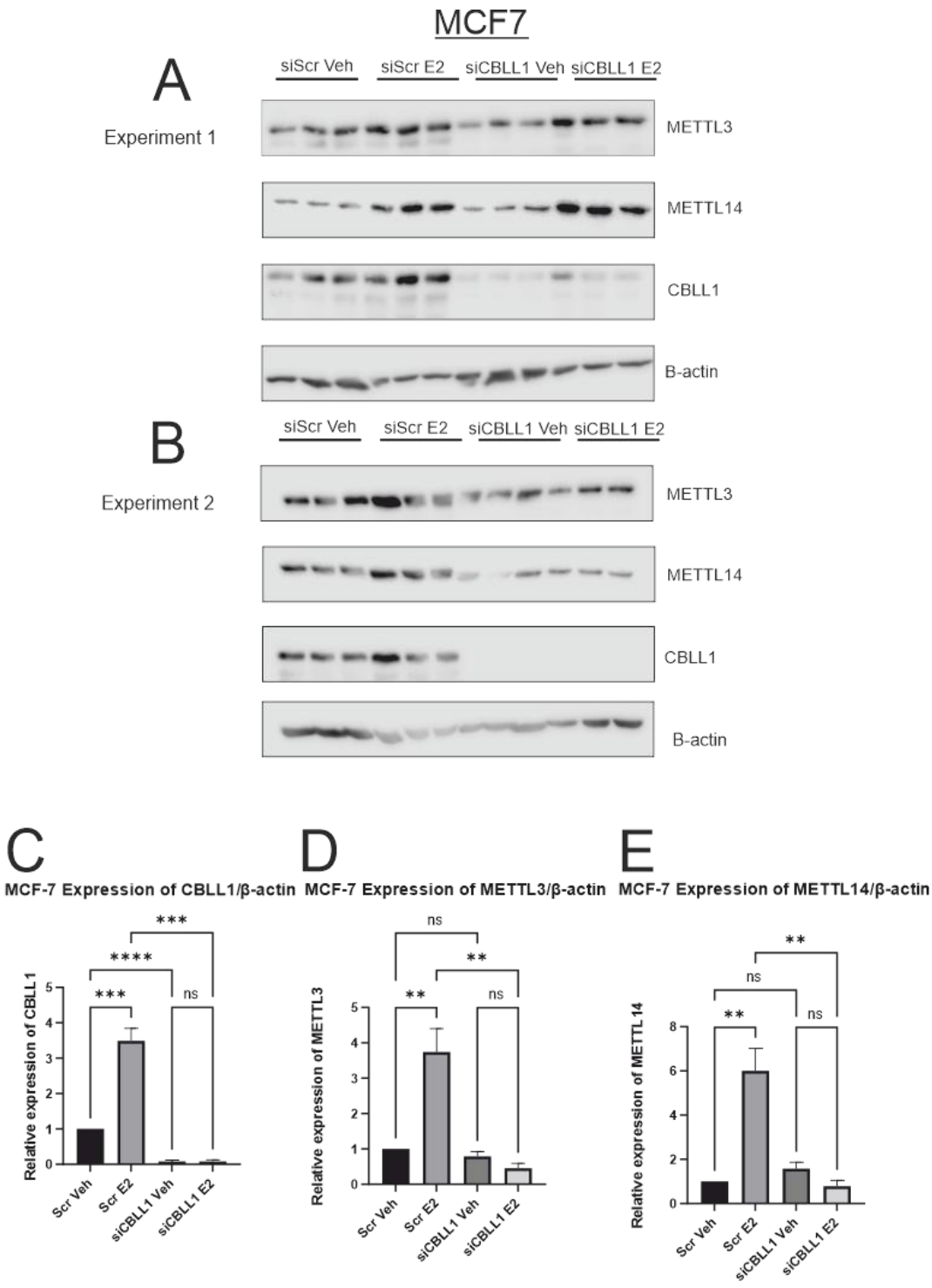


Figure 10. Representative western blots and quantification graphs to show the siRNA mediated knockdown of CBLL1 with E2 treatment.

The western blots were completed in duplicate with (**A**) as experiment 1 and (**B**) as experiment 2. All quantification graphs are relative to β-actin and are shown as representative for each protein sample siScr Veh, siScr E2, siCBLL1 Veh and siCBLL1 E2 with (**C**) expression of CBLL1/β-actin (**D**) expression of METTL3/β-actin (**E**) expression of METTL14/β-actin. For graphs * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.001$, ****$p < 0.0001$, ns = not significant.

3.2.2 siRNA-mediated depletion of CBLL1 on differential gene expression in vehicle and estrogen treated

RNA sequencing was completed to further understand the function of CBLL1 in the MCF7 breast cancer cell line. The significantly differentially expressed genes (DEGs) upon CBLL1 siRNA-mediated depletion followed with subsequent treatment of vehicle or estrogen were identified using DESeq2 and KEGG pathway analysis was carried out using WebGestalt to identify the significantly enriched KEGG pathways.

There was a number of significant DEGs identified from analysis (**Figure 11-11**). The volcano plot shows the top by fold change 10 DEGs from downregulated (blue) to upregulated (red) genes. In both volcanoes METTL3 and METTL14 have been added as genes of interest assessed further in this study, but were shown to be unchanged.

In this analysis, five downregulated genes (*HIST2H2BE, CBLL1, PPP2R1B, NTN4 and AC1444831.1*) and five upregulated DEGs (*ABHD17C, KIAA1324, GDAP1, CRISPLD1 and CPE*). were identified in vehicle treated siScr as compared to vehicle-treated CBLL1 depleted cells. No significantly enriched KEGG pathways were identified but the top 10 pathways were identified (**Figure 11**).

For estrogen-treated siScr as compared to estrogen treated CBLL1 depleted cells, downregulated DEGs were *CBLL1*, *HIST2H2BE*, *PPP2R1B*, *ZNF33B*, *RPS6KA3*, *MAL2*, *KRT81*, *ZHX1* and the upregulated genes were *MGP* and *STC2*. *METTL3* and *METTL14* are also shown to be higher in estrogen treated siScr as compared to estrogen treated CBLL1 depleted MCF-7 cells. For this condition there were not significantly enriched KEGG pathways but the top 10 KEGG pathways were identified (**Figure 12**).

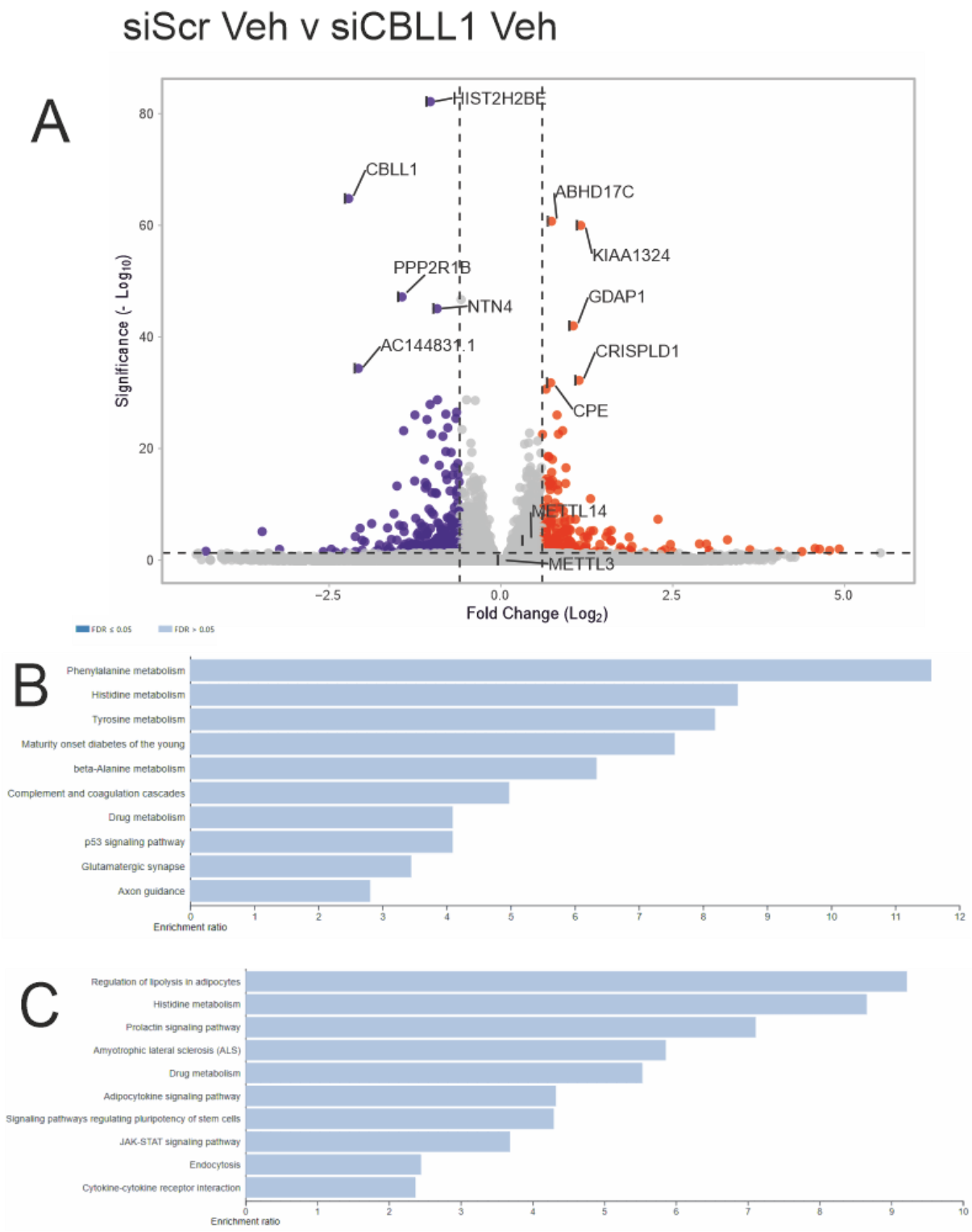


Figure 11. Differentially expressed genes from siScr Veh verses siCBLL1 Veh shown as significant represented in volcano plot and the KEGG pathway analysis.

Volcano plots representing the log2 fold change and KEGG pathway analysis of differentially expressed genes (DEGs) in vehicle treated CBLL1 depleted cells as compared with vehicle treated non-targeting control MCF-7 cells. Genes were considered significant with a fold

change (FC) of ≥1.5 or ≤-1.5 and a false discovery rate (FDR) of <0.05. (**A**) shows the volcano plot of the top 10 significant DEGs. KEGG pathway analysis is represented with (**B**) downregulated DEGs and (**C**) upregulated DEGs following the siRNA mediated knockdown of CBLL1.

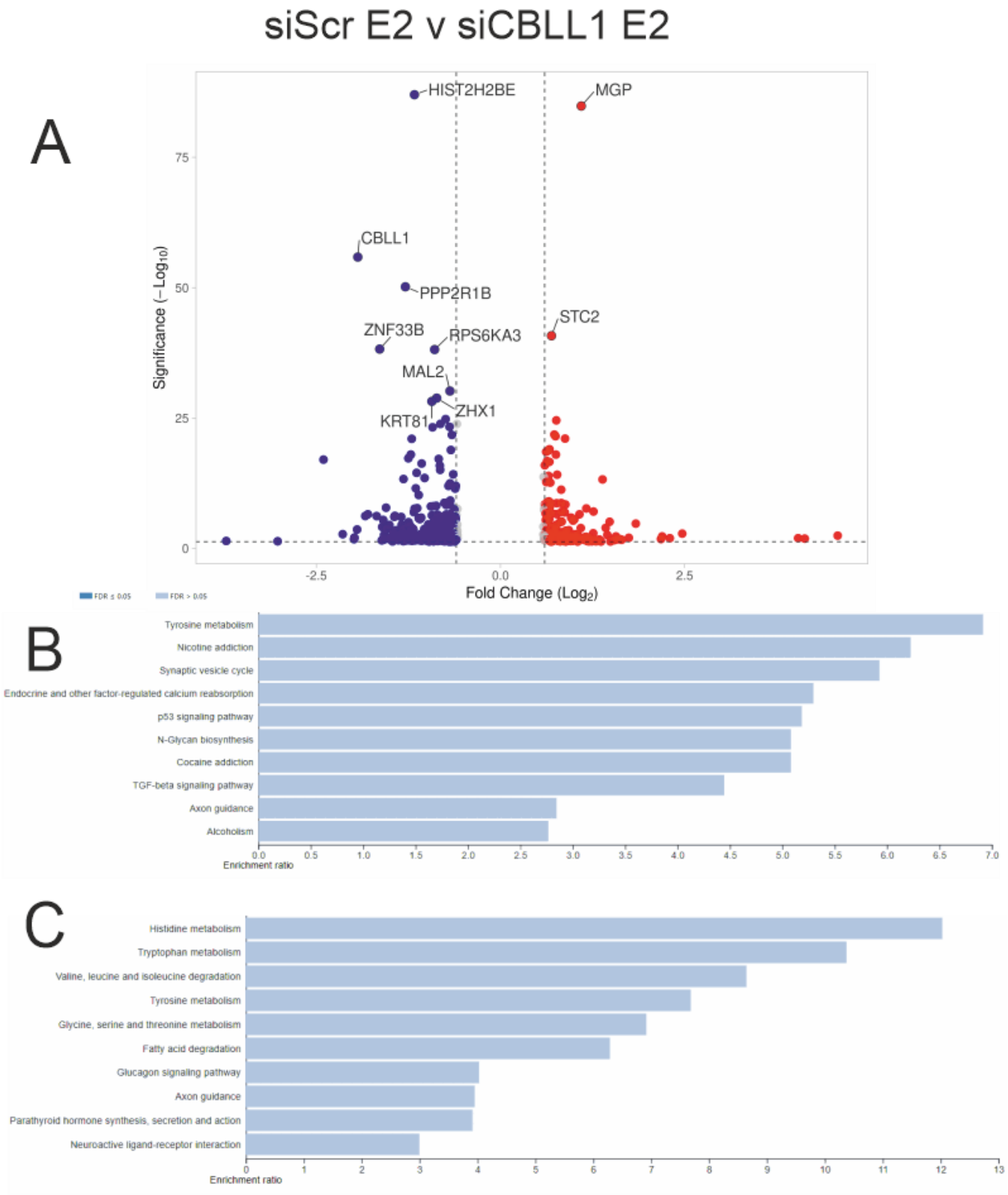


Figure 12. Differentially expressed genes from siScr E2 verses siCBLL1 E2 shown as significant represented in volcano plot and the KEGG pathway analysis.

Volcano plots representing the log2 fold change and KEGG pathway analysis of differentially expressed genes (DEGs) in vehicle treated CBLL1 depleted cells as compared with vehicle treated non-targeting control in the MCF-7 cell line. Genes were considered significant with a fold change (FC) of ≥1.5 or ≤-1.5 and a false discovery rate (FDR) of <0.05. (**A**) shows the volcano plot of the top 10 significant DEGs. KEGG pathway analysis is represented with (**B**) downregulated DEGs and (**C**) upregulated DEGs following the siRNA mediated knockdown of CBLL1.

3.2.3 The effect of CBLL1 siRNA mediated knockdown on splicing events in MCF7 with and without estrogen treatment

Preliminary alternative splicing analysis was completed by Dr. Jennifer Lothion-Roy (personal communication). To extend this study, a comprehensive pathway analysis of transcripts and genes with estrogen and CBLL1-regulated alternative splicing was completed using WebGestalt (**Figure 13-14**). Pathway analysis was completed for each pairwise comparison (i) siScr Veh vs siCBLL1 Veh and (ii) siScr E2 vs siCBLL1 E2, iii) combined splicing events, for each of the alterative splicing events (alternative 5' splice site (A5SS), retained intron (RI), skipped exon (SE), mutually exclusive exon (MXE), alternative 3' splice site (A3SS)). Pathway analysis was also completed for the combined alternative splicing events in each of the different cell line conditions.

For all alternative splicing events for the cell line condition siScr E2 verses siCBLL1 E2, there were no significantly enriched pathways we found under the FDR<0.05 threshold. For A5SS, colorectal cancer was observed as one of the top 10 pathways. For MXE alternative splicing event a number of metabolism pathways were observed which include biotin, nucleotide, purine and other metabolic pathways. From the top 10 pathways identified within the RI alternative splicing events the hedgehog signalling pathway has been shown to have an association with cancer. This pathway was also observed as one of the top 10 within the SE splicing event alongside N-glycan biosynthesis, tight junction and ribosome pathways. For the A3SS splicing event within this condition, the results observed showed proteoglycans in cancer pathway as well as spliceosome and base excision repair pathways.

For all alternative splicing events for siScr Veh verses siCBLL1 Veh cell line condition, there were no significantly enriched pathways we found under the FDR<0.05 threshold. For A5SS splicing event, the ribosome pathway was observed which has overall been found in several of the MCF7 cell line conditions and splicing events described. For MXE one of the top 10 pathways seen was choline metabolism in cancer. Furthermore, a key cancer pathway observed in the RI splicing event was p53 signalling pathway. For the SE splicing event RNA degradation was observed as well as prolactin signalling pathway (hsa04917). Lastly, for A3SS splicing event, endometrial cancer (hsa05213) was one of the top 10 pathways and was observed to have the highest enrichment ratio of 4.9132. Also, in this splicing event the key cancer pathway of transcriptional misregulation in cancer was identified.

This study then went on to investigate the combined splicing events within each of the MCF7 conditions. For combined splicing events observed within the siScrE2 verses siCBLL1E2 condition 5 significantly enriched pathways were seen. Lastly for siScr Veh verses siCBLL1 Veh

condition there were no significant results found under threshold FDR 0.05 so the top ten pathways were selected.

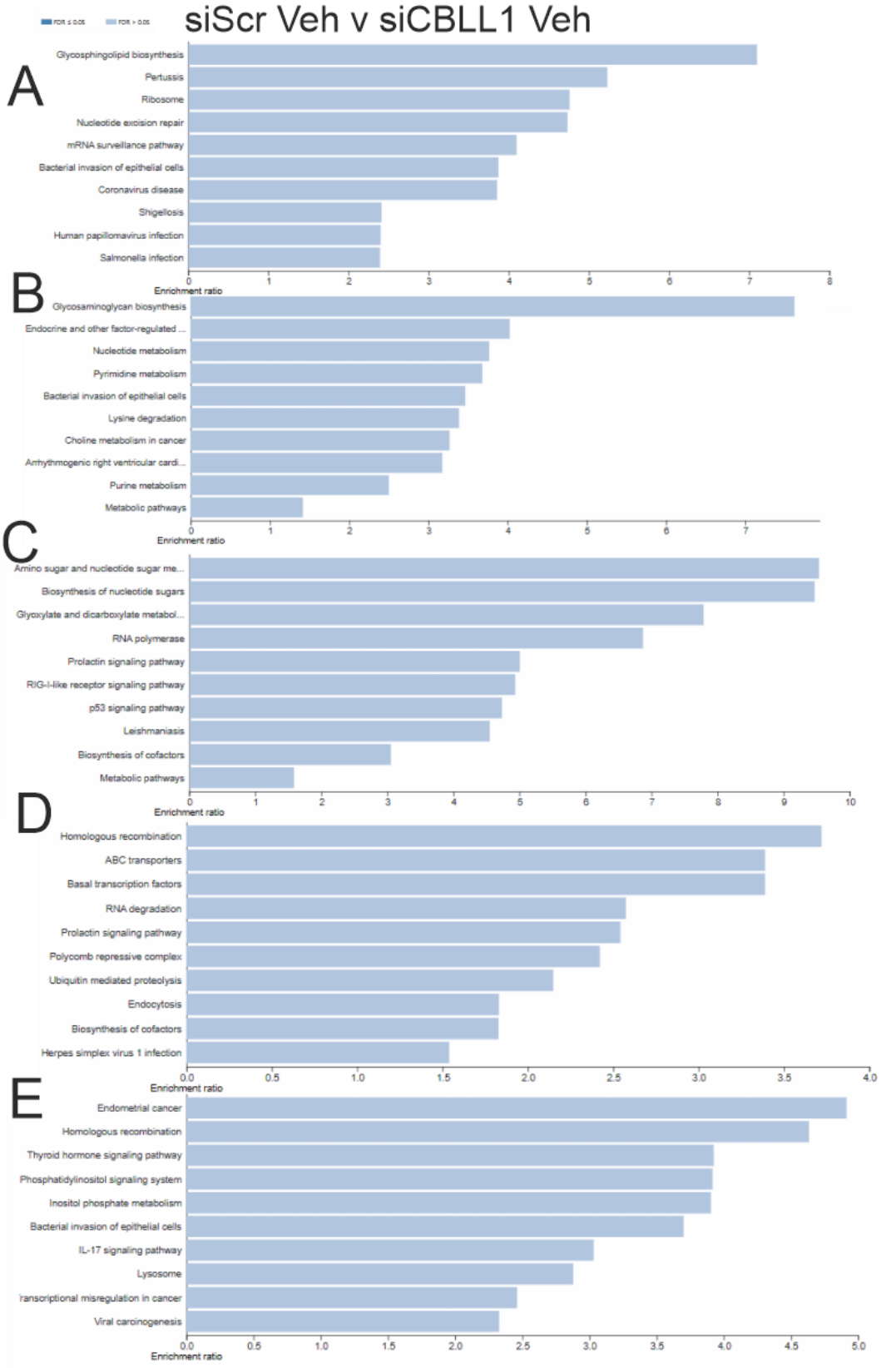


Figure 13. siScr Veh verses siCBLL1 Veh rMATs pathway analysis

Pathway analysis for condition: siScr Veh verses siCBLL1 vehin the breast cancer cell line MCF7. (**A**) alternative 5' splice site (A5SS), (**B**) retained intron (RI), (**C**) skipped exon (SE), (**D**) mutually exclusive exon (MXE), (**E**) alternative 3' splice site (A3SS).

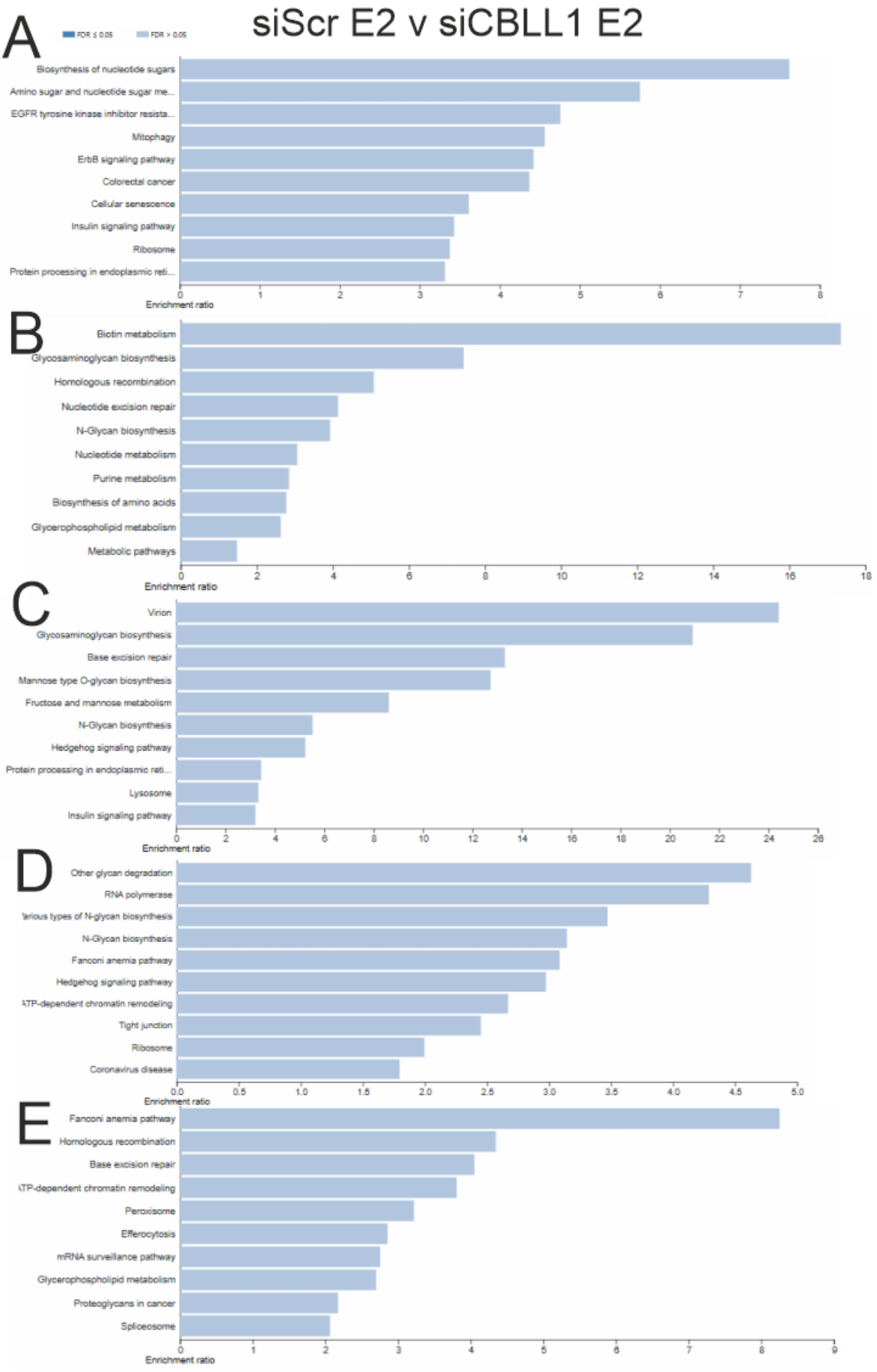

A
siScr E2 v siCBLL1 E2
FDR ≤ 0.05
FDR > 0.05
Biosynthesis of nucleotide sugars
Amino sugar and nucleotide sugar me...
EGFR tyrosine kinase inhibitor resista...
Mitophagy
ErbB signaling pathway
Colorectal cancer
Cellular senescence
Insulin signaling pathway
Ribosome
Protein processing in endoplasmic reti...
0 1 2 3 4 5 6 7 8
Enrichment ratio
B
Biotin metabolism
Glycosaminoglycan biosynthesis
Homologous recombination
Nucleotide excision repair
N-Glycan biosynthesis
Nucleotide metabolism
Purine metabolism
Biosynthesis of amino acids
Glycerophospholipid metabolism
Metabolic pathways
0 2 4 6 8 10 12 14 16 18
Enrichment ratio
C
Virion
Glycosaminoglycan biosynthesis
Base excision repair
Mannose type O-glycan biosynthesis
Fructose and mannose metabolism
N-Glycan biosynthesis
Hedgehog signaling pathway
Protein processing in endoplasmic reti...
Lysosome
Insulin signaling pathway
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Enrichment ratio
D
Other glycan degradation
RNA polymerase
arious types of N-glycan biosynthesis
N-Glycan biosynthesis
Fanconi anemia pathway
Hedgehog signaling pathway
TP-dependent chromatin remodeling
Tight junction
Ribosome
Coronavirus disease
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0
Enrichment ratio
E
Fanconi anemia pathway
Homologous recombination
Base excision repair
TP-dependent chromatin remodeling
Peroxisome
Efferocytosis
mRNA surveillance pathway
Glycerophospholipid metabolism
Proteoglycans in cancer
Spliceosome
0 1 2 3 4 5 6 7 8 9
Enrichment ratio

Figure 14. siScr E2 verses siCBLL1 E2 rMAT pathway analysis

Pathway analysis from different alternative splicing events within siScr E2 verses siCBLL1 E2 condition in the breast cancer cell line MCF7. (**A**) alternative 5' splice site (A5SS), (**B**) retained intron (RI), (**C**) skipped exon (SE), (**D**) mutually exclusive exon (MXE), (**E**) alternative 3' splice site (A3SS).

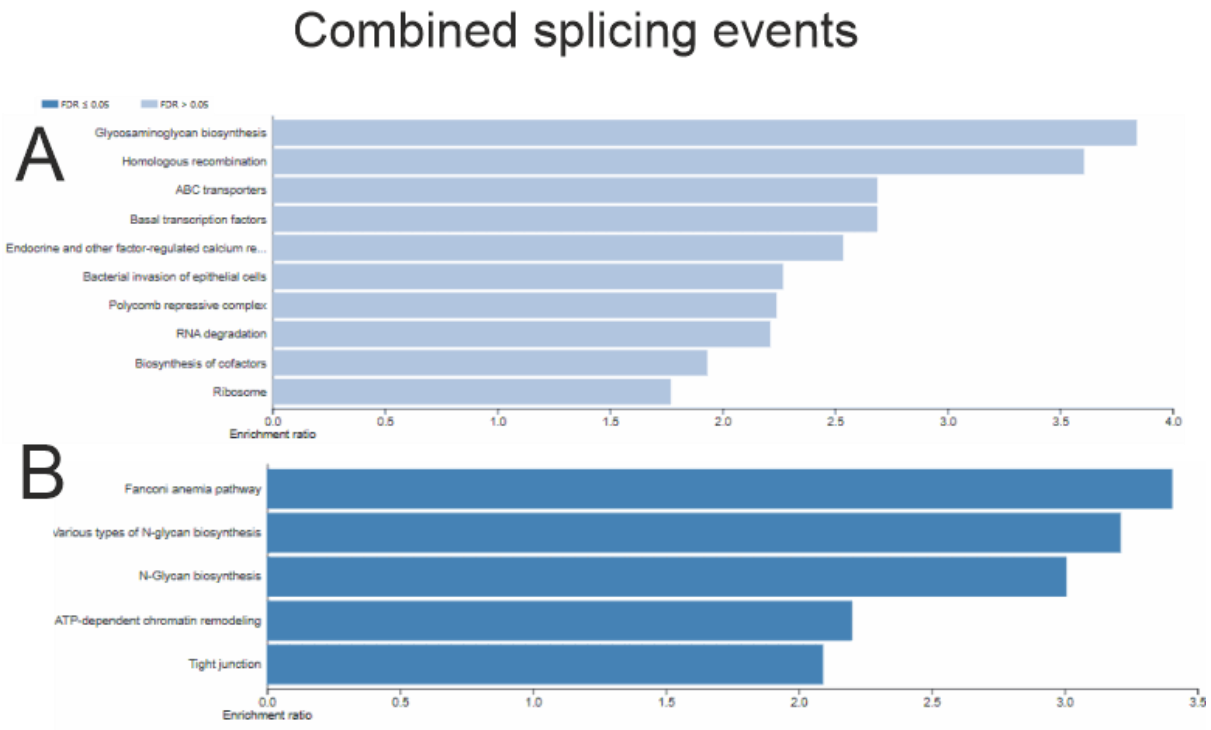


Figure 15. rMATs pathway analysis of all combined MCF7 conditions

Pathway analysis for combined alternative events including alternative 5' splice site (A5SS), retained intron (RI), skipped exon (SE), mutually exclusive exon (MXE), alternative 3' splice site (A3SS)) for each condition in the breast cancer cell line MCF7. (**A**) siScrVeh verses siCBLL1Veh. (**B**) siScrE2 verses siCBLL1E2

3.3 The functional role of CBLL1 in breast cancer cell line MDA-MB-231

3.3.1 siRNA mediated depletion of CBLL1 in MDA-MB-231

To investigate the role of CBLL1 in TNBC, the MDA-MB-231 TNBC cell line was used to complete an siRNA mediated knockdown of CBLL1. Successful CBLL1 knockdown was confirmed by western blot (**Figure 16**). The quantification further supported this as the relative expression of CBLL1 when comparing the siScr verses siCBLL1 was shown to be significant. METTL3 and METTL14 expression were unaffected by CBLL1 knockdown.

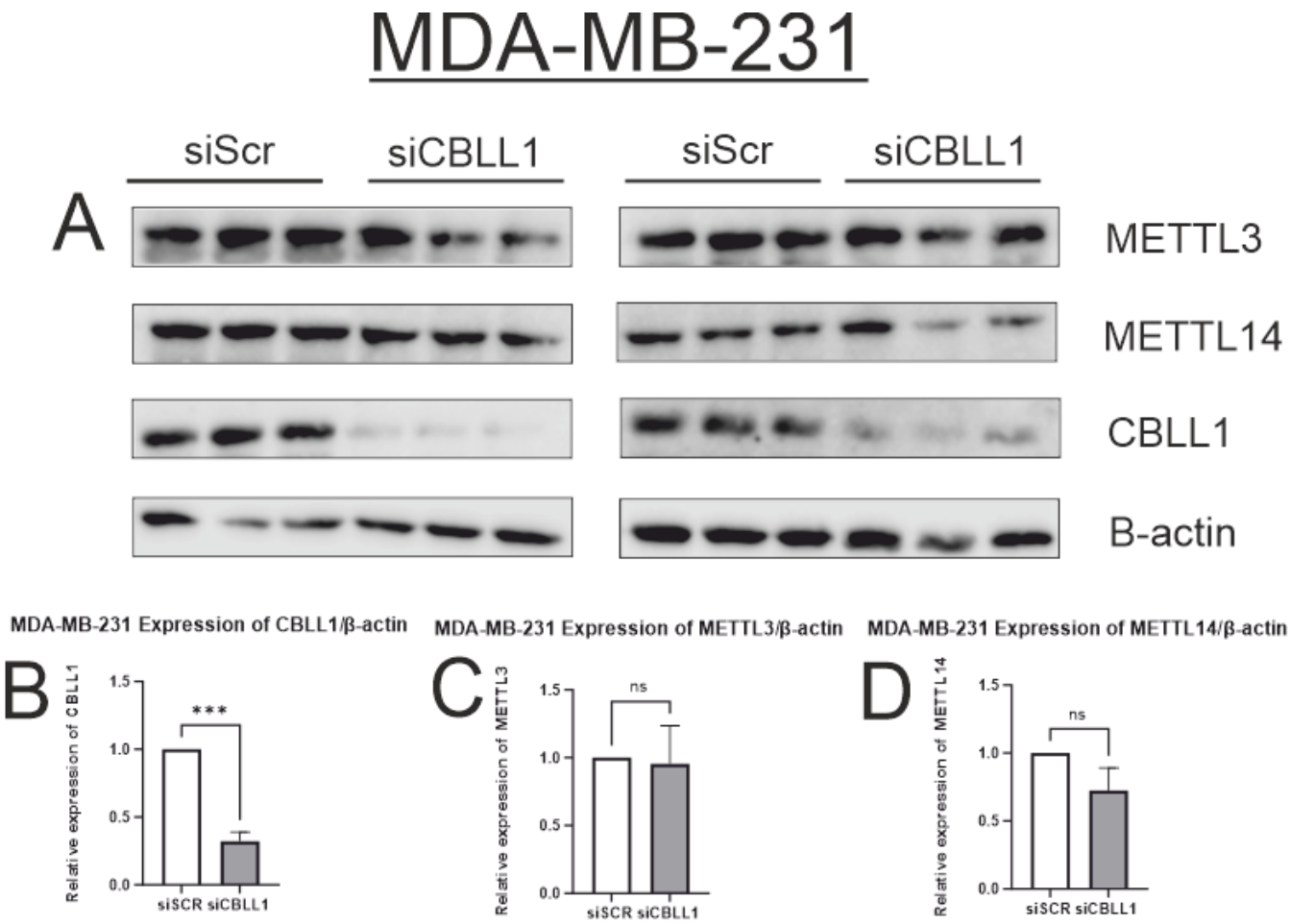


Figure 16. siRNA-mediated knockdown of CBLL1 in the triple negative breast cancer cell line MDA-MB-231.

(**A**) siRNA mediated knockdown of CBLL1 was completed in duplicate with experiment 1 and experiment 2 displayed side by side. After completion, the quantification of the western blot was carried out with (**B**) expression of CBLL1/β-actin, (**C**) expression of METTL3/β-actin and (**D**) expression of METTL14/β-actin shown in graphs with significance. For graphs * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.001$, ****$p < 0.0001$, ns = not significant.

3.3.2 The effect of siRNA-mediated depletion of CBLL1 on differential gene expression

RNA sequencing was completed to further understand the function of CBLL1 in the breast cancer cell line MDA-MB-231. The DEGs were identified upon CBLL1 siRNA-mediated knockdown analysis.

A volcano plot (**Figure 17A**) showing the significant DEGs, with *NORAD, MT2A, CBLL1, MT1E, CHML* and *PURA* shown as downregulated with the siRNA mediated knockdown of CBLL1, whereas expression of *IFIT2, AGR2 and ITGB2* have been shown to be upregulated in the siRNA

mediated knockdown of CBLL1 condition in MDA-MB-231 RNA-seq.

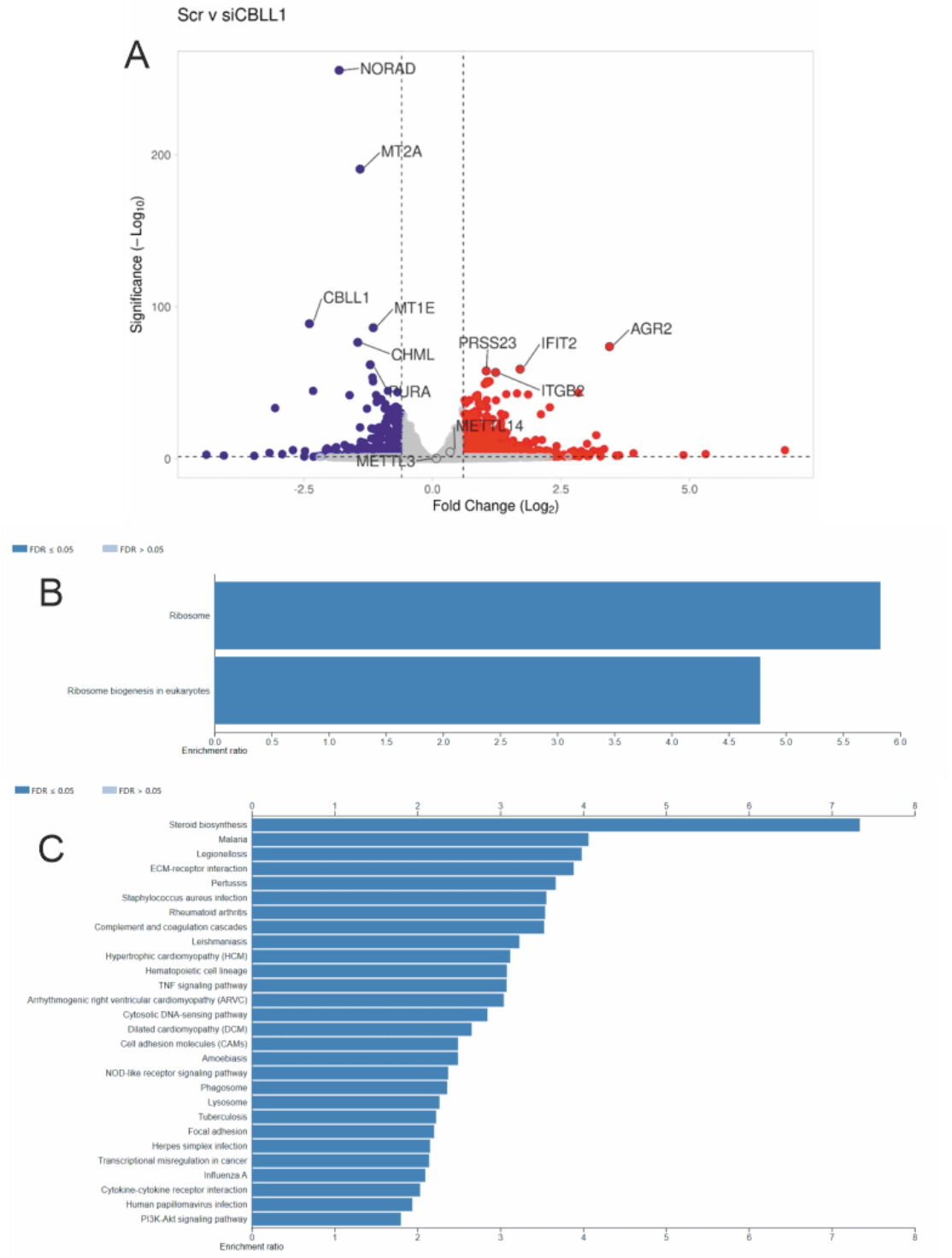


Figure 17. RNA seq analysis from the siRNA mediated knockdown of CBLL1 in MDA-MB-231

(**A**) volcano plot representation of the effect of siRNA mediated knockdown of CBLL1 with the significantly differentially expressed genes. Significant differentially expressed genes (DEGs) were identified and KEGG pathways analysis was completed, (**B**) shows the significant KEGG pathways from the downregulated DEGs and (**C**) upregulated DEGs following the siRNA mediated knockdown of CBLL1.

3.3.3 The effect of CBLL1 siRNA mediated knockdown on splicing events in MDA-MB-231

For alternative 5' splice sites (A5SS) there were no significant KEGG pathways (FDR≤0.05), but the top 10 enriched pathways included ribosome (hsa03010), basal transcription factors (hsa03022), base excision repair (hsa03410), glucagon signalling pathway (hsa04922), protein processing in endoplasmic reticulum (hsa04141), insulin signalling pathway (hsa04910), terpenoid backbone biosynthesis (hsa00900), proteosome (hsa03050), folate biosynthesis (hsa00790), phosphatidylinositol signalling system (hsa04070).

For retained introns (RI) there was one significantly enriched KEGG pathway which was spliceosome (hsa03040). For skipped exon (SE) there were 5 significantly enriched KEGG pathway of which are the NOD-like receptor signalling pathway (hsa04621), Fanconi anemia pathway (hsa03460), glycerolipid metabolism (hsa00561), other glycan degradation (hsa00511) and RNA degradation (hsa03018).

For mutually exclusive exons (MXE) and A3SS, the top 10 pathways were identified. In the MXE splicing event, the top 10 pathways were identified as hippo signalling pathway (hsa04392), shigellosis (hsa05131), metabolic pathways (hsa01100), epithelial cell signalling in Helicobacter pylori infection (hsa05120), AMPK signalling pathway (hsa04152), endocrine resistance (hsa01522), ferroptosis (hsa04216), ubiquitin mediated proteolysis (hsa04120), central carbon metabolism in cancer (hsa05230) and RNA transport (hsa03013). A3SS shows the 10 top pathways of ubiquitin mediated proteolysis (hsa04120), thyroid cancer (hsa05216), one carbon pool by folate (hsa00670), amyotrophic lateral sclerosis (ALS) (hsa05014), RNA transport (hsa03013), Parkinson disease (hsa05012), fanconi anemia pathway (hsa03460), base excision repair (hsa03410), basal cell carcinoma (hsa05217) and mitophagy (hsa04137).

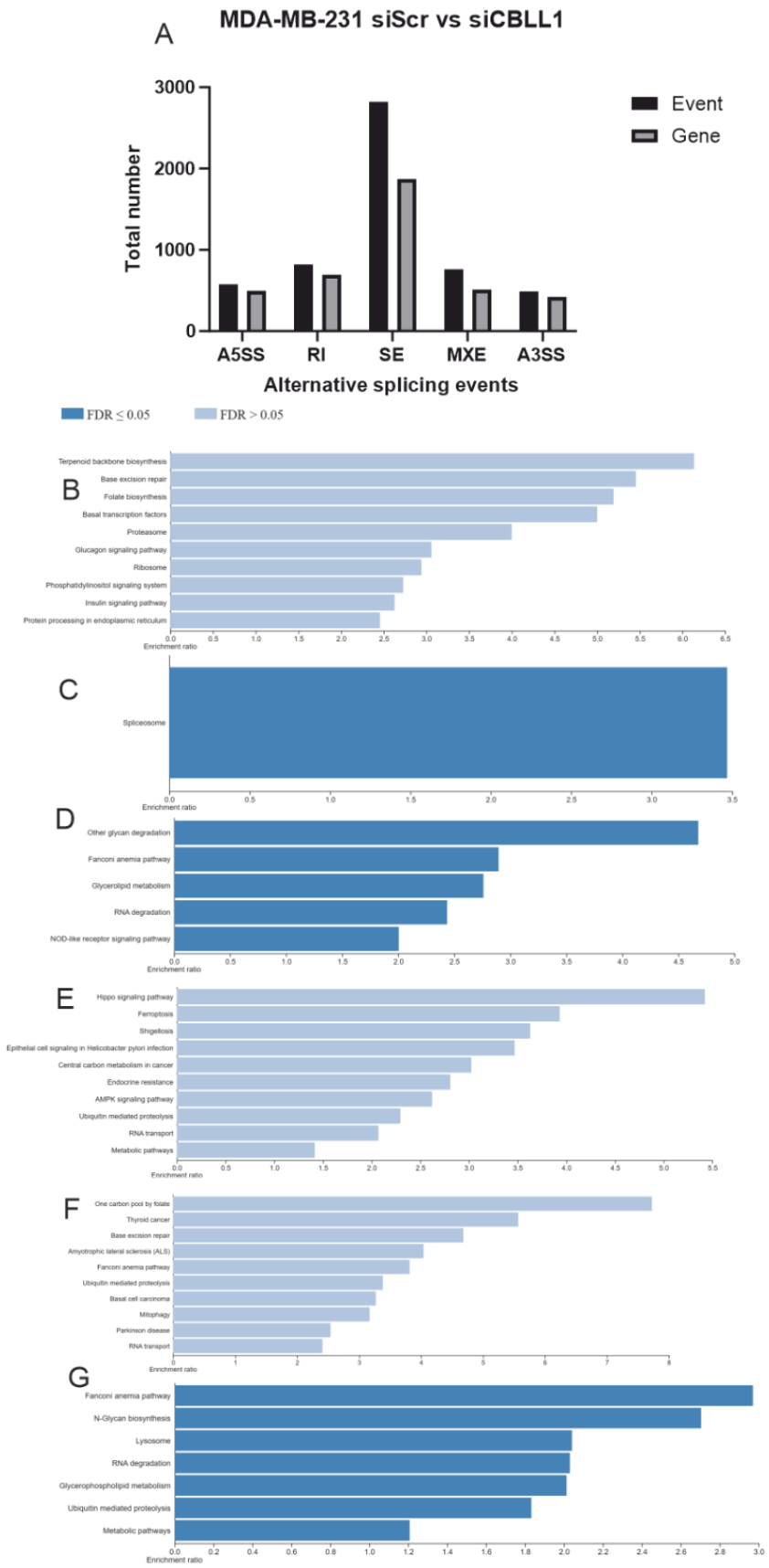


Figure 18. Alternative splicing in cell line MDA-MB-231 after the siRNA mediated CBLL1 knockdown

RNA-seq was carried out to determine the alternative splicing events following siRNA mediated CBLL1 knockdown in the cell line MDA-MB-231. To be significant the differential splicing events were difference in percentage spliced in (dPSI) ≥5% and FDR <0.05. (**A**) For each alterative splicing event the differentially spliced transcript and differentially spliced gene numbers were plotted. (**B**) Enriched KEGG pathways in the differentially spliced gene set of siRNA mediated CBLL1 knockdown in MDA-MB-231 in the alternative splicing events: A5SS (**C**) RI, (**D**) SE (**E**) MXE (**F**) A3SS and (**G**) all combined genes.

3.3.4 Transcriptomic analysis of CBLL1 in the TCGA dataset

To determine the clinical significance of CBLL1, RNA-seq data obtained from TGCA-BRCA primary BCa cohort (n=1076) was dichotomised on the basis of low as compared to high *CBLL1* expression. To do this, normalized expression counts were calculated and quartiles determine and patients dichotomised into low (Q1) versus high (Q4) CBLL1 expression. Differential gene expression analysis between the two quartiles was performed using DESeq2.

This analysis revealed 4002 significantly differentially expressed genes identified to be decreased in patients with increased CBLL1 expression. Of these genes no significantly enriched KEGG pathways were found under the threshold FDR 0.05. However, the top 10 pathways FDR > 0.05 were observed (**Figure 19**) which includes pentose and glucoronate interconversions, ascorbate and aldarate metabolism, homologous recombination, nicotine addition, taste transduction, cell cycle, nucleocytoplasmic transport, chemical carcinogenesis, Herpes simplex virus 1 infection and neuroactive ligand-receptor interaction.

Increased in Q4 were 7061 significantly differentially expressed genes. After analysis using WebGesalt there were 32 significant KEGG pathways of which key cancer pathways included, cytokine-cytokine receptor interaction, chemokine signalling pathways and phagosome as (**Figure 19**).

As shown in the volcano plot there are a number of genes which are upregulated which includes *CBLL1, THAP5, TMEM209, ZNF800, WASL, COG5, CNOT4, SRPK2, PNPLA8* and *AL035425.4* (**Figure 19**).

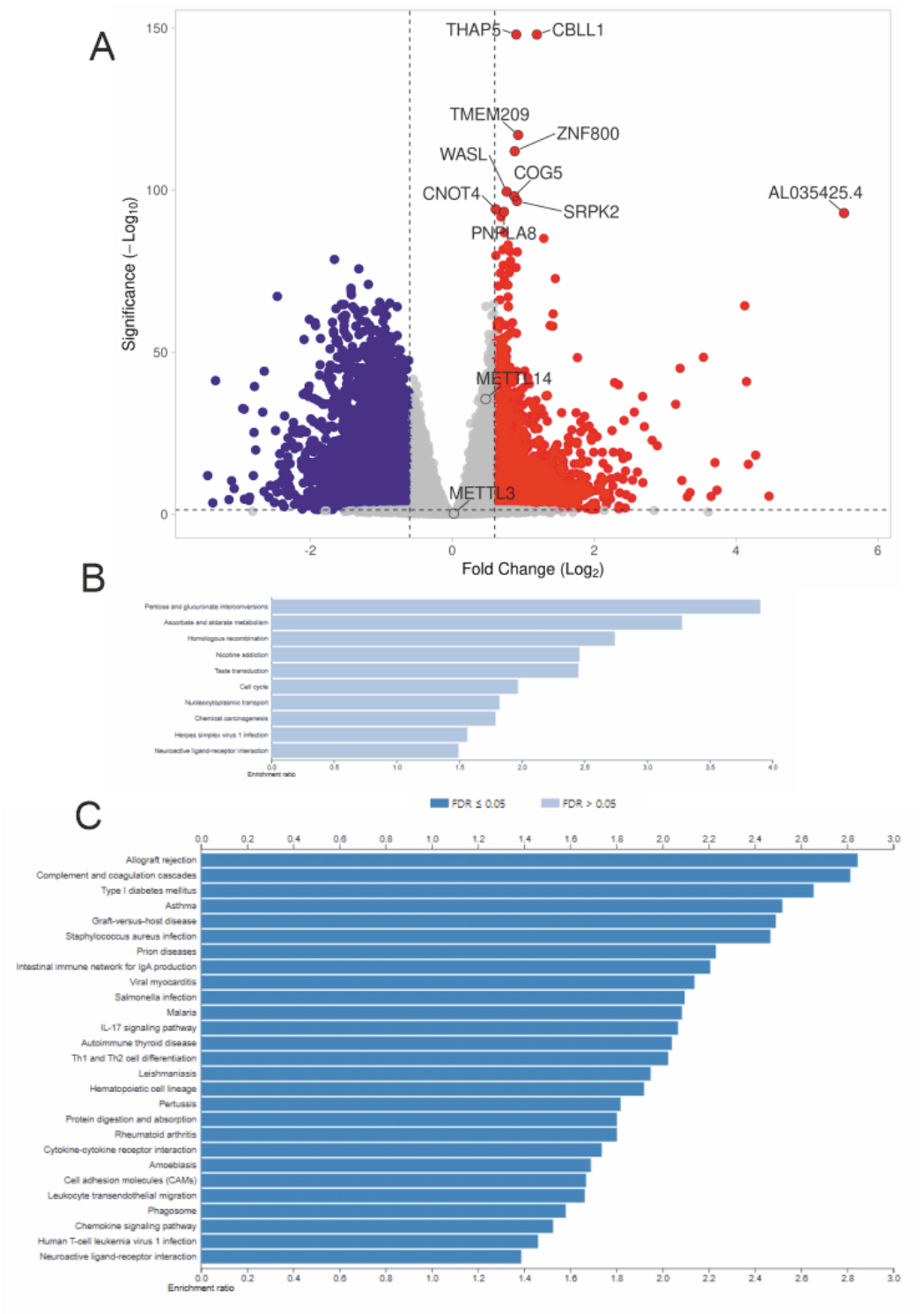


Figure 19. Differential gene expression between high and low *CBLL1* expression in BCa specimens.

The differential gene expression of *CBLL1* is quartile 1 (Q1) where there is low expression of *CBLL1* and quartile 4 (Q4) where there is high expression of *CBLL1* (**A**) Volcano plot showing the DEGs with Q1 (blue) and Q4 (red) of *CBLL1* expression. (**B**) Enriched KEGG pathways from significant DEGs that were increased in Q1 are lower with *CBLL1* expression (**C**) and significant DEGs increased in Q4 which are higher with *CBLL1* expression.

3.3.5 siRNA-mediated depletion of CBLL1&METTL3 and CBLL1&METTL14

To further the understanding the function of CBLL1 association in the methyltransferase complex, simultaneous siRNA mediated knockdown of siCBLL1 and siMETTL3 together and siCBLL1 and siMETTL14 together was completed. The results show a successful siRNA mediated knockdown of CBLL1, METTL3 and METTL14 as intended. Interestingly knockdown of METTL3 resulted in significant reduction in METTL14 expression, and conversely knockdown of METTL14 also resulted in significant knockdown of METTL3, and this occurred whether CBLL1 was present (**Figure 20**).

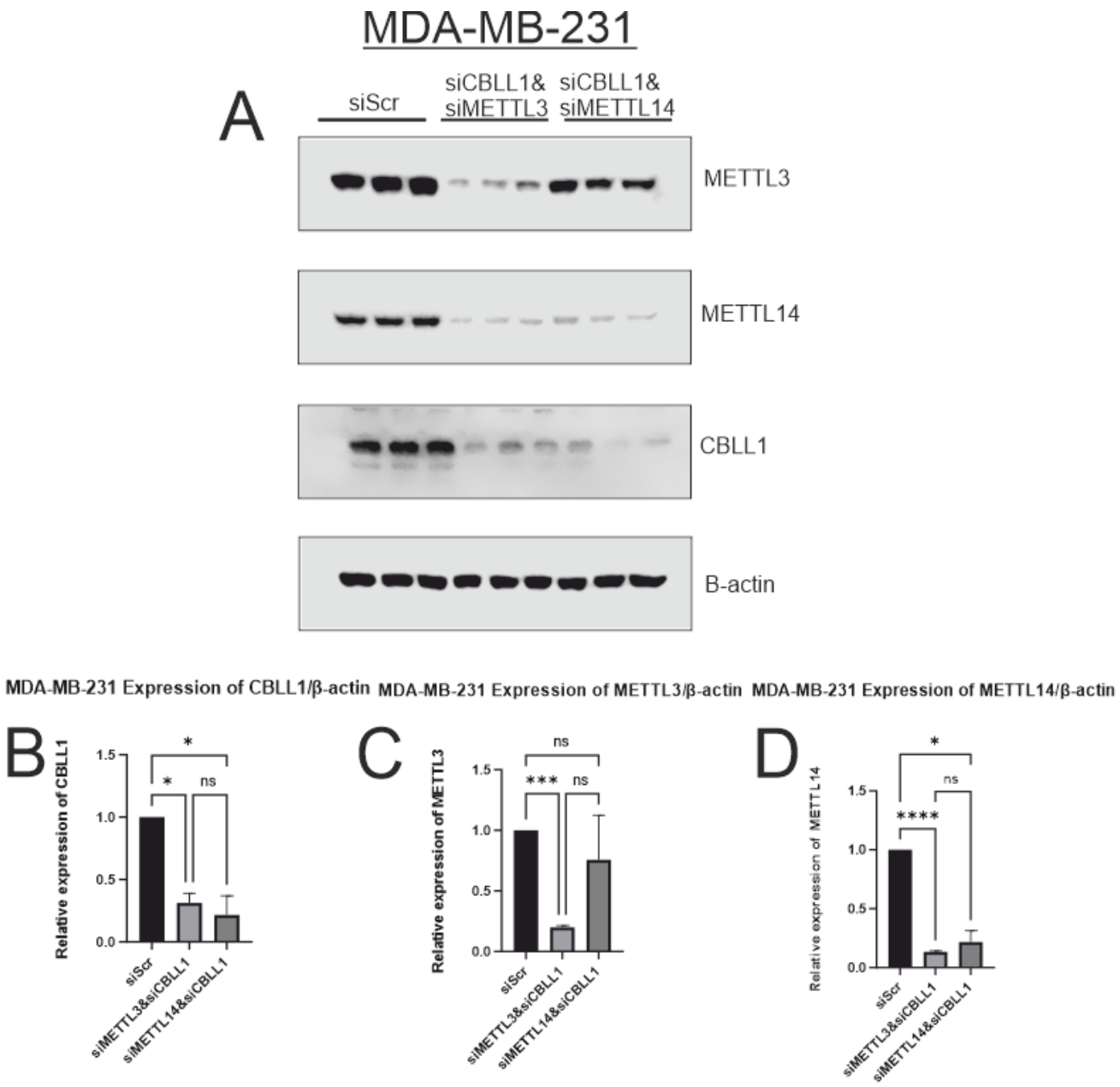


Figure 20. The siRNA-mediated depletions of siCBLL1 & siMETTL3 and siCBLL1 & siMETTL14 in the breast cancer cell line MDA-MB-231

Completed in breast cancer cell line MDA-MB-231, METTL3, METTL14 and CBLL1 protein expression was analysed by western blot which was then analysed relative to β-actin. (**A**) western blot showing the siScr, siCBLL1&siMETTL3 and siCBLL1&siMETTL14 siRNA mediated knockdowns. After completion of the western blot, quantification was carried out with (**B**) the expression of CBLL1/ β-actin, (**C**) the expression of METTL3/ β-actin, (**D**) the expression of METTL14/ β-actin shown in the graphs. For graphs * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.001$, ****$p < 0.0001$, ns = not significant.

3.3.6 siRNA-mediated depletion of METTL3, CBLL1&METTL3, METTL14 and CBLL1&METTL14

To test whether the CBLL1 ubiquitin ligase component of the methylation complex plays a role in the observed mutual regulation of METTL3 and METTL14, combinatorial knockdowns of CBLL1 and METTL3, and CBLL1 and METTL14 were performed, and the effect of METTL3 and METTL14 expression determined using western blot (**Figure 21**).

The results obtained confirmed CBLL1, METTL3 and METTL4 were successfully knocked down by siRNA-mediated depletion in breast cancer cell line MDA-MB-231. However, knockdown of CBLL1 had no significant effect on METTL3 or METTL14 expression (**Figure 21**).

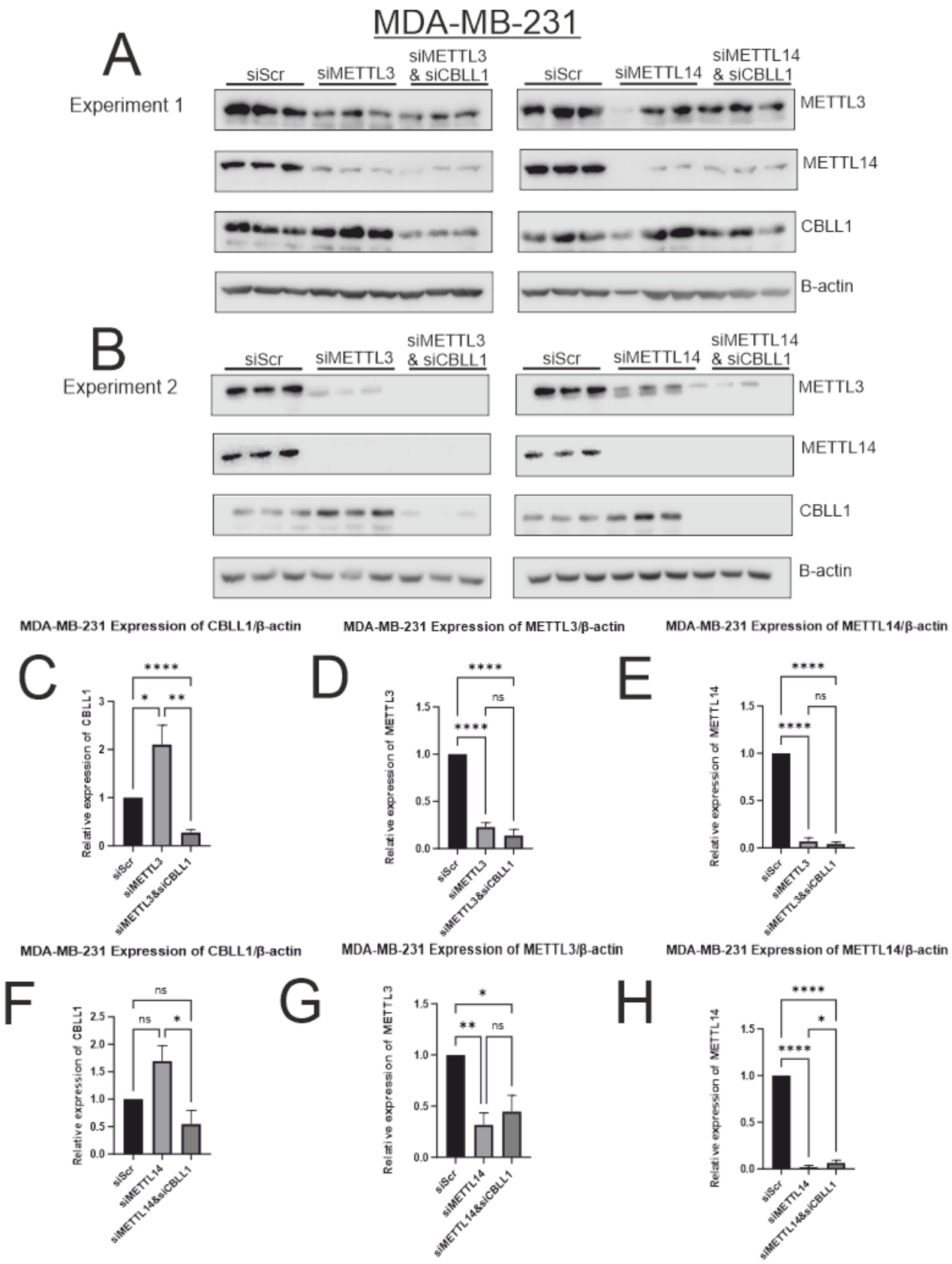


Figure 21. The siRNA mediated knockdowns of siMETTL3, siCBLL1 & siMETTL3, siMETTL14 and siCBLL1 & siMETTL14

After siRNA mediated knockdown, expression of siMETTL3, siMETTL3&CBLL1, siMETTL14 and siMETTL14&CBLL1 were analysed using western blot. Both repeats are shown with (**A**) experiment 1 and (**B**) experiment 2. Western blot analysis and quantification was carried out with the combined results from the analysis of the two independent (experiment 1 (A) and experiment 2 (B)) experiments to produce the graphs with combinatorial knockdown of CBLL1 (**C, F**), METTL3 (**D, G**) and METTL14 (**E, H**). * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.001$, ****$p < 0.0001$, ns = not significant.

3.4 Comparison of the siRNA mediated knockdown of CBLL1 in MDA-MB-231 verses MCF7

Next, the effect of CBLL1 knockdown in MCF-7 (luminal A) and MDA-MB-231 (TNBC) cells was compared using RNAseq. Venn diagrams were created (Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/) to identify downregulated and upregulated DEGs from MDA-MB-231 siRNA mediated knockdown of CBLL1 in comparison with MCF-7 siRNA mediated knockdown of CBLL1 with estrogen (E2) treatment and without (Veh). CBLL1 regulated 67 downregulated (**Figure 22A)** and 30 upregulated DEGs (**Figure 22B)** commonly in MCF-7 and MDA-MB 231 following the siRNA mediated knockdown of CBLL1**.** In **Figure 22C** there were 234 for MCF-7 and 428 for MDA-MB-231 with 46 shared. In **Figure 22D** there were 167 for MCF-7 and 942 for MDA-MB-231 with 48 shared genes.

CBLL1 regulated alternatively spliced genes were identified using Venn diagrams (**Figure 23)**. The results for all three comparisons of the CBLL1 knockdown in MDA-MB-231 verses MCF7 without estrogen and estrogen treated MCF7 revealed 208 spliced genes common to all conditions. These 208 spliced genes found in common have then undergone manual analysis using PubMed, to identify if any had an association with RNA:m^6A. Of these 208, 38 genes, were found to have been previously associated with RNA:m^6A (**Table 3**) (**Figure 23**).

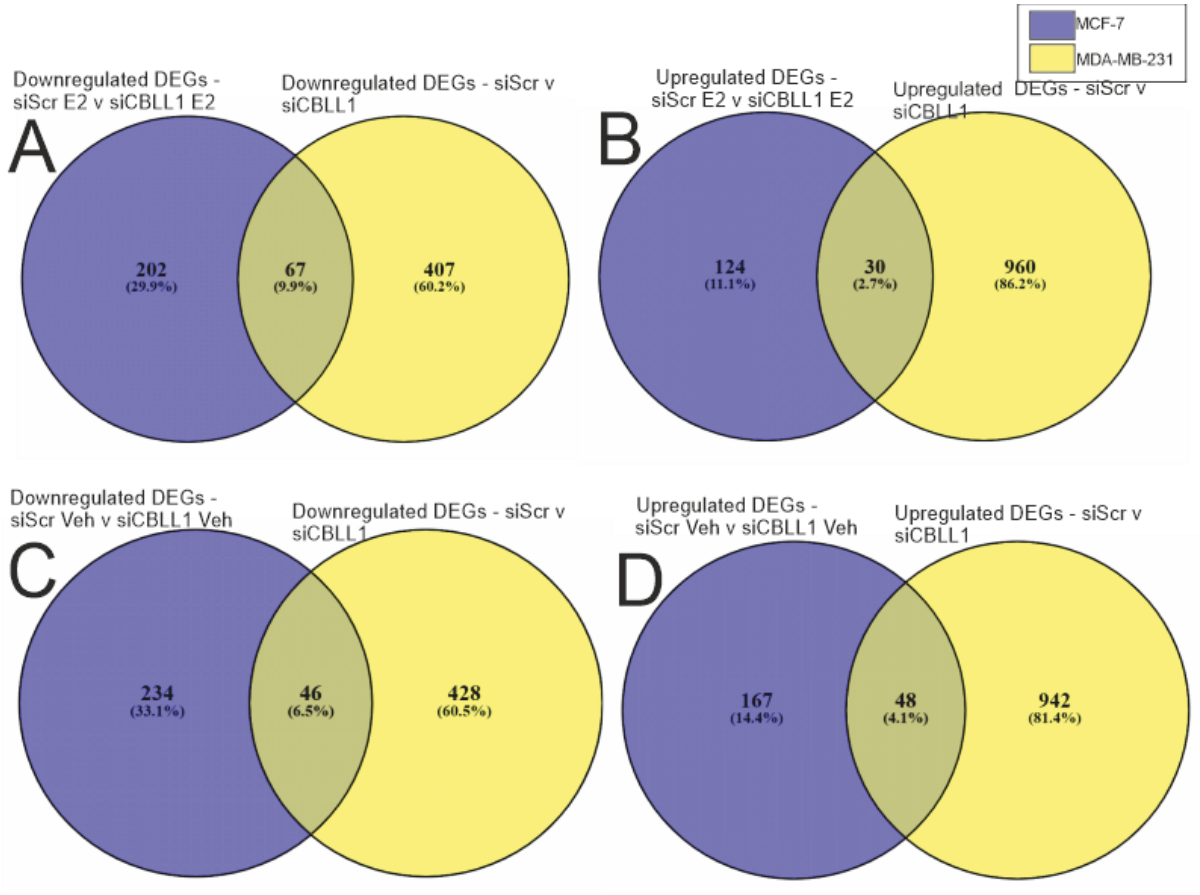


Figure 22. Downregulated and upregulated DEGs associated with the MCF7 siCBLL1 knockdown with estrogen treatment as compared with the MDA-MB-231 siCBLL1 knockdown.

Venn Diagrams show the downregulated and upregulated significantly differentially expressed genes (DEGs) following siRNA mediated knockdown of CBLL1 expression and in vehicle and estrogen treated MCF-7 cells and in vehicle treated MDA-MB-231 cells. Comparison of these cell lines with MCF-7 in purple and MDA-MB-231 in yellow consists of (**A**) MCF7 downregulated DEGs – siScr E2 v siCBLL1 E2 compared to MDA-MB-231 downregulated DEGs – siScr v siCBLL1, (**B**) MCF7 upregulated DEGs – siScr E2 v siCBLL1 E2 compared to MDA-MB-231 upregulated DEGs – siScr v siCBLL1, (**C**) MCF7 downregulated DEGs – siScr Veh v siCBLL1 Veh compared to MDA-MB-231 downregulated DEGs – siScr v siCBLL1, (**D**) MCF7 upregulated DEGs – siScr Veh v siCBLL1 Veh compared to MDA-MB-231 upregulated DEGs – siScr v siCBLL1.

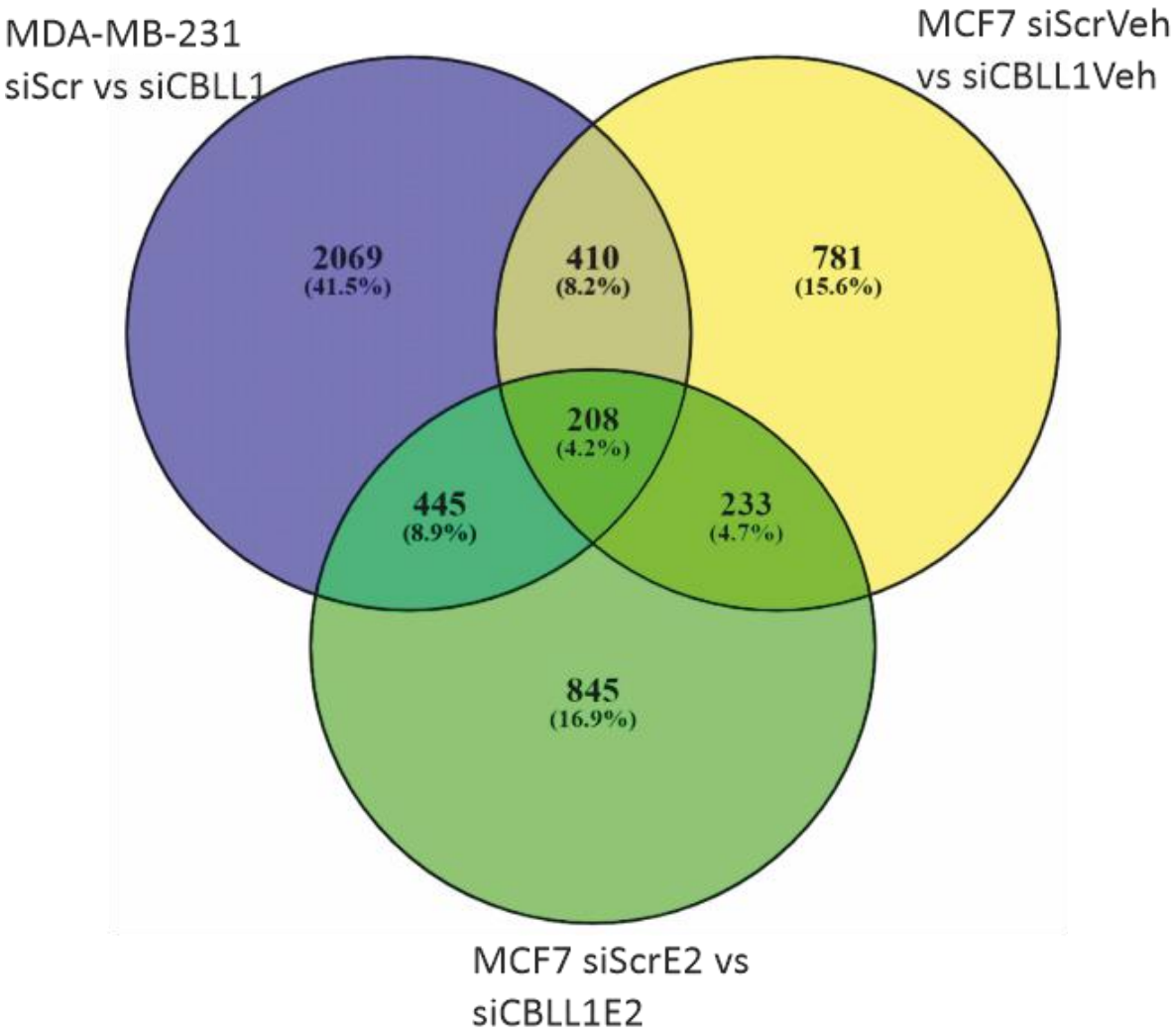


Figure 23. Comparison of rMATs in MDA-MB-231 siCBLL1 knockdown and MCF7 siCBLL1 knockdown with and without estrogen treatment.

Combined alternative splicing events were compared for each condition, MDA-MB-231 siScr verses siCBLL1, MCF7 siScr Veh verses siCBLL1 Veh and MCF7 siScr E2 verses siCBLL1 E2.

Table 3. Genes associated with RNA:m^{6}A that are shown to be common with the CBLL1 knockdown in MDA-MB-231 verses MCF7 without estrogen treatment verses MCF7 with estrogen treatment.

Name of Gene	Reference
TAF15	(Zheng et al., 2023b)
EEF1D	(Tong et al., 2023)
ZDHHC16	(Liu et al., 2024e)
OCIAD1	(De Marchi et al., 2016)
HNRNPH1	(Yan et al., 2024)
LAMA5	(Gu et al., 2021)
LRRC75A-AS1	(Sui et al., 2024)
EWSR1	(Xu et al., 2023a)
MOK	(Myint et al., 2022)

CPSF1	(Xia et al., 2022)
GBAP1	(Liu et al., 2023)
USP10	(Shi et al., 2023)
C1QTNF6	(Chu et al., 2022)
EIF4G1	(Wang et al., 2021a)
RHPN1	(Cui, 2022)
PFKL	(Zhou et al., 2022)
THRAP3	(Chen et al., 2023a)
LARP7	(Warda et al., 2017)
FANCD2	(Kui et al., 2022)
PHF3	(Appel et al., 2023)
SLC38A1	(Liu et al., 2024a)
TROAP	(Li et al., 2022d)
MAPKAPK5	(Tao et al., 2022)
MPDZ	(Chen et al., 2024)
GALNT6	(Zou et al., 2022)
RAD54B	(Ma et al., 2022)
NRCAM	(Wang et al., 2024)
ATRX	(Yu et al., 2021)
CERS4	(Wu et al., 2021)
ANXA2	(Miao et al., 2023)
CCDC18	(Junli et al., 2024)
CCDC18-AS1	(Junli et al., 2024)
UBA1	(Wang et al., 2023b)
PVT1	(Bao et al., 2024)
DISC1	(Tu et al., 2023)
HNRNPA1	(Xie et al., 2024)
RAB5A	(Shen et al., 2023)
CTNNB1	(Yang et al., 2023a)

3.5 The effect of the STM2457 METTL3 inhibitor on MDA-MB-436 BCa cell proliferation

CyQUANT™ Direct Cell proliferation assays were performed on the breast cancer cell line MDA-MB-436 to assess the effect on cellular proliferation of increasing concentrations (0µm, 10µm, 20µm, 30µm and 40µm) of the STM2457 METTL3 inhibitor as previously described (Haigh et al., 2022, Yankova et al., 2021).

This experiment was repeated, as technical replicates, three times (**Figure 24**). The results show that when n=9 there is a significant reduction in proliferation as the concentration of STM2457 increases. At 40µm STM2457 concentration the MDA-MB-436 cells were observed under a light microscope to have the majority of cells having undergone apoptosis.

After completing the experiments, the data was collated into a graph using GraphPad Prism. When the experiment data from the three experiments was utilised to create **Figure 24A** all STM2457 concentrations were significant as compared to the vehicle.

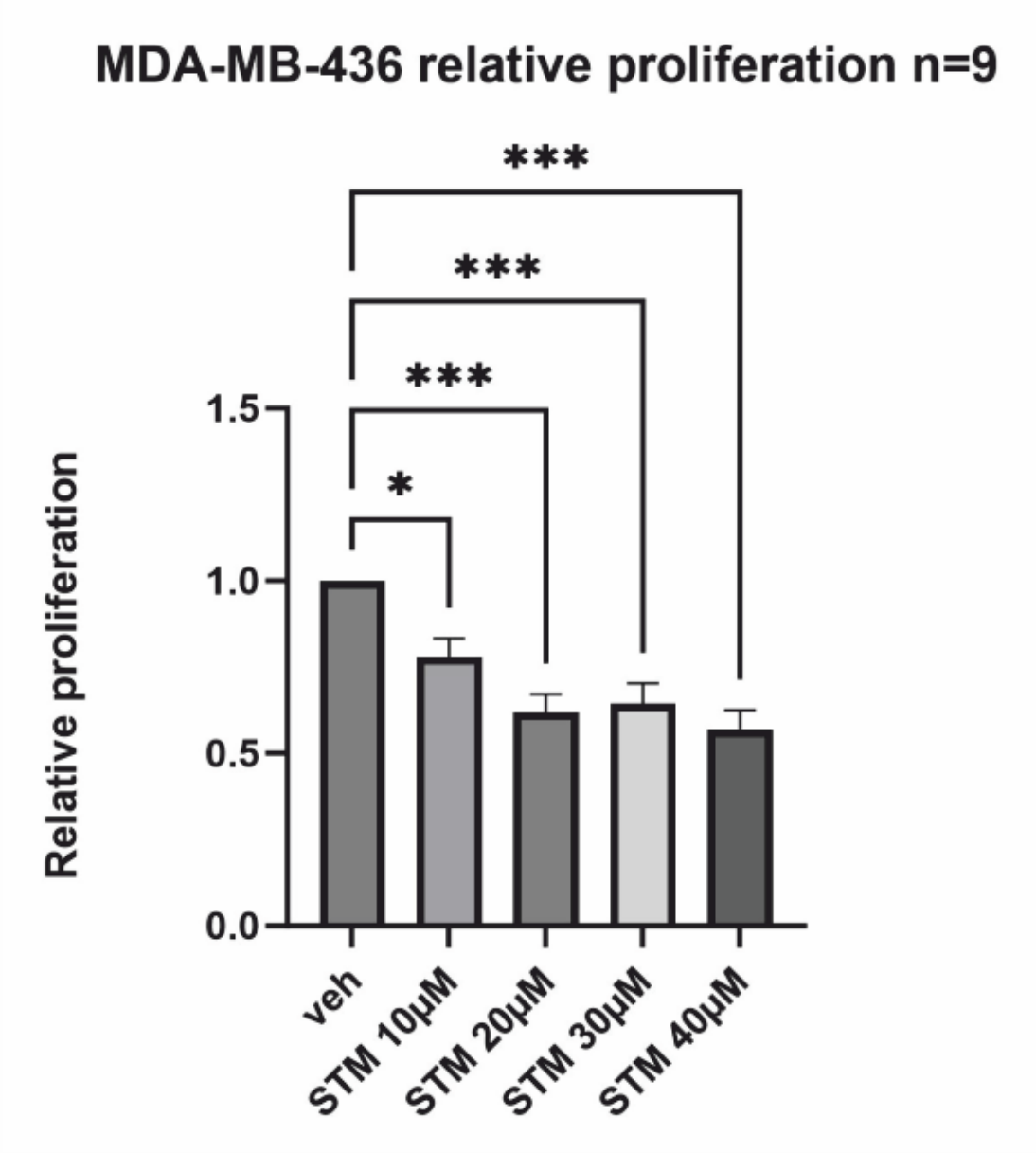


Figure 24. Proliferation assays using STM2457 on the breast cancer cell line MDA-MB-436

Proliferation assay of STM2457 treatment was completed in the TNBC cell line MDA-MB-436 (**A**) combined statistical analysis show in the graph (n=9). This experiment was repeated, as technical replicates, on three independent occasions.

Chapter 4

Discussion

4 Discussion

4.1 Clinical significance of *CBLL1*, *METTL3* and *METTL14* expression

4.1.1 Genetic alterations and mutations of CBLL1, METTL3 and METTL14 using CbioPortal

The genetic alternations and mutations affecting *CBLL1*, *METTL3* and *METTL14* were analysed using cBioPortal using the Breast Invasive Carcinoma (TCGA, PanCancer Atlas) (n=996) cohort (**Figure 3**-**5**). The single *CBLL1* mutation consisted of a missense mutation and found present in the sample taken from a 61-year-old white female with basal BCa subtype (**Figure 3**). Of the three *METTL3* mutations, two basal and one luminal A BCa subtypes were identified (**Figure 4**). For METTL3 the M393V protein change is within the MT-A70 domain. This protein change may have an effect on METTL3 function. Furthermore, it has been shown that the catalytic MT-A70 domains of METTL3 and METTL14 are able interact (Schöller et al., 2018), any mutations with the MT-A70 domain may have an impact on methyltransferases interaction and subsequently the methyltransferase complex (MTC). Five *METTL14* mutations were identified in which two were observed in the MT-A70 domain (**Figure 5**). However, no *METTL14* genomic deletions were reported in the GISTIC databases suggesting it may be essential for cellular viability.

4.1.2 Clinical significance of *CBLL1*, *METTL3* and *METTL14* expression at transcriptomic levels

The clinical relevance of *CBLL1, METTL3* and *METTL14* expression was analysed.

METTL3 was found to have the highest expression in PR and ER expressing BCa, which may indicate a functional association between estrogen and progesterone signalling and *METTL3*. Expression levels of *METTL3* was higher in non-TNBC and non-basal like tumours (**Figure 6**). When considering different subtypes, the *METTL3* mRNA expression levels were significantly (<0.001) highest in luminal B as compared with all other PAM50 subtypes (**Figure 6K-L**). There is more *METTL3* expression in the HER2 negative tumours (**Figure 6C**).

Consistent with results obtained for *METTL3, METTL14* expression was associated with PR and ER positivity. This is supported as METTL14 expression was positively associated with estrogen and progesterone receptor (Dong et al., 2020) (**Figure 7A-B**). *METTL14* was also found to be higher in non-TNBC and non-basal like. Similar to METTL3 there were greater expression levels for *METTL14* in the HER2 negative subtype (**Figure 7C**).

CBLL1 has been reported to function as an ERα co-regulator in BCa via interaction with the DNA-binding domain of ERα and therefore enhancing its transcriptional activity (Gong et al., 2010). This is consistent with results obtained in this study as CBLL1 siRNA mediated knockdown effected estrogen signalling in MCF7 (**Figure 10**). *CBLL1* expression however is higher in HER2 negative samples (**Figure 8C**). Furthermore, in the different PAM50 subtypes *CBLL1* expression is significantly higher (<0.0001) in luminal A subtype as compared with basal-like and HER2-E (**Figure 8L**).

For all three components of the MTC studied here, the age at which has the greatest expression levels was between the ages of 40 to 70 (**Figure 6F, 6F, 7F**). Furthermore, considering that 51 years of age median age women experience menopause the results show that there is a higher expression for CBLL1, METTL3 and METTL14. This contrasts with observations found in this study which show E2 upregulation.

4.1.3 Kaplan-Meier survival estimates for the expression of *CBLL1*, *METTL3* and *METTL14*

From **Figure 9**, Kaplan-Meier plots show the association of the expression levels to patient outcomes. High expression levels of *CBLL1* are observed to be associated with a better patient survival. Higher CBLL1 expression in BCa is associated with delayed relapse-free survival in TCGA cohort as well as better overall survival in METABRIC cohort (Zheng et al., 2021). The same is observed for *METTL14* with high expression levels showing an association with better patient survival. In contrast, low *METTL3* expression has been shown to have a better patient survival with levels.

4.2 The functional role of CBLL1 in breast cancer cell line MCF7

4.2.1 siRNA-mediated depletion of CBLL1 following estrogen treatment

MCF7 is a luminal-A like cell line and therefore ERα signalling is intact (Holliday and Speirs, 2011). CBLL1, METTL3 and METTL14 (**Figure 10**) are upregulated by E2 treatment. This might suggest CBLL1 may regulate the expression of METTL3 and METTL14. However, no significant change in METTL3 and METTL14 expression was observed following CBLL1 knockdown. This might potentially suggest that METTL3 and METTL14 relies on both estrogen and CBLL1 being present to have upregulated protein expression, but this would need further understanding. These results overall show complex relationships between CBLL1, METTL3 and METTL14 in MCF7.

4.2.2 siRNA-mediated depletion of CBLL1 on differential gene expression in vehicle and estrogen treated

Analysis of the CBLL1 knockdown without estrogen treatment, identified expression of the top ten significant genes by fold change (**Figure 11**). Expression of *METTL3* and *METTL14* were observed to be unchanged, therefore this might indicate that *CBLL1* has no direct effect one each of the genes. For this condition there were no significantly enriched pathways found to be associated with the upregulated or downregulated significant DEGs, but the top ten non-significant pathways have been analysed. The absence of significant pathways might possibly suggest CBLL1 is involved in the regulation of genes in a wide number of cellular processes and may not regulate specific mechanisms. *NTN4* was identified to be downregulated following the siRNA mediated knockdown of *CBLL1*, without estrogen treatment (**Figure 11A**). Furthermore, this knockdown under estrogen treatment also resulted in *NTN4* downregulation therefore this might indicate that the depletion of *CBLL1* may consequently cause this affect. Netrin-4 (NTN4) is a member of the neurite guidance factor family, and is secreted by breast epithelial cells (Yi et al., 2022b). Low expression of NTN4 in BCa has been shown to be associated with poorer survival (Yi et al., 2022b). Therefore, the depletion of

CBLL1 expression may therefore lead to poorer survival due to the consequential downregulation of NTN4 expression.

RNAseq analysis of siRNA mediated CBLL1 knockdown and the E2 treatment of the MCF-7 cells was completed. Within this condition the top ten significant by fold change DEGs identified (**Figure 12A**). *Stanniocalcin 2* (*STC2),* a secreted glycoprotein, was shown to be upregulated (**Figure 12A**) following CBLL1 knockdown in estrogen treatment. STC2 has been shown to increase proliferation in the MCF7 cell line (Di et al., 2022), therefore implying that the upregulation may increase proliferation of BCa. On the contrary, *MAL2* was downregulated following CBLL1 knockdown with estrogen treatment (**Figure 12A**). In addition to this, following the CBLL1 knockdown without estrogen treatment *MAL2* was also shown to be down regulated. Therefore, this might indicate that the knockdown of CBLL1 is causing the downregulation of MAL2. Myelin and lymphocyte protein 2 (MAL2) is a transmembrane protein which has been shown to be upregulated in BCa and is associated with poor prognosis. Furthermore, mechanistically MAL2 has shown to interact with β-catenin, with the silencing of MAL2 resulting in the reduction of β-catenin and c-MYC (An et al., 2023). Therefore, following CBLL1 knockdown, the downregulation of MAL2 would have a positive effect on prognosis but the extent of this would need to be further investigated. In addition to this *MYCL* was also shown to be downregulated following CBLL1 knockdown under estrogen treatment. MYCL has been shown to be upregulated and associated with poor prognosis in TNBC (Jiang et al., 2022) therefore meaning the downregulation of MYCL following the knockdown may have positive effects on prognosis.

Analysis of significant genes show that the CBLL1 knockdown may have positive and negative effects on BCa prognosis. Therefore, indicating that this could have positive and negative effects if considered as a therapeutic target.

Table 4. A table to summarise key differential findings of siRNA-mediated depletion of CBLL1 on differential gene expression in vehicle and estrogen treated in MCF7 cell line.

Gene name	Description	Summary of findings	References
NTN4	Secreted by breast epithelial cells	Downregulated following CBLL1 knockdown, without estrogen treatment	(Yi et al., 2022b)
STC2	Secreted glycoprotein	Upregulated following CBLL1 knockdown with estrogen treatment	(Di et al., 2022)
MAL2	Myelin and lymphocyte protein 2 – a transmembrane protein	Downregulated following CBLL1 knockdown without estrogen treatment	(An et al., 2023)

MYCL	MYCL proto-oncogene	Downregulated following CBLL1 knockdown with estrogen treatment	(Jiang et al., 2022)

4.2.3 The effect of CBLL1 siRNA mediated knockdown on splicing events in MCF7 with and without estrogen treatment

As described in the results following the CBLL1 knockdown in MCF7 without estrogen in **Figure 13** within the SE alternative splicing event analysis the prolactin signalling pathway was observed, which has been shown to have an association with BCa (Standing et al., 2022). Prolactin is a peptide hormone which is mainly secreted from the anterior pituitary gland and therefore has been shown to play a role in pregnancy and mammary gland development (Standing et al., 2022). Furthermore, prolactin signalling has been shown in the BCa cell line MCF7 to upregulate prolactin receptor when these cells are overexpressing prolactin (Kavarthapu and Dufau, 2022). Included in this pathway is *ERS1*, an estrogen receptor 1, which when mutated, has been shown to naturally occur in the MCF7 cell line (Martin et al., 2017). These mutations which occur after gaining resistance to long term estrogen deprivation, are $ESR1^{Y537C}$ and $ESR1^{Y537S}$ (Martin et al., 2017). This might therefore not be associated with the siRNA mediated knockdown of CBLL1. ERS1 was observed to be included in the RI alternative splicing event and had an inclusion level difference of 0.081 therefore indicating that the intron has not been retained.

When analysing the combined alternative splicing events for the CBLL1 knockdown with estrogen treatment there were 5 significantly enriched KEGG pathways which included in these is the Fanconi anaemia pathway. The gene BRCA1 has been observed to be included in this pathway, and from further analysis have been observed in the MXE alternative splicing event. This splicing event was found on chromosome 11 on the positive strand with an inclusion level difference value of 0.223. Therefore, as BRCA1 is involved in DNA repair (Somasundaram, 2003), the effect of the siRNA mediated knockdown of CBLL1 with E2 treatment, resulting in a MXE splicing event may have a negative effect on patient outcome, however further investigation would need to be considered.

4.3 The functional role of CBLL1 in breast cancer cell line MDA-MB-231

4.3.1 siRNA mediated depletion of CBLL1 in MDA-MB-231

These results shown in the western blots and quantification (**Figure 16**) shows CBLL1 is expressed in the TNBC cell line MDA-MB-231. As described, MDA-MB-231 is a triple negative breast cancer cell line and therefore is negative for PR, ER and HER2 (Holliday and Speirs, 2011). Therefore, as it is known that CBLL1 interacts with E-cadherin, this cell line can be used to investigate the effect the siRNA mediated knockdown of CBLL1 has without the presence of E-cadherin. The interaction between CBLL1 and the MTC still needs further investigation to distinguish the role CBLL1 plays in TNBC.

4.3.2 The effect of siRNA-mediated depletion of CBLL1 on differential gene expression

Bioinformatic analysis of the CBLL1 siRNA mediated knockdown in MDA-MB-231 identified a number of significant DEGs (**Figure 17**). One of these genes (**Figure 17A**), NORAD, a long non-coding RNA, is downregulated when CBLL1 is knocked down (**Figure 17**). NORAD has an association with the progression of several diseases (Liao et al., 2024). Recent research has shown that this lncRNA, which is activated by DNA damage, is differentially expressed in breast cancer (Capela et al., 2024) with high expression of NORAD observed in MDA-MB-231 and other triple negative breast cancer cell lines (Alves-Vale et al., 2023). The downregulation of NORAD has been shown to sensitise MDA-MB-231 to chemotherapy (Alves-Vale et al., 2023). Therefore, if there is the possibility that CBLL1 downregulates NORAD then targeting CBLL1 could be promising therapeutic, to use this treatment in combination with chemotherapy for triple negative breast cancer patients, however this would need further investigation.

Furthermore, *AGR2* is upregulated following CBLL1 knockdown (**Figure 17A**). Anterior gradient-2 (AGR2) expression has been shown to have a significant correlation with ER expression (Fletcher et al., 2003). However, in this study the RNA-seq was completed in TNBC. Following siRNA mediated knockdown of AGR2 resulting in the inhibition of cell growth and reduces the expression of c-MYC in T47D and ZR-75-1 BCa cell lines which are both ER positive (Vanderlaag et al., 2010). However, it has been shown that AGR2 may have an ER-independent pathway for downregulating cyclin D1 (Vanderlaag et al., 2010). Mechanistically it seems AGR2 plays different roles in different subgroups, but the research is limited. Following CBLL1 knockdown, *AGR2* upregulation may have a negative effect as ER positive BCa is positively correlated with poor prognosis (Vanderlaag et al., 2010) and therefore there is potential TNBC may or may not coincide with this. Further research would be needed to investigate the role of AGR2 in TNBC.

Following KEGG pathway analysis, key cancer pathways were identified including cytokine-cytokine receptor interaction and PI3K-Akt signalling pathway. The cytokine-cytokine receptor interaction pathway involves a number of significant upregulated DEGs. These upregulated DEGs were researched and shown to have an association with RNA:m^6A such as *CX3CL1, CCL20* and *TLR4*. CX3CL1, C-X3-C motif ligand 1, has been shown to be a direct downstream target of FTO-mediated m^6A modification (Liu et al., 2024c). Recent research has identified that when tumoral YTHDF2 is depleted this leads to the promotion of recruitment of macrophages and does so via CX3CL1 (Xiao et al., 2024). Furthermore, in non-small-cell lung cancer (NSCLC), METTL3 has been shown to be associated with another significant cytokine gene which from analysis was a significant upregulated DEG found in the cytokine-cytokine receptor interaction pathway analysis, named *CCL20*. METTL3 promotes the expression of CCL20 and therefore when inhibited using STM2457, it has been shown to downregulate CCL20 and other pro-tumorigenic cytokines (Yu et al., 2024). This inhibition using STM2457 then sensitises the NSCLC tumours to anti-PD-1 immunotherapy (Yu et al., 2024). TLR4 also has an association with METTL3 as METTL3 plays a role in regulating neutrophil mobility and activation specifically by inducing TLR4 protein expression in these neutrophils. METTL3 does this by simultaneously increasing translation transcriptionally and reducing the degradation of TLR4

mRNA (Luo et al., 2024). CCL20 has also been researched in breast cancer, with a promising natural (one of the main ingredients of ginseng) therapeutic target named Ginsenoside Rh1 (Zheng et al., 2023a, Jin et al., 2022). Rh1 is a novel casein kinase II subunit alpha (CK2α) inhibitor which promotes the nuclear translocation of HHEX which in turn resulted in disrupting the crosstalk between CCL20 and CCR6 (C-C motif receptor 6) therefore reducing the invasion of breast cancer cells (Zheng et al., 2023a). Rh1 has also be shown have an effect on mitochondrial dysfunction in TNBC cells, therefore indicating further its potential use for treating breast cancer (Jin et al., 2022). Overall, the result of this pathway following CBLL1 knockdown, might indicate that targeting CBLL1 as a potential therapeutic target might be beneficial in combination with other therapeutics such as Rh1.

From this analysis, the PI3K-Akt signalling pathway has several significant upregulated DEGs including the mesenchymal epithelial transition factor (MET). MET expression was reduced following siRNA mediated knockdown of CBLL1. MET is a proto-oncogene and receptor tyrosine kinase (Mohan et al., 2024) which regulates several biological processes, including cell proliferation and survival. In terms of m^6A, MET has been shown to have an association with METTL14 (Miyake et al., 2023). Specifically, this occurs when the expression of cellular-mesenchymal epithelial transition factor (c-MET) mRNA is affected by the methylation of the mutant METTL14 (R298P) and in turn is destabilised (Miyake et al., 2023). This destabilisation could potentially occur as there is increased methylation efficiency at canonical motifs which are close to aberrant m^6A modification sites of which is the location that the mutant METTL14 induces m^6A modification (Miyake et al., 2023). For patients with TNBC, high expression of MET has been shown to be associated with shorter overall survival (Hsu et al., 2014). Overall, the reason it may be downregulated when CBLL1 is knocked down is that when MET is localised to the membrane it is tightly associated with E-cadherin (Reshetnikova et al., 2007). In this study *MET* is upregulated following CBLL1 knockdown, and therefore this might have a negative on patient outcome if CBLL1 was considered as a therapeutic target.

Overall, the RNA-seq analysis of the siRNA mediated knockdown in MDA-MB-231 showed a number of significant DEGs of which could be considered for future investigation for mechanistic associations with RNA:m^6A and BCa, potentially leading to therapeutic targets.

Table 5. A table to summarise the key findings of the effect of siRNA-mediated depletion of CBLL1 on differential gene expression in MDA-MB0231 cell line.

Gene name/ key cancer pathways	Description	Summary of findings	References
NORAD	long non-coding RNA	Downregulated following CBLL1 knockdown	(Liao et al., 2024)
AGR2	Anterior gradient-2	Upregulated following CBLL1 knockdown	(Vanderlaag et al., 2010)

Cytokine-cytokine receptor interaction pathway	Pathway involved in cancer	Three genes in this pathway have been shown to have an association with RNA:m^{6}A such as *CX3CL1, CCL20* and *TLR4*	(Liu et al., 2024c), (Xiao et al., 2024), (Yu et al., 2024), (Luo et al., 2024), (Jin et al., 2022)
PI3K-Akt pathway	Pathway involved in cancer	This pathway has been shown to involve the MET proto-oncogene	(Miyake et al., 2023), (Hsu et al., 2014), (Reshetnikova et al., 2007).

4.3.3 The effect of CBLL1 siRNA mediated knockdown on splicing events in MDA-MB-231

As described in section **3.3.3 The effect of CBLL1 siRNA mediated knockdown on splicing events in MDA-MB-231** the splicing events were analysed for key KEGG pathways (**Figure 18**). Identified in this discussion are the significant KEGG pathways from the RI and SE splicing events which have been shown to have an association with RNA:m^{6}A and/or cancer.

The RI splicing event only resulted in a single significantly enriched KEGG pathway: Spliceosome (**Figure 18**). This pathway involves key genes such as *LSM8* and *LSM7* of which are homologs of each other and are U6 small nuclear RNA associated and *LSM7* is also associated with mRNA degradation. U6 spliceosomal small nuclear RNA (snRNA) has been shown to be associated with METTL16, a long-unknown methyltransferase and part of the RNA:m^{6}A modification (Pendleton et al., 2017). Recent research has shown that LSM7 has been found to be upregulated in BRCA patients and therefore may play a role in BRCA development (Ta et al., 2021). These proteins are more widely studied in yeast with the Lsm2-8 complex shown to bind and stabilise the spliceosomal U6 snRNA and Lsm1-7 complex plays roles in the decay of mRNA (Fernandez et al., 2004). Other genes associated with the spliceosome include *U2AF1*, *U2AF1L5*, *U2AF1L4* which are all U2 small nuclear RNA auxiliary factor 1 with like 5 and like 4 respectively. Alternative splicing can be coupled with nonsense mediated decay which is an efficient strategy to regulate gene expression (da Costa et al., 2017). Furthermore, it has been shown that nonsense mediated mRNA decay is able to mute the splicing defects of the spliceosome mutations, and that the full extent of the effect on these spliceosome mutations can only be seen once the nonsense mediated mRNA decay is inactivated (Kawashima et al., 2009).

Within the SE alternative splicing event, the significant KEGG pathway, Fanconi anaemia pathway has shown to include *BRCA1*, a key BCa gene. Following CBLL1 knockdown the *BRCA1* gene was shown to have an inclusion level difference of -0.083. This would indicate that *BRCA1* is not skipped in this splicing event, therefore emphasising, as is well known, its importance in BCa. Furthermore, *YTHDF2* is identified with an inclusion level difference of -0.106 and therefore also is not being spliced. This might indicate the m^{6}A binding proteins importance following the CBLL1 knockdown or just within the TNBC cell line.

4.3.4 Transcriptomic analysis of CBLL1 in the TCGA dataset

Significant differential gene expression identified between patients stratified on the basis of low, quartile 1 (Q1) *CBLL1* mRNA expression as compared to high, quartile 4 (Q4) *CBLL1* expression in BCa specimens (**Figure 19**), including *CBLL1*, *THAP5*, *TMEM209*, *ZNF800*, *WASL*, *COG5*, *CNOT4*, *SRPK2*, *PNPLA8* and *AL035425.4*. These genes give insight into the improved prognosis observed with increased CBLL1 expression.

Genes associated with high CBLL1 include *THAP5*, a substrate originally identified as involved in the mitochondrial pro-apoptotic Omi/HtrA2 protease. Shown in melanoma cell lines, *THAP5* induction correlates with a significant increase in apoptotic cell death (Balakrishnan et al., 2011). Furthermore, shown in non-small lung cancer, *THAP5* was identified as a *MET* fusion protein partner in patient samples (Kang et al., 2023). This fusion might play a role in tumorigenesis since there is a kinase domain on exon 15 (Kang et al., 2023).

Similarly expression of the transmembrane protein 209 (*TMEM209*) which encodes a nuclear envelope protein, has been shown to have an association with lung cancer (Fujitomo et al., 2012) and found in this study to be associated with high CBLL1. TMEM209 functions by regulating the nuclear levels of the c-MYC protein as does so through the interaction with NUP205 a component of the nuclear pore complex (NPC) (Fujitomo et al., 2012). Therefore when TMEM209 was knocked down this resulted in the inhibition of cancer cell growth (Fujitomo et al., 2012).

ZNF800 is a long non-coding RNA has been shown to repress the AKT/mTOR/HIF-1α pathway through its interaction with PTEN (Lu et al., 2020) also found in this study to be associated with high CBLL1. *ZNF800* was shown to have no effect on *PTEN* mRNA expression but did in fact have an effect on protein levels, subsequently increasing the protein expression of PTEN (Lu et al., 2020). However, the mechanism in which this occurs is still unknown. In colorectal cancer, circular ZNF800 (circZNF800) has been shown in late-stage colorectal cancer tumours to have upregulated expression (Rengganaten et al., 2023). In breast cancer, the loss of PTEN activity and the activation of PI3K signalling are associated with endocrine therapy resistance (Xing et al., 2019). The AKT inhibitor, MK-2206 has shown that when PTEN is lost the cell lines are more sensitive to this inhibitor, also with sensitivity to *PTEN* mutations but less so than loss of (Sangai et al., 2012, Xing et al., 2019). Therefore, there may be potential that ZNF800 could be a potential therapeutic target in cancer and breast cancer, but this would need further investigation. When considering that this gene is positively correlated with CBLL1 and ZNF800 may have a negative impact on patients with BCa, the knockdown of CBLL1 might therefore reduce the expression of ZNF800. However, from analysis of the DEGs from RNA-seq when the siRNA mediated knockdown of CBLL1 was completed, there was no significant change to ZNF800. The complete inhibition however may still have an effect though this would need to be investigated further.

Furthermore, there were a number of enriched KEGG pathways in these gene sets (FDR <0.05) in patients with low (Q1) as compared with high (Q4) CBLL1 expression. The KEGG pathways shown in high CBLL1 patients include 32 significant pathways identified in which breast cancer is included in these as shown in **Figure 19**, including the allograft rejection pathway which has

known associations with both breast cancer (Oshi et al., 2021, Uddin and Wang, 2022) and RNA:m^{6}A (Liu et al., 2022, Li et al., 2022c, Wang et al., 2022b). Allograft rejection pathways may be related to the involvement of RNA:m^{6}A in a diverse range of immunoregulation (Wang et al., 2022b). In mouse models, the overexpression of WTAP, a MTC component, has been shown to alleviate allograft rejection and therefore reduces the inflammatory response and increased Treg population (Wang et al., 2022b). Moreover, METTL3 inhibition using STM2457, reduced the m^{6}A levels, inhibited the proliferation of the T-cells and supressed effector differentiation of polyclonal CD4$^+$ T cells, of which these CD4$^+$ T cells play a central role in allograft rejection (Li et al., 2022c). Overall, this further indicates that the methyltransferases play a role in the immune response.

Within the allograft rejection KEGG pathway there 18 significantly enriched DEGs identified so which the majority of (human leukocyte antigen) HLA genes within the major histocompatibility complex (MHC). The m^{6}A binding protein YTHDF1, when tumour intrinsic, has been shown to drive immune evasion and resistance to immune checkpoint inhibitors (Lin et al., 2023). When considering YTHDF1 mechanistic involvement, it has been shown that deficiency of YTHDF1 inhibits translation of lysosomal genes and in turn limits lysosomal proteolysis of the MHC class I (MHC-I), therefore resulting in restoring tumour immune surveillance (Lin et al., 2023). Key genes found in the Q4 allograft rejection KEGG pathway and are part of MHC-I include HLA-A, HLA-B, HLA-C, HLA-E, HLA-F, HLA-G.

In the significantly enriched breast cancer KEGG pathway 36 DEGs were identified from patients with high CBLL1 expression. These included several Wnt family members and fibroblast growth factors. Also included was JUN, a proto-oncogene and AP-1 transcription factor subunit, has been shown to be involved in breast cancer. Protein levels of c-Jun have been shown to be upregulated in characterised bone metastatic MCF7-BM cell lines, as compared with the parental cells (Han et al., 2023). Furthermore, c-Jun deficiency has been shown to result in the suppression of tumour cell migration (Han et al., 2023). Overall this could indicate c-Jun as a potential therapeutic target for preventing bone metastasis in luminal breast cancer (Han et al., 2023). In terms of m^{6}A, the FZD10–β-catenin/c-Jun axis has been shown to transcriptionally activate the expression of METTL3 (Wang et al., 2023c). Another proto-oncogene and AP-1 transcription factor subunit was identified in the breast cancer KEGG pathway. This gene named FOS has been shown to be implicated in cellular processes such as transformation, proliferation, differentiation, and apoptosis and shown to have an association with breast cancer (Chang et al., 2023).

In patients with low CBLL1 expression, the top ten enriched KEGG pathways, pentose and glucoronate interconversions, ascorbate and aldarate metabolism, homologous recombination, nicotine addition, taste transduction, cell cycle, nucleocytoplasmic transport, chemical carcinogenesis, Herpes simplex virus 1 infection and neuroactive ligand-receptor interaction. Within Q1 the homologous recombination KEGG pathway, the genes BRCA1 and BRCA2 were observed which, as described in the introduction, are key BCa genes (Sun et al., 2017). These genes are both associated with DNA repair and do so by encoding proteins which are critical for homologous recombination DNA repair (Tutt et al., 2021). Therefore, patients

with low CBLL1 expression levels, they may have a potentially higher risk of BCa as BRCA1 and BRCA2 are associated with low CBLL1 expression levels.

To conclude it can be argued that higher CBLL1 expression levels seem to potentially have a positive impact for patients with BCa, therefore the complete inhibition of CBLL1 as a potential therapeutic target could still possibly be considered. On the other hand, CBLL1 may potentially be a good biomarker as high expression levels might have an association with improved patient outcome and low expression levels might have a negative patient impact. Although to be sure of this, further investigation would be necessary.

4.3.5 siRNA-mediated depletion of CBLL1&METTL3 and CBLL1&METTL14

This western blot experiment (**Figure 20**) was completed to compare the combinatorial knockdown of CBLL1 with METTL3, and CBLL1 with METTL14. The original hypothesis was the ubiquitin-ligase function of CBLL1 may regulate MTC complex composition by responding to changes in the stoichiometry of METTL3 and/or METTL14 expression as observed when knockdown of METTL3 results in reduced METTL14 expression, and conversely knockdown of METTL14 also results in reduced METTL3 expression. To test this hypothesis, the effect of knockdown of CBLL1 and METTL3 or METTL14 were examined using western blot analyses.

4.3.6 siRNA-mediated depletion of METTL3, CBLL1&METTL3, METTL14 and CBLL1&METTL14

To build upon the previous western blot (**Figure 20**), the knockdowns of METTL3 and METTL14 individually were completed to access the comparison between the simultaneous knockdown of CBLL1 and METTL3 as compared with CBLL1 and METTL14 (**Figure 21**). Interestingly, simultaneous knockdown of METTL3 and CBLL1 increased the reduction in METTL3 expression as compared to METTL3 knockdown alone. Simultaneous knockdown of CBLL1 and METTL14 reduced METTL3 expression to a similar level of METTL14 knockdown alone. These results suggest a complex relationship between METTL3 and CBLL1 expression and suggests the observed effect of METTL3 knockdown on METTL14 expression. Conversely METTL14 knockdown on METTL3 expression is not mediated by CBLL1. One possible mechanism is that expression of METTL3 and METTL14 is regulated by RNA:m^6A and thus loss of either METTL3 and METTL14 disrupts MTC function and in turn affects expression of both METTL3 and METTL14.

4.4 Comparison of the siRNA mediated knockdown of CBLL1 in MDA-MB-231 verses MCF7

4.4.1 Comparison of the upregulated and downregulated DEGs between MDA-MB-231 and MCF7

One of the downregulated DEGs when comparing the CBLL1 knockdown in MDA-MB-231 against the estrogen treated MCF7 knockdown (**Figure 22A**) was the RRN3P3 tumour suppressor (Tan et al., 2022b). Described as a pseudogene *RRN3P3* has been shown to be associated with RNA:m^6A which more specifically has been demonstrated that METTL3

significantly methylates RRN3P3 mRNA at the Chr16:22431201 site (Tan et al., 2022b). There is limited information on RRN3P3 and therefore further research would be needed to fully understand its function and association with RNA:m^{6}A. However, as *RRN3P3* was downregulated following the knockdown, this might indicate that inhibiting CBLL1 may result in increased tumour growth.

Also found to be common in the downregulated DEGs (**Figure 22A**) was EFNA3, which although has no published association with RNA:m^{6}A but it has been shown to be associated with BCa (Mendonça et al., 2024). EFNA3 is a Ephrin-A3 protein and part of a family of cell surface ligands, which when induced by HIF, has been shown to lead to increased protein expression in BCa tumour cell lines (Mendonça et al., 2024, Gómez-Maldonado et al., 2015). Furthermore, when EFNA3 was observed to be positive in BCa tumours this was linked to an increased likelihood of metastasis (Mendonça et al., 2024, Gómez-Maldonado et al., 2015). Therefore, as the knockdown of CBLL1 causes a decrease of EGNA3 mRNA expression this may have a positive impact on patients, therefore potentially lowering their risk of metastasis, although this would need to be further investigated.

On the contrary, MET was shown to be a common upregulated DEG (**Figure 22B, D**). As described before (**4.3.3** The effect of CBLL1 siRNA mediated knockdown on splicing events in MDA-MB-231 , c-MET has been shown to be associated with METTL14. Of these common upregulated DEGs, *FOXO4* was found to be common between these cell lines and therefore showing that its expression is decreased when CBLL1 is knocked down. This may have positive implications as FOXO4 is part of the forkhead box O (FOXO) transcription factor family it is considered a tumour suppressor and therefore limits cell proliferation and induces apoptosis (Coomans de Brachène and Demoulin, 2016). FOXO4 has been shown that it is a downstream target from PI3K/AKT and in prostate cancer and when there is a loss of FOXO4 expression this is associated with an increased PISK/AKT-mediated metastatic invasiveness (Su et al., 2014). Therefore, the knockdown of CBLL1 may have a positive impact on BCa patients however further research would be needed to be confident of this.

Another of the common upregulated DEGs included in these experimental conditions (**Figure 22B, D**), is NOX5 which is a NADPH oxidase which is known to stimulate cell proliferation through the generation of reactive oxygen species (ROS) (Dho et al., 2015). The long form of NOX5 (NOX5-L) depletion has been shown to decrease BCa cell proliferation, invasion and migration in vitro, with this expression regulated by SAT5A in BCa cell lines (Dho et al., 2017). This therefore indicates that the knockdown of CBLL1, and subsequent upregulation of the NOX5-L gene might lead to decreased BCa cell death, however this would need further investigation, specifically considering the short and long forms, to analyse further the implications of using CBLL1 as a potential therapeutic target.

When considering the downregulated DEGs (**Figure 22A, C**), the gene EYA3 has been observed to be common. In human breast tumours, EYA3 has been shown to correlate with PD-L1 and when the expression levels of EYA3 are high there is decreased CD8$^+$ T cell signature in these tumours (Vartuli et al., 2018). This would have a negative impact on patients with ER-negative breast cancer as the presence of these CD8$^+$ T cells has been shown to be associated with

significant reduction in relative risk of death (Ali et al., 2014). This therefore would mean that potentially using CBLL1 as a therapeutic target may have a negative effect.

Overall, there are positive and negative implications to using CBLL1 as a therapeutic target, with a number of significant DEGs observed to be upregulated and downregulated when the siRNA mediated knockdown of CBLL1 is completed. There are also a few variations between different cell lines which would need to be taken into consideration.

4.4.2 Comparison of CBLL1 regulated splicing events in MDA-MB-231 and MCF7

When comparing the combined splicing events rMATs between the CBLL1 knockdown in MDA-MB-231 verses MCF7 without estorgen treatment verses MCF7 with estorgen treatment the results identified 208 shared spliced genes with 38 of these manually identified to have an association with RNA:m^{6}A. From these 38 (**Figure 23**), several genes have been identified and have been described in this study in more detail.

Derived from CTNNB1, circular RNA (circ-CTNNB1) which has been shown to play a role in cancer progression (Yang et al., 2023a). When considering circ-CTNNB1 association with m^{6}A, it has been shown to interact with RBM15 (Yang et al., 2023a). This allows RBM15 to raise 3'UTR m^{6}A levels for hexokinase 2 (HK2), glucose-6-phosphate isomerase (GPI) and phosphoglycerate kinase 1 (PGK1) of which are all glycolysis genes (Yang et al., 2023a). When utilising hepatoblastoma cells, it has been shown that there is a reduction in m^{6}A methylation which leads to the decrease in CTNNB1 expression and stability (Liu et al., 2019). From rMATs analysis CTNNB1 was found within the A3SS splicing event in all three conditions (**Figure 23**). Therefore, this indicates further that CTNNB1 may be functioning at the 3'UTR.

Another of the common genes identified was eukaryotic translation initiation factor gamma 1 (EIF4G1). 5'-tRF-GlyGCC has been shown to reduce the methylation of eIF4G1 and does this by binding to FTO which in turn decreases its activity and reducing the methylation levels of eIF4G1(Chen et al., 2023b). In BCa this methylation reduction of eIF4G1 has shown to promote proliferation and metastasis (Chen et al., 2023b). YTHDF2 has been shown to favour binding with the coding sequence of *eIF4G1* mRNA, in turn triggering the degradation of eIF4G1 (Chen et al., 2023b). From the results of a dual-luciferase reporter and mutagenesis assay completed by Wang et al., the mutated site 1 of exon 11 of eIF4G1 has been shown to be the key target of METTL14 (Wang et al., 2021b). EIF4G1 (ENSG00000114867) was identified in all conditions to be found in the SE alternative splicing event, with MCF7 siScrE2 verses siCBLL1E2 and MDA-MB-231 siScr verses siCBLL1 both showing EIF4G1 in the RI splicing event also. The MCF7 siScrVeh verses siCBLL1Veh and MDA-MB-231 siScr verses siCBLL1 also located EIF4G1 in the MXE alternative splicing event.

Annexin A2 (ANXA2), one of the 38 common spliced genes, has been shown to interact with DBT via DBT's lipoyl-binding domain which in turn activates Hippo signalling (Miao et al., 2023). Furthermore, in clear cell renal cell carcinoma ANXA2 has been shown to be downregulated by METTL3-mediated m^{6}A modification (Miao et al., 2023). In addition, in non-small cell lung cancer it has been shown that METTL3-mediated m^{6}A modification decreased *HAR1A* and in turn, stimulated tumour growth and metastasis by activating the ANXA2/p65 axis (Ling et al., 2024). This research might suggest a link, whether indirect or

direct, between ANXA2 and METTL3-mediated m^{6}A. Within the rMAT analysis ANXA2 (ENSG00000182718) was found in the MXE and SE alternative splicing events for MDA-MB-231 siScr verses siCBLL1, and just the MXE splicing event for MCF7 siScrVeh verses siCBLL1Veh. When under the estrogen treatment condition, MCF7 siScrE2 verses siCBLL1E2, ANXA2 is found in the A5SS and SE alternative splicing events.

Another of the identified common splicing gene, TAF15 a TATA-box binding protein associated factor, is a transcription factor found to interact directly with *LINC00839* (Zheng et al., 2023b). TAF15 is a member of the FET family and has several processes included within transcription and the initiation of RNA polymerase II (Wang et al., 2023a). Furthermore, in breast cancer, the signal transducer and activator of transcription 1 (STAT1) has been shown to mediate the upregulation of LINC00504 which in turn stabilised the expression of cytoplasmic polyadenylation element-binding protein 2 (CPEB2) and does so via the binding to TAF15 (Feng et al., 2021). It has also been shown that circDNAJC11 interacts with TAF15 which leads to the progression of BCa (Wang et al., 2023a). In triple-negative breast cancer tissues and cells it has been shown that circDNAJC11 is significantly upregulated and that this high expression correlates with poor patient prognosis (Wang et al., 2023a).

The eukaryotic translation initiation, *EIF4A1* (ENSG00000161960), was found to be a common rMAT gene between the CBLL1 knockdown in MDA-MB-231 verses MCF7 with estrogen treatment. EIF4A1 was found within the RI splicing event with an inclusion level difference value of -0.073 therefore indicating that the intron has been retained. However, in the MCF7 siScrE2 verses siCBLL1E2 condition EIF4A1 was observed to be within the A3SS splicing event with an inclusion level difference value of 0.107. As the intron is retained within the TNBC (MDA-MB-231) cell line this might indicate that this gene has an important function. To further support this, research has shown that eIF4A1 has a regulatory role in translating a number of oncoproteins (Raman and Tiwari, 2022). Furthermore, in terms of clinical relevance, eFT226 has been identified as an inhibitor for eIF4A1 which has entered human clinical trials (Ernst et al., 2020). In association with RNA:m^{6}A, IGF2BP2 (a reader protein) has been shown to facilitate CDK6 translational output and does so by recruiting EIF4A1 in TNBC (Xia et al., 2024). Therefore, by targeting IGF2BP2 this increases the sensitivity of CDK4/6 inhibitors indicating that IFG2BP2 may be a potential therapeutic target used in combination with these CDK4/6 inhibitors (Xia et al., 2024). Also, eFT226 could potentially be used in combination with CDK4/6 inhibitors, however further research would be needed.

Although identifying and describing, *CTNNB1*, *ANXA2*, *TAF15*, *YTHDF2* and *EIF4A1* further indicates the potential association and function within the experimental conditions, there is a need for validation of these splicing variants. Therefore, the resulting effect on protein function would need to be determined to indicate whether these could be potential therapeutic targets.

4.5 The effect of the STM2457 METTL3 inhibitor on MDA-MB-436 BCa cell proliferation

The MDA-MB-436 BCa cell line derived from a triple negative BCa lesion and is a widely used model for TNBC studies (Matysiak-Kucharek et al., 2020, Elstrodt et al., 2006). In this study

increasing concentrations of STM2457 were used on the TNBC cell line MDA-MB-436 to observe the change in proliferation. The increasing concentrations show an incremental and significant reduction in relative proliferation and therefore indicating that STM2457 inhibition of the METTL3 activity reduces the proliferation of this TNBC cell line. This is a promising therapeutic approach for TNBC treatments if the phase 1 trial shows favourable tolerability.

Further BCa cell lines were used by Dr. Anna Harris (personal communication), to analysis the effect of STM2457 treatment on different BCa subtypes which in turn further supports the findings presented in this study.

Furthermore, the specificity of STM2457 should be considered and if there are potential off-target effects of the inhibitor especially in the context of clinical application. Using surface plasmon resonance, the direct binding to METTL3-METTL14 heterodimer was shown to be confirmed (Yankova et al., 2021). This may highlight the specificity to the heterodimer. However, it is still important to consider any off-target implications, however, there has been no publications to date suggesting any possible off-target effects of the inhibitor.

Furthermore, it would be interesting to look at further if there is potential to therapeutically target CBLL1, METTL3 and METTL14, particularly in TNBC. This would be interesting future work, with the hypothesis that targeting these three with inhibitors would reduce the proliferation of the TNBC cell line. These could also be considered for other BCa subtypes.

Chapter 5

Conclusions and Future Work

5 Conclusions and Future Work

BCa remains a clinical problem as it one of the most commonly diagnosed cancers (Siegel et al., 2024). Therefore, there is a need for future treatments and novel therapeutics, especially for more aggressive BCa types such as TNBC and metastasis. This is because for TNBC treatment resistance is common which therefore indicates the need for novel therapies such as utilising Epitranscriptomics.

Targeting epitranscriptomics, specifically including RNA:m^{6}A, is an exciting and promising approach for new therapeutics in which this study has addressed. RNA:m^{6}A has been shown to be dysregulated in cancer with several of the regulators involved in this modification and as described, potentially being utilised as therapeutic targets or biomarkers. For example, METTL3 and FTO have been shown to be therapeutic targets with several potential drugs targeting these. METTL3, one of the methyltransferases, has had a large amount of interest with the development of the novel METTL3 inhibitor STM2457 (Yankova et al., 2021). Moreover, STC-15, an oral METTL3 inhibitor and derivative of STM2457, is in ongoing clinical trials (clinicaltrials.gov accession#: NCT05584111). Within this study, STM2457 treatment experiment was conducted in a TNBC BCa cell line which resulted in a significant reduction of proliferation consistent with other studies (Yankova et al., 2021, Haigh et al., 2022).

This study has focussed on the main protein of interest CBLL1 and its association with the methyltransferase complex (MTC) specifically the two methyltransferases METTL3 and METTL14. The original hypothesis was that CBLL1 can detect the stoichiometric imbalance of METTL3 and METTL14, following the knockdown of METTL3 or METTL14, and target the more abundant MTC component for degradation. However, siRNA mediated knockdown of CBLL1 did not affect the knockdown of METTL3 and METTL14 also affecting METTL14 and METTL3 respectively, indicating a CBLL1-independent mechanism. Further investigation would be needed to fully understand whether CBLL1 regulates MTC composition and function.

Bioinformatic analysis of the RNA-seq and rMAT alternative splicing events was completed to investigated genes which have an association with RNA:m^{6}A and/or BCa. From this bioinformatic analysis of the DEGs and rMATs creates a discussion of whether CBLL1 would be a good potential therapeutic target. There are several genes, following siRNA mediated knockdown of CBLL1, in which have negative implications and several which have positive implications on potential patient outcome.

To conclude the comparison between the MCF7 and MDA-MB-231 cell lines, CBLL1 could be considered to have a positive patient impact when there are high expression levels as shown in the Kaplan Meier plots (**Figure 9**). This indicates that inhibiting CBLL1 might have negative effects on the patient outcome. However, there are a number of DEGs from the RNA-seq analysis (**Figure 11**, **Figure 17**) which suggest that CBLL1 may have positive effects on BCa patient outcomes following siRNA mediated knockdown. Furthermore, CBLL1 may have different roles in different BCa subtypes as seen with luminal A and TNBC in this study, as several different significant differential genes were present in the cell lines. However, as CBLL1

has E-cadherin function this should be considered when estrogen is present in the cells as in non-TNBC types. This would be interesting to consider further with the potential use of a CBLL1 therapeutic and hormone treatment (estrogen) combinations. This would need to be considered when addressing non-TNBC cell types. Even though it is still not fully understood how CBLL1 precisely functions with METTL3 and METTL14, their interaction might inform the potential therapeutic approaches for other breast cancer types such as luminal B, but this would need further investigation.

Limitations in this study include the siRNA mediated knockdowns of CBLL1, METTL3 and METTL14. This is considered a limitation as they have not therefore been fully knocked out and thereby may still have some level of function. CRISPR knockouts may be a potential method to consider in the future however as RNA:m^6A is fundamental knockout cells may not survive. In addition to this, the use of more BCa cell lines would further add to this study, particularly utilising these cell lines that represent different BCa subtypes. Future work could utilise the sequencing techniques, methylated RNA immunoprecipitation sequencing (MeRIP-seq) or Nanopore Direct RNA sequencing (DRS) (Mason et al., 2019) to analyse the distribution of m^6A on a transcriptome-wide basis. By utilising this methodology this will allow the development of future exploration into the m^6A_m modification (Mason et al., 2019). Overall, either of these methodologies can be used to compare the distribution of RNA:m^6A in the control and the siRNA mediated knockdown of CBLL1 in different breast cancer cell lines. Considering future methodology, DART-FISH has recently been developed to visualise m^6A-modfied and unmodified transcripts of interest (Sheehan and Meyer, 2024). This builds upon the development of DART-seq which works by using APOBEC1-YTH. This method could be utilised as a visualisation technique. Furthermore, further validation methods could be used for RNA-seq results to further identify and confirm the significance of the differential gene expression. This would also be interesting to further investigate these.

Overall, this study contributes to the understanding of CBLL1 and its relationship with the two methyltransferases, METTL3 and METTL14.

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This record is to be maintained by the postgraduate student who will retain it. It is to be used by supervisors and internal assessor at the Annual Review.

STUDENT DETAILS			
Name of Student	Rachel Thompson	Start Date:	29.09.23
Student ID	20232593	Current year of registration:	1
Main Supervisor	Prof. Nigel Mongan		

SKILLS PORTFOLIO

The level of competency is SELF ASSESSED and rated on a scale of 1 to 5: 1 = Unsatisfactory, 2 = Needs development, 3 = Adequate, 4 = Proficient, 5 = Competent

Skill Description	Level of Competency					Date	Comments
	1	2	3	4	5		
Critique of scientific papers					X	24.06.24	
Use of reference manager					X	24.06.24	
Communication skills					X	24.06.24	
Team working abilities					X	24.06.24	
Practical skills of lab, or other, methods					X	24.06.24	
Poster design and presentation					X	24.06.24	
Verbal presentation					X	24.06.24	
Written presentation					X	24.06.24	
Writing skills					X	24.06.24	
Clinical skills (if applicable)					N/A	N/A	N/A

School of Veterinary Medicine and Science

Training

This record is to be maintained by the Postgraduate Student. It is to be submitted each year to accompany the written report, as part of the Annual Review process.

I declare that I have undertaken mandatory research integrity training ☒ Date: 24/06/2024

List of Courses/Presentations/Conferences/Seminars/Scientific Meetings etc.	Date Attended	Credits	Cumulative credits
Machine Learning approaches for the understanding inter- and intra- patient heterogeneity in breast cancer by Syed Haider (Institute of Cancer Research)	29.09.23	1	1
Modulation of the pre-metastatic bone niche by Vicky James	19.10.23	1	2
Breast Cancer bone metastasis – what do we know about the biology and therapeutic targeting? By Prof Ingunn Holen	27.10.23	1	3
The clinical and pharmacological significance of the RNA methyltransferase complex in breast cancer by Anna Harris (NBCRC seminar)	09.11.23	1	4
Bench to bedside: discovering CDK7 inhibitors as new cancer therapeutics by Prof Simak Ali, Imperial College London.	24.11.23	1	5
Winter symposium	05.12.23	2	7
RNA Interdisciplinary Cluster at Nottingham Symposium	17.01.24	2	9
Presented a poster at Cancer Research Nottingham Symposium	16.01.24	5	14
Prostate cancer support group 7-9pm	08.02.24	2	16
Presentation at NBCRC student seminar	22.02.24	3	19

Demonstrating -ONCG/3005/01/C/01/01 Cancer Biology and Molecular Therapeutics Understanding Genomics and Bioinformatics	07.03.24 (2-5)	3	22
Western Blot & Immunoprecipitation Workshop	25.03.24	1	23
Demonstrating – Yr3 Apr-VETS3013-VRP-Oncology Rotation-Prac-Cancer Genomics Bioinformatics Practical	15.04.24 (2-5)	3	26
BBSRC DTP Spring Conference	16.04.24	2	28
Attended Ideas to Innovation, Prostate Cancer UK conference	17.04.24-18.04.24	4	32
Spatial proteomics to decipher the tumour immune microenvironment – Judith Ramage	24.04.24	1	33
Demonstrating - Yr3 Apr-VETS3013-VRP-Aging and Cardiovascular Rotation-Workshop-Learning some key techniques and understanding critique analysis	29.04.24 (2-5)	3	36
10 min presentation at NBCRC research day	15.05.24	3	39
Co-author on published manuscript – 'Immunohistochemical Investigation into Protein Expression Patterns of FOXO4, IRF8 and LEF1 in Canine Osteosarcoma'	20.05.24	3	42
March for Men – PCUK walk	02.06.24	1	43
Prostate cancer support group 7-9pm	13.06.24	2	45
NBCRC Dragon Boat Race engagement and outreach	23.06.24	1	46
3 min thesis presentation for Summer Symposium	24.06.24	5	51
Attendance of day 2 of the Summer Symposium	25.06.24	2	53