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**Investigating the mechanisms of action of
cyclin-dependent kinase 7 (CDK7) inhibitors in
breast cancer**

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Thesis submitted for the degree of Doctor of Philosophy
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Abstract

Breast cancer is the most common cancer worldwide, with tumour development and progression driven by the estrogen receptor (ER) in most cases. ER inhibitors are now standard-of-care therapies for ER+ breast cancer and have been successful in reducing mortality since their introduction five decades ago. However, many patients relapse with advanced disease necessitating the development of alternative therapies. Previously, our lab identified cyclin-dependent kinase 7 (CDK7) as a potential cancer therapeutic target. CDK7 controls expression of protein coding genes by phosphorylating RNA polymerase II (Pol-II). CDK7 is also required for phosphorylation of CDKs that control appropriate cell cycle progression. Because transcriptional and cell cycle processes are both commonly dysregulated in cancer, our lab has developed selective small molecule inhibitors of CDK7, demonstrating potent tumour growth inhibition. Samuraciclib has emerged from this as an orally bioavailable CDK7 inhibitor that has demonstrated encouraging levels of efficacy in ER+ breast cancer patients in a Phase I clinical trial. A fundamental understanding of the mechanisms of sensitivity and resistance to samuraciclib is essential for guiding appropriate and informed treatment regimens. In this work, I explore mechanisms of samuraciclib-induced cell growth arrest and cell death using a combination of CRISPR-Cas9 genomic and quantitative proteomics screens. I show that samuraciclib-induced cell growth arrest is highly dependent on a functional PI3K/Akt/mTOR pathway such that PIK3CA-activating mutations amplify growth arrest. To profile the molecular response to samuraciclib, I performed a high-throughput, quantitative mass spectrometry analysis of ER+ breast cancer cells treated with samuraciclib. In addition to confirming alterations in proteins in PI3K/Akt/mTOR, global proteomics identified altered levels of proteins implicated in ferroptosis, a recently discovered non-apoptotic cell death pathway. I demonstrate that samuraciclib sensitizes breast cancer cells to ferroptosis-directed cell death and highlight new combination therapies that could enhance the clinical utility of CDK7 inhibitors in cancer patients.

Declaration of originality

This thesis is submitted in fulfilment of the requirements for the degree of Doctor of Philosophy. I declare that the work reported in this thesis is my own. Where results generated by a colleague are included, this is clearly stated and fully acknowledged. Cell lines that were stably transduced with lentivirus encoding nuclear-localized GFP were generated by Dr Chun-Fui Lai and Prof Laki Buluwela. MCF10A parental, and PIK3CA mutant (E545K and H1047R) cell lines were kindly provided by Dr George Poulogiannis, (Institute of Cancer Research, UK).

In the introduction section, data for Figure 1.9 were obtained from CRISPR-Cas9 screens undertaken by the Tyers lab (Montreal, Canada) or generated by Dr G. Sava (Imperial College London) in collaboration with Dr S. Omarjee and Prof J. S. Carroll (Cambridge, UK). In the results section, Figures 3.1 and 3.2 were also generated from data collected by Dr G. Sava. Sample preparation, collection of quantitative mass spectrometry data, and computational analysis of proteomics results was performed in collaboration with Dr T. de Marchi (Lund University, Sweden).

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Abbreviations

AI	Aromatase Inhibitor
ATP	Adenosine Triphosphate
BC	Breast Cancer
CAK	CDK-activating Kinase
CCND1	Cyclin D1
CCNE1	Cyclin E1
CDK	Cyclin-Dependent Kinase
CDK4/6i	Cyclin-Dependent Kinase 4/6 inhibitor
CDK7i	Cyclin-Dependent Kinase 7 inhibitor
cDNA	Complementary DNA
CoA	Co-activator
CoR	Co-repressor
CTD	C-Terminal Domain
CycH	Cyclin H
ddH ₂ O	Double-Distilled Deionized Water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DSB	Double-Strand Break
E2	Estrogen
E2F	E2F Transcription Factors
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
ER α	Estrogen Receptor alpha
ERE	Estrogen Response Element
FDA	Food and Drug Administration
gDNA	Genomic DNA
gRNA	Guide RNA
GTF	General Transcription Factor
HR	Hormone Receptor
MAPK	Mitogen-Activated Protein Kinase
NHEJ	Non-Homologous End Joining

NMD	Nonsense-Mediated Decay
ORC	Origin Recognition Complex
PARP	Poly (ADP-Ribose) Polymerase
PCR	Polymerase Chain Reaction
PL	Phospholipid
Pol-II	RNA Polymerase II
PR	Progesterone Receptor
PUFA	Polyunsaturated Fatty Acid
qPCR	Quantitative Polymerase Chain Reaction
RB/RB1	Retinoblastoma Protein
RCD	Regulated Cell Death
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPA	Replication Protein A
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
SERD	Selective Estrogen Receptor Downregulator
SERM	Selective Estrogen Receptor Modulator
SRB	Sulforhodamine B Assay
TBE	Tris-Borate Buffer
TCA	Trichloroacetic Acid
TF	Transcription Factor
TFII	Transcription Factor II
TKI	Tyrosine Kinase Inhibitor
TNBC	Triple-Negative Breast Cancer
WT	Wild Type

1. Introduction

1.1 Breast cancer overview

1.1.1 Breast cancer epidemiology and prevalence

Breast cancer (BC) is the most prevalent cancer and the most common malignancy in women worldwide, accounting for almost one in four new cancer diagnoses in women (Sung *et al*, 2021). One in eight women will be diagnosed with BC in their lifetime. There are over 600,000 deaths from BC and over 2.2 million new BC cases diagnosed annually. BC typically develops later in life with most diagnosis (>80%) being in women over the age of 50 (Anders *et al*, 2009; Howlader *et al*, 2021; McGuire *et al*, 2015). Despite improvements in diagnosis and treatment, BC remains the leading cause of cancer death in women (Howlader *et al*, 2021). Localized BC with an early diagnosis has a five-year survival rate of 90% (DeSantis *et al*, 2014). However, metastatic, and advanced stage BC has poor survival prognosis with a five-year survival rate of 30%, demonstrating the highly aggressive potential of the disease.

1.1.2 Breast cancer risk factors, common mutations, and patient prognosis

Known risk factors for BC consist of modifiable and non-modifiable factors. Modifiable factors include lifestyle choices and environmental carcinogens such as obesity, alcohol, smoking, radiation exposure, and hormonal replacement therapy (Bagnardi *et al*, 2001; Bhatia *et al*, 1996; Colditz, 1998; Gaudet *et al*, 2013; Hsieh *et al*, 1990). Less than 1% of BC cases are in men, and most cases in males are associated with older age, genetic mutations, elevated estrogen levels, radiation exposure, Klinefelter syndrome, and a family history of BC (Anderson *et al*, 2004; Bhatia *et al*, 1996; Fentiman *et al*, 2006; Harnden *et al*, 1971; Mc & Higgins, 1951; Sasco *et al*, 1993; Thorlacius *et al*, 1997). Non-modifiable BC risk factors in women include age, pregnancy, menopause, and genetic predispositions (Anders *et al*, 2009; Britt *et al*, 2007; Collaborative Group on Hormonal Factors in Breast, 2012; McGuire *et al*, 2015; Stephens *et al*, 2012). Levels of circulating estrogens and progesterone in females are associated with increased BC risk, especially for hormone receptor-positive (HR+) BCs (Anderson *et al*, 2002; Colditz *et al*, 2004; Mc & Higgins, 1951).

Pregnancy has been shown to have a dual effect in terms of the risk of BC. Geriatric pregnancy increases the risk of BC while full-term pregnancy before menopause and earlier age at pregnancy are associated with reduced risk of both hormone receptor-positive and -negative BCs (Britt *et al*, 2007; Clavel-Chapelon & Gerber, 2002; Clemons & Goss, 2001;

Collaborative Group on Hormonal Factors in Breast, 2012; Lipworth *et al*, 2000; Qiu *et al*, 2022). Breastfeeding is also associated with the frequency of HR+ BCs as it has been found to reduce the risk of BC. Compared to women who never breastfed, those who breastfed for 25 months or more had a significantly reduced risk of developing BC (Collaborative Group on Hormonal Factors in Breast, 2002). In postmenopausal women, higher levels of estrogens and progesterone, which may be present with postmenopausal hormone treatment, were associated with an increase in BC risk (Collins *et al*, 2005). Similarly, a later age of menopause and an earlier age of menarche (puberty onset) are associated with greater BC risk (Clavel-Chapelon & Gerber, 2002).

In addition to lifestyle risks, germline and somatic mutations are strongly predictive of BC risk. Inherited mutations in several genes are associated with a substantially increased risk of BC. Foremost among these are the *BRCA* gene 1 and 2 (*BRCA1/2*) genes, responsible for >80% of familial BC cases and many HR+ BCs (Natrajan *et al*, 2012; Struwing *et al*, 1997). Inactivating germline mutations in *BRCA1* and *BRCA2* tumour suppressor genes increase the risk of BC by approximately 65% and 45%, respectively (Anglian Breast Cancer Study, 2000; Ford *et al*, 1998; Struwing *et al*, 1997). *BRCA1/2* genes are important for the homologous recombination repair pathways, which is important for error-free DNA repair (Moynahan *et al*, 1999; Moynahan *et al*, 2001; Patel *et al*, 1998; Wang *et al*, 2000). After a double-stranded break, the replication protein A (RPA) complex at the site of the double-stranded break invades the homologous DNA site in a *BRCA1/2*-dependent mechanism to facilitate DNA polymerase activity and DNA ligation. *BRCA1* is also important for non-homologous end joining, and thus genomic stability. *BRCA1* interacts with Ku80 at DNA double-stranded break sites to facilitate the precise non-homologous end joining DNA repair (Zhang *et al*, 2004b; Zhuang *et al*, 2006). Thus, mutations in *BRCA1/2* can lead to genomic instability and error-prone DNA repair, increasing BC risk (Tutt *et al*, 1999).

Breast cancer is often associated with somatic mutations and gene rearrangements, most frequently in cancer driver genes. Most prominent are activating mutations and/or amplifications in the *PIK3CA*, *MYC*, and *CCND1* genes (Nik-Zainal *et al*, 2016). *PIK3CA* mutations are among the most common genetic alterations in BC and occur in 20-40% of cases (Campbell *et al*, 2004). The most frequent mutations are found in the helical and kinase domains, which result in activation of the PI3K/Akt/mTOR pathway, with E545K and H1047R being the most common mutations (Martinez-Saez *et al*, 2020). *PIK3CA* mutations correlate

with HR expression, lymph node positivity, and overexpression of ERBB2 (HER2), which is another major driver gene of BC (Saal *et al*, 2005). Interestingly, *PIK3CA* mutations are nearly mutually exclusive with the loss of the tumour suppressor *PTEN*, which inhibits the PI3K/Akt/mTOR pathway (Sansal & Sellers, 2004). This indicates that a single event is required to activate the PI3K/Akt/mTOR pathway and drive oncogenesis. *PIK3CA* mutations have been associated with various clinical features and responses to therapy (Andre *et al*, 2019; Guo *et al*, 2020; Ippen *et al*, 2019).

MYC is a transcription factor that regulates the expression of genes involved in cell proliferation, differentiation, self-renewal, and apoptosis (Dang, 2012). Elevation of *MYC* levels and *PIK3CA* mutations occasionally coexist in BC, contributing to oncogenesis and potentially influencing resistance to PI3KCA pathway inhibitors (Dey *et al*, 2015). *CCND1* encodes cyclin D1, which is a key regulator of cell cycle progression and elevated expression, and is associated with greater BC recurrence and poor therapy response (Bieche *et al*, 2002; Ortiz *et al*, 2017; Yang *et al*, 2019).

BC is also driven by the overexpression of growth factor receptor genes, particularly *ERBB2* which is canonically known as HER2. HER2 is a member of the epidermal growth factor receptor (EGFR) family and is involved in the signalling pathways that regulate cell growth and differentiation (Akiyama *et al*, 1986). HER2 overexpression increases the formation of heterodimers, leading to enhanced responsiveness to growth factors and oncogenic transformation (Yarden, 2001). HER2 amplification is observed in 15-20% of cases and defines a distinct BC subtype (HER2+) (Pahuja *et al*, 2018).

Genes whose loss of function is strongly associated with BC include the tumour suppressors *TP53*, *CDH1*, and *PTEN*, increasing the risk of BC formation up to 85%, 83%, and 85%, respectively (Lukasiewicz *et al*, 2021). Mutations in *TP53* are among the most common genetic alterations in BC, occurring in 20 – 40% of cases and contributing to tumour progression and resistance to therapy (Borresen-Dale, 2003). *CDH1* encodes E-cadherin, which is a cell adhesion molecule critical for maintaining epithelial cell integrity (Pecina-Slaus, 2003). Loss of function mutations or reduced expression of *CDH1* disrupts cell adhesion, promoting tumour invasiveness and metastasis. *PTEN* is a tumour suppressor gene that negatively regulates the PI3K/Akt signalling pathway (Depowski *et al*, 2001; Georgescu, 2010; Sansal & Sellers, 2004). Loss of *PTEN* function, whether through mutations or deletions, leads to

hyperactivation of this pathway, promoting tumorigenesis and cancer progression. Regular screening for mutations associated with BC risk and frequent testing for BC (*i.e.*, mammograms) have improved BC prognosis and cure rates (Fracheboud *et al.*, 2004; Hortobagyi *et al.*, 2005).

BC patient prognosis is associated with several clinical factors. BC clinical subtype and tumour grade, both of which are histopathological measurements, are the most important factors for informing patient prognosis (Haque *et al.*, 2012; Saadatmand *et al.*, 2015). These prognostic factors can also inform on the utility of BC therapies. Tumour stage, which describes the dissemination of the disease to other tissues and organs, is measured on a scale of zero to four and provides a strong association with patient survival. Tumour stage significantly influences therapeutic regimens, therapeutic efficacy, and progression-free survival. Stage 1 refers to a small, localized tumour while stage 4 refers to a metastatic/disseminated, aggressive, and inflamed tumour. Consequently, patients with higher stage tumours tend to have worse survival prognosis than lower stage tumours. Other factors affecting BC patient prognosis include patient age, pre-existing health conditions (*e.g.*, obesity), tumour complete resection, and therapeutic biomarkers, such as the tumour clinical subtype (Anders *et al.*, 2009; Bleyer *et al.*, 2008; Fortner *et al.*, 2016; Howlader *et al.*, 2021; Parker *et al.*, 2009; Rapiti *et al.*, 2006), as discussed below. Younger patients (*i.e.*, less than 40 years) typically have worse survival prognosis than older patients due to the greater prevalence of more therapy-resistant BC subtypes in younger patients (Anders *et al.*, 2009; Bleyer *et al.*, 2008). Obesity is associated with worse response to therapies and greater overall mortality (Fortner *et al.*, 2016).

1.1.3 Breast cancer subtyping methods and clinical subtypes

Subtype classification is important for understanding the disease heterogeneity and for tailoring effective therapeutic strategies. There are several methods for classifying BC into defined subtypes including clinical, based on tumour histology, and molecular, based on genetics and transcriptomics (**Figure 1.1**) (Cancer Genome Atlas, 2012; Harris *et al.*, 2007). Clinical subtypes, as determined using immunohistochemical staining and histological analysis, are important for informing patient prognosis and treatment regimens (Harris *et al.*, 2007). Extensive molecular profiling resulted in the development of the PAM50 model for predicting BC patient survival, which was used to help classify the BC expression subtypes (Cancer Genome Atlas, 2012; Parker *et al.*, 2009). The clinical and expression classification schemes are typically used because they provide the most therapeutic and prognostic information.

Clinical BC subtypes may be categorized into three major subtypes. The first of these is characterised by immunohistochemical detection of the HRs, estrogen receptor- α (ER) and/or the progesterone receptor (PR) (Harris *et al.*, 2007). The second characterisation is for human epidermal growth factor receptor 2 (HER2), encoded by the *ERBB2* gene, which is amplified in HER2+ BC (Figure 1). Approximately 80% of BCs are classified as the HR+ subtype (Kohler *et al.*, 2015). The majority of HR+ BCs are ER+ and PR+ (65%), while the rest are exclusively ER+ (Lim *et al.*, 2012). PR+ and not ER+ BC is extremely rare and typically considered a histopathological false positive for PR (Kohler *et al.*, 2015; Lim *et al.*, 2012). The HER2 positive subtype (HER2+) is characterized by overexpression of HER2 and either high or low expression of ER $\pm$ PR (Loibl & Gianni, 2017; Wolff *et al.*, 2018). The HER2+ subtype accounts for ~15 – 20% of BC patients. Triple-negative BC (TNBC) shows low/absence of ER and PR and no overexpression of HER2 (Foulkes *et al.*, 2010). Around 15% of BCs are characterised as TNBC (Reis-Filho & Tutt, 2008). These subtypes form the basis for selection of adjuvant drug treatments for BC patients.

Gene expression microarray analysis was originally used to segregate BCs based on differential gene expression patterns (Perou *et al.*, 2000; Sorlie *et al.*, 2001). Based on the differential gene expression analysis, 6 BC subtypes were identified, subsequently reduced to five subtypes, namely basal-like, normal-like, HER2-enriched, luminal A and luminal B (**Figure 1.1**). These five subtypes could be classified on the basis of the expression of just 50 genes, leading to development of the PAM50 gene signature for BC subtyping model and include basal-like, normal-like, HER2-enriched, luminal A and luminal B (Nielsen *et al.*, 2010; Paik *et al.*, 2004). A 70-gene signature test known as MammaPrint has also been developed to improve the prediction of clinical outcomes in women with early-stage BC (Cardoso *et al.*, 2016). Expanding on subtype classification beyond strictly gene expression, BC tumours have also been classified according to a combination of gene copy number, mutations, and gene expression, resulting in a panel of ten subtypes (Curtis *et al.*, 2012). These subtypes provide insights into the molecular pathways driving tumour growth and potential therapeutic vulnerabilities.

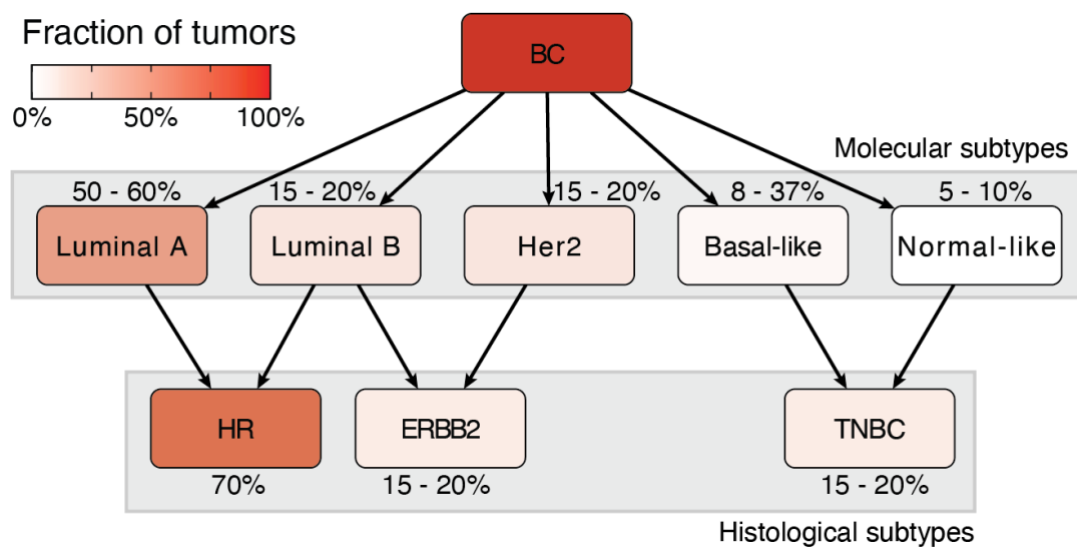


Figure 1.1 Relative composition and classification of breast cancer (BC) subtypes.

Breast cancer can be classified according to molecular (top) and histological (bottom) classification methods. Within each subtype class, node colour reflects the percentage of BC tumours comprising each subtype and the real value or range is also shown. Arrows indicate the major overlap between the two classification methods, which are not mutually exclusive. BC (breast cancer); HR (hormone receptor); TNBC (triple-negative breast cancer). Classifications and fractions of each subtype of BC tumours are defined in literature (Cancer Genome Atlas, 2012; Yersal & Barutca, 2014).

1.1.4 Breast cancer expression subtypes

The Luminal A and B subtypes constitute the majority (~70%) of BC tumours and have extensive genetic and gene expression heterogeneity compared to other subtypes (Cancer Genome Atlas, 2012; Yersal & Barutca, 2014). Luminal A, comprising 50-60% of all tumours, is slow-growing, low grade, and has fewer mutations than other subtypes, with the tumour driver *PIK3CA* mutated in ~45% of luminal A BC (Yersal & Barutca, 2014). Luminal A tumours are characterized by the presence or overexpression of ER, PR, and other proliferation-associated genes, while not overexpressing HER2 (Cancer Genome Atlas, 2012; Yersal & Barutca, 2014). Luminal A tumours generally have good prognosis with lower relapse rates than other subtypes and metastatic recurrences occurring in less than 10% of patients (Kennecke *et al*, 2010).

Luminal B, comprising 15 – 20% of all BC, has worse prognosis than Luminal A (Cancer Genome Atlas, 2012; Creighton, 2012). Typically, luminal B presents with a higher stage due to the higher presentation of proliferation pathways, which is reflected in higher expression of genes such as Ki67 and cyclin E (Reis-Filho *et al*, 2010). Molecularly, Luminal A and B tumours are similar, with *PTEN* and *PI3K* mutations also driving luminal B pathogenicity (Cancer Genome Atlas, 2012; Yersal & Barutca, 2014). Luminal A and B tumours are typically classified as the HR+ clinical subtype, with some tumours of the luminal B subtype clinically also classified as HER2+ (de Ronde *et al*, 2010). HER2+ tumours of the luminal B subtype show high expression of *GATA3*, *BCL2*, and *ESR1* (Prat & Perou, 2011; Yersal & Barutca, 2014).

The basal-like BC subtype is most analogous to the TNBC clinical subtype with 75% of TNBC tumours presenting a basal-like gene expression patterns (Foulkes *et al.*, 2010). Basal-like tumours comprise 8 – 37% of all BCs and are classified as having a basal-like gene expression signature, including expression of CK5 or EGFR (Cancer Genome Atlas, 2012). Basal-like tumours are more common in younger women and are driven by many different pathways such as inactivation of *TP53*, *BRCA1/2*, and *RBI*, and the phosphoinositide-3-kinase (PI3K) pathway activity (Alluri & Newman, 2014; Cancer Genome Atlas, 2012). Patients with basal-like tumours have poor prognosis despite their chemosensitivity and cause a disproportionate number of deaths compared to other subtypes (Bertucci *et al*, 2012).

The normal-like subtype constitutes 5 – 10% of BC tumours and presents an intermediate prognosis between the luminal and basal expression subtypes (Cancer Genome Atlas, 2012; Creighton, 2012). The subtype name reflects the poor characterization as they appear histologically similar to fibroadenomas and normal breast samples (Yersal & Barutca, 2014). All normal-like tumours can be classified as the TNBC clinical subtype (Foulkes *et al.*, 2010). The existence of the normal-like subtype has been challenged by histological studies suggesting this subtype exists due to sample contamination with normal tissue (Weigelt *et al.*, 2010). However, this subtype is populated with tumours that are clinically TNBC samples and stain negatively for the Basal-like markers CK5 and EGFR (Foulkes *et al.*, 2010).

HER2 (HER2-enriched) tumours constitute 15 – 20% of BCs and are generally characterized by HER2 overexpression and low ER and PR expression, except in some luminal B ER+ cases (Cancer Genome Atlas, 2012). HER2+ patients have worse prognosis than those with luminal tumours due to their high proliferation rates and activation of several transcription factors via the HER2 downstream signalling pathways (Gutierrez & Schiff, 2011; Tsutsui *et al.*, 2003). HER2+ tumours show a higher mutation frequency than all other expression subtypes, with *TP53* mutations common and approximately half of all HER2+ tumours also being ER+ (Tsutsui *et al.*, 2003; Yersal & Barutca, 2014). All tumours of the HER2 molecular subtype are of the HER2+ clinical subtypes but only ~50% of clinical HER2+ subtypes are of the HER2-enriched molecular subtype, with the rest being Luminal B (de Ronde *et al.*, 2010; Prat & Perou, 2011).

1.1.5 General breast cancer therapies and therapies by subtype

Currently, BC therapies are personalized according to clinical subtype and patient clinical factors (Moo *et al.*, 2018). BC clinical subtypes present different vulnerabilities that can predict treatment efficacy (de Ronde *et al.*, 2010). Patient clinical factors, such as age and Karnofsky performance scores (*e.g.*, patient quality of life), are considered when deciding on therapies due to the consequences therapies have on patient health (O'Connor *et al.*, 2013; Wang *et al.*, 2019a).

BC therapies can be classified as either localized or systemic. Effective therapy regimens include combinations of multiple localized and systemic therapies (Fisher *et al.*, 2002; Valero *et al.*, 1996). Localized therapies typically consist of surgery and radiation therapy (Fisher *et al.*, 2002; Tyagi & Dhesy-Thind, 2018). Surgery involves either breast-conserving surgery,

with localized tumour excision, or radical mastectomy, which is excision of all breast tissue (Veronesi *et al*, 2002). Surgery is often accompanied with axillary lymph node biopsies to investigate for disease dissemination (McCartan *et al*, 2016). Generally, neoadjuvant therapy is applied first to minimize tumour size prior to surgical resection (van der Hage *et al*, 2001). Neoadjuvant therapies consist of endocrine therapy, such as tamoxifen (Spring *et al*, 2016). Following surgical resection, adjuvant therapies are applied that may continue for years (Early Breast Cancer Trialists' Collaborative, 2005). Localized radiation therapy after surgery reduces the risk of recurrence, improves patient survival, and is especially useful in advanced metastatic BC where a large amount of residual disease may remain (Tyagi & Dhesy-Thind, 2018). However, radiation therapy is not used in pregnant patients or in older patients with advanced disease where radiation therapy may unjustifiably diminish their quality of life (Fenig *et al*, 2001; Stueber *et al*, 2020).

Systemic therapies include endocrine therapies, chemotherapies, targeted therapies for other genes (e.g. for HER2), and immunotherapies (Emens, 2018; Lumachi *et al*, 2011; O'Shaughnessy, 2005). Endocrine, or hormonal, therapies are focussed on limiting estrogen action in cancer cells to impair the growth of malignant cells (Collaborative Group on Hormonal Factors in Breast, 2019; Draganescu & Carmocan, 2017). Endocrine therapies consist of drugs that competitively bind ER to inhibit its activity, and drugs that reduce circulating estrogen levels, preventing ER activation (Lumachi *et al*, 2011). Endocrine therapies are typically adjuvant therapies following surgery and radiation (Early Breast Cancer Trialists' Collaborative *et al*, 2011). Chemotherapies target cancer cells through cytostatic and cytotoxic mechanisms. However, they often have more severe side-effects compared to targeted therapy options (O'Shaughnessy, 2005). Chemotherapy is often used both pre- and post-operatively to minimize tumour size prior to excision and to target residual disease (Early Breast Cancer Trialists' Collaborative, 2005). Other small molecule inhibitors include inhibitors of tyrosine kinase receptor targeting precision inhibitors and CDK4/6 inhibitors, while immunotherapies include chimeric antigen receptor T-cells (CAR-T), and general immune checkpoint blockade inhibitors (Kwa & Adams, 2018; Piccart-Gebhart *et al*, 2005; Yang *et al*, 2022; Zhang *et al*, 2021).

TNBC provides limited treatment options due to the lack of targetable and/or ubiquitous cell surface markers and the heterogeneity of genetic drivers (Yam *et al*, 2017). Aside from surgical resection, chemotherapy remains the standard of care for TNBC with regimens including

anthracyclines and taxanes (Won & Spruck, 2020). Anthracyclines, such as doxorubicin, intercalate DNA, inhibiting topoisomerase II to disrupt DNA replication and repair (McGowan *et al*, 2017; Speth *et al*, 1988). Taxanes, such as paclitaxel, stabilize microtubules and prevent their depolymerization during cell division, leading to cell cycle arrest and apoptosis (Alqahtani *et al*, 2019). Platinum-based chemotherapy agents, like cisplatin and carboplatin, are also used, especially in *BRCA1/2*-mutated TNBC (Tutt *et al*, 2018). These agents form intra- and inter-strand cross-links in the DNA, hindering DNA repair and replication, and thereby inducing cell death (Dasari & Tchounwou, 2014; Fox, 2000). Poly-ADP-ribose polymerase (PARP) inhibitors, such as olaparib and talazoparib, have shown promise in *BRCA1/2*-mutated TNBC (Golan *et al*, 2019; Litton *et al*, 2018). PARP is involved in the single-strand DNA repair pathway (Cortesi *et al*, 2021). *BRCA1/2* are implicated in homology-directed DNA repair (HDR) of double-strand DNA breaks. Inactivating mutations in *BRCA1* or *BRCA2* impair HDR. Inhibiting PARP results in loss of single-strand DNA repair, causing “synthetic lethality” in *BRCA*-deficient cells. Other targeted treatments for TNBC include immune checkpoint inhibitors, such as pembrolizumab, which helps to promote the activation of leukocytes in the tumour microenvironment (Golan *et al*, 2019; Litton *et al*, 2018). Pembrolizumab targets the interaction between PD-1 on immune cells and PD-L1, which helps prevent exhaustion of T-cells within the tumour immune microenvironment (Kwok *et al*, 2016).

HER2+ BC treatments have been revolutionized by the discovery of the HER2 receptor's role in tumour progression (Schechter *et al*, 1984). This discovery has led to the development of HER2-targeted therapies that have markedly improved patient outcomes in this subgroup by targeting HER2 enzymatic activity or with antibody-directed targeting of HER2 (Cameron & Stein, 2008; Molina *et al*, 2001). Trastuzumab (Herceptin) and Pertuzumab are monoclonal antibodies that bind to the extracellular domain of the HER2 receptor to prevent its dimerization and inhibit downstream signalling pathways including the PI3K/Akt and MAPK pathways that regulate cell proliferation and survival (Molina *et al*, 2001; von Minckwitz *et al*, 2017). Humanized trastuzumab also engages the immune system through its Fc region to induce antibody-dependent cellular cytotoxicity in cancer cells (Molina *et al*, 2001). Combination therapies have further enhanced treatment efficacy. For example, the use of trastuzumab in conjunction with chemotherapy has demonstrated synergistic effects, leading to better tumour regression and prolonged survival rates (Tan *et al*, 2021). Pertuzumab, another monoclonal antibody, targets a different region of the HER2 receptor and is often co-administered with trastuzumab to enhance the therapeutic impact (von Minckwitz *et al*, 2017).

Some therapies also consist of a combination of targeted monoclonal antibodies and cytotoxic agents, such as with trastuzumab deruxtecan, which is an antibody-drug conjugate that combines an anti-HER2 antibody, a cleavable linker, and a cytotoxic topoisomerase I inhibitor (Modi *et al*, 2022). This phase 2 study by Modi *et al* on trastuzumab deruxtecan reported a response rate of 60.9%, with a median response duration of 14.8 months and median progression-free survival of 16.4 months. However, trastuzumab deruxtecan is associated with notable adverse events, including anaemia and decreased immune cell counts.

In addition to monoclonal antibodies, small molecule inhibitors have emerged as crucial players in HER2+ BC therapy. Tyrosine kinase inhibitors (TKIs) work by inhibiting the kinase activity of receptor tyrosine kinases, thereby disrupting downstream signalling cascades (Cameron & Stein, 2008). Lapatinib is a small molecule tyrosine kinase inhibitor that targets the intracellular kinase domain of both HER2 and EGFR (HER1). Lapatinib prevents the autophosphorylation and activation of these receptors, thereby disrupting their signalling pathways. Lapatinib is often used in combination with a chemotherapeutic agent and is used particularly for patients who have progressed despite being on trastuzumab-based therapy (Guarneri *et al*, 2022). Combining TKIs with monoclonal antibodies or chemotherapy has demonstrated promising results in both early and advanced HER2+ BC cases (Guarneri *et al*, 2022; Xu *et al*, 2021a).

1.1.6 Resistance to breast cancer therapies

Resistance to trastuzumab and other HER2-targeted therapies is a common clinical issue. Mechanisms of resistance include mutations in HER2, alterations in downstream signalling components, and activation of alternative signalling pathways (Vernieri *et al*, 2019). HER2 isoform expression, typically from alternative splicing, results in impaired targeting by extracellular HER2 inhibitors, such as antibodies like trastuzumab (Scaltriti *et al*, 2007). Constitutive activation of intracellular signalling pathways, often through single-nucleotide variants of signal-transducing factors, can overcome both intra-cellular and extra-cellular HER2-targeted therapies (Berns *et al*, 2007; Trowe *et al*, 2008). As BC develops, alternative and redundant mitogenic signals often become activated, reducing the reliance of the tumour on single growth and proliferation pathways (Wilson *et al*, 2012). Due to the prevalence of acquired resistance to HER2-targeted therapies, novel BC therapies are continuing to be developed for treatment of the HER2+ clinical subtype.

HR+ BCs are most frequently treated with hormone therapy with the primary target being ER+ tumours because all HR+ tumours express the ER. The steroid hormone estrogen can drive BC by activating the ER (**Figure 1.2**) (Fuentes & Silveyra, 2019). ER targeting hormone therapies work through two distinct mechanisms. In the first, levels of circulating estrogen are inhibited using aromatase inhibitors. For postmenopausal women, aromatase inhibitors (AIs) such as letrozole, anastrozole, and exemestane are used to reduce estrogen levels systemically (Kharb *et al*, 2020). Aromatase inhibitors work by blocking the enzyme aromatase, which is responsible for the peripheral conversion of adrenal androgens to estrogens, particularly significant in postmenopausal women where the ovaries have ceased estrogen production (Miller, 1991). In pre-menopausal women, where most estrogen is produced in the ovaries, an ovarian suppression approach is used, in which LHRH agonists are used to inhibit gonadotrophin release from the pituitary gland, resulting in inhibition of estrogen release by the ovaries (Emons & Schally, 1994; Forward *et al*, 2004). The high level of aromatase activity in the ovaries has precluded the use of AIs in pre-menopausal women. However, recent data demonstrate the efficacy of combining AIs with ovarian suppression (Early Breast Cancer Trialists' Collaborative, 2022).

The second approach in ER+ BC is the use of anti-estrogens, which compete with estrogen for binding to ER. The first of these is tamoxifen. Tamoxifen was the first targeted hormone therapy drug for ER+ BC (Jordan & Koerner, 1975). Tamoxifen acts by competitively binding to the ER to inhibit expression of ER target genes and consequently estrogen/ER-directed cell growth and proliferation (**Figure 1.2**). Tamoxifen is a frequently employed anti-cancer drug for both pre- and post-menopausal women. In pre-menopausal women, tamoxifen is used in combination with ovarian suppressing LHRH agonists (Francis *et al*, 2015). Interestingly, while tamoxifen acts as an estrogen antagonist in the mammary epithelium and BC, it exhibits agonistic properties in the uterine epithelium and bone (Gallo & Kaufman, 1997). This mixed estrogenic/antiestrogenic action of tamoxifen has led to its designation as a selective ER modulator (SERM) (O'Regan & Jordan, 2002). Several other SERMs have been developed, although none are commonly used for treatment of ER+ BC.

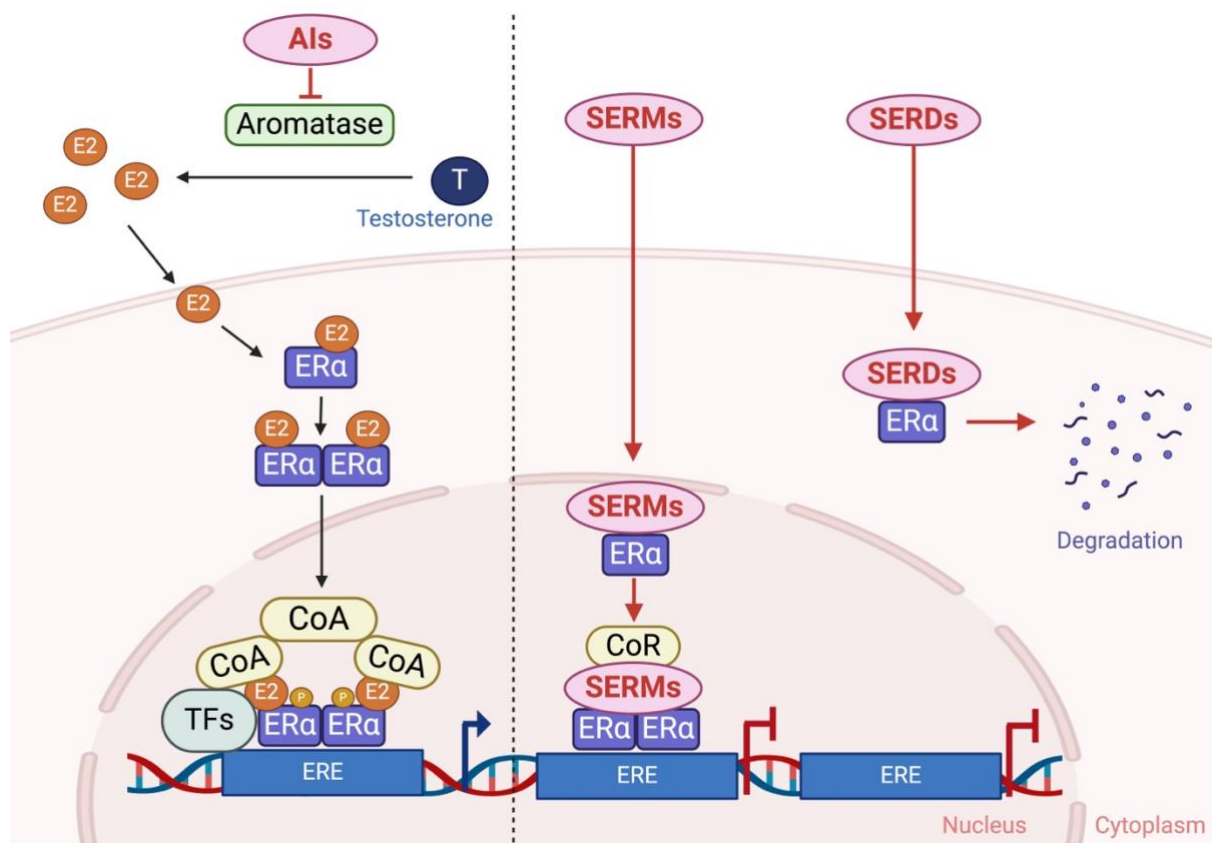


Figure 1.2 Mechanisms of endocrine therapy disrupting the estrogen receptor (ER) signalling pathway

(Left) Estrogen (E2) binding to estrogen receptor- α (ER α) promotes its dimerization and translocation into the nucleus, where it binds to estrogen response elements (EREs) and recruits coactivators (CoA) and transcription factors (TFs), leading to the transcription of estrogen-responsive genes. E2 can be synthesized from testosterone by aromatase, which is inhibited by aromatase inhibitors (AI; *e.g.* anastrozole, letrozole, exemestane). (Right) Selective estrogen receptor modulators (SERMs; *e.g.*, tamoxifen) and selective estrogen receptor degraders (SERDs; *e.g.*, fulvestrant) compete with E2 to bind ER α . SERM binding, which may lead to the recruitment of corepressors (CoR), inhibits gene transcription from the EREs. SERDs binding promotes proteasomal ER α degradation. Figure was generated using BioRender.

A second class of anti-estrogens, typified by fulvestrant, bind to the ER and trigger a conformational change, resulting in the degradation of the receptor via endocytosis and proteolysis (Osborne *et al*, 2004), resulting in their designation as selective ER degraders (SERDs). Due to its action in degrading ER, fulvestrant is used as a treatment option for patients who have developed resistance to other forms of hormone therapy (Osborne *et al*, 2004). One of the issues with fulvestrant is the fact that it is not orally bioavailable, necessitating regular intramuscular injection and thus limiting its use. Its desirable activities as an ER degrader has led to the development of a new generation of oral SERDs, such as elacestrant, which has recently been approved by the FDA for patients in which resistance arises due to activating ER mutations (Patel *et al*, 2023).

Resistance to hormone therapies is an important clinical problem. As many as 30% of patients diagnosed with localised, primary ER+ BC, and almost all patients diagnosed with metastatic disease fail on hormone therapies (Early Breast Cancer Trialists' Collaborative *et al*, 2011). Further, for ER+ BC patients, risk of relapse does not fall over time, even for patients who initially presented with the smallest tumours and localised (lymph node-negative) disease and successfully completed five years of hormone therapy (Pan *et al*, 2017).

Recent sequencing analysis of metastatic ER+ BC revealed new insights into genetic changes that accompany acquired resistance to hormone therapies (Bertucci *et al*, 2019; Razavi *et al*, 2018). A major discovery has been the identification of mutations in the ER (*ESR1*) gene (Jeselsohn *et al*, 2015; Katzenellenbogen *et al*, 2018; Will *et al*, 2023). The overwhelming majority of these mutations map to the ER hormone binding domain (Rinaldi *et al*, 2020) and every mutation that has been functionally assessed has been shown to be active in the absence of estrogen. The mutations also show reduced sensitivity to anti-estrogens, with the greatest resistance to anti-estrogens being observed for the Y537S and D538G mutations. Interestingly, *ESR1* mutations are rare in primary, pre-hormone treatment BC but are detected in 20 – 40% of metastatic BC, implying enrichment of rare cells with pre-existing *ESR1* mutations, or acquisition of new mutations in the *ESR1* gene. These observations indicate that *ESR1* mutations are clearly selected for by the hormonal therapy.

Other possible mechanisms of resistance resulting in altered regulation of ER target genes include overexpression or altered expression of ER co-activators, such as the p160 co-activator family members, in particular amplified in breast cancer 1 (AIB1) (Anzick *et al*, 1997) and

decreased expression of co-repressors such as NCoR and SMRT (Graham *et al*, 2000; Mottis *et al*, 2013; Smith *et al*, 1997). Altered balance of expression between these co-activators and co-repressors has been associated with reduced activities of anti-estrogens such as the SERM tamoxifen (Blackmore *et al*, 2014; Frasor *et al*, 2005; Kiliti *et al*, 2023; Li *et al*, 2021c; Walsh *et al*, 2012).

Resistance to hormone therapies may also be achieved independently of ER expression. Bypass signalling pathways, such as *EGFR* and *HER2* which may be upregulated or hyperactive in hormone therapy-resistant BCs, provide alternative methods for tumour survival and proliferation by activating the PI3K/Akt/mTOR and MAPK/ERK pathways (Campbell *et al*, 2001; Kato *et al*, 1995; Miller, 2013). Sequencing of advanced, hormone-therapy-resistant ER+ BC revealed amplification and mutations that result in increased MAPK activity, observed in nearly 20% of cases (Razavi *et al*, 2018). Diverse genetic alterations that would increase MAPK pathway activity were observed, including loss-of-function *NF1* mutations, activating mutations in *ERBB2* (*HER2*), and high expression of *EGFR* and *KRAS*. Also interesting was the fact that *ESR1* mutations and MAPK pathway alterations were not generally observed in the same metastatic tumours. Although MAPK hyperactivation can promote tumour survival and aid proliferation independent of ER, many studies have shown that EGFR/MAPK-directed ER phosphorylation can cause estrogen-independent ER activation, raising the possibility that in metastatic BC MAPK alterations may function, at least in part, through activation of ER and the ER transcriptome (Murphy *et al*, 2011). In strong support of this possibility, uterine proliferation which is estrogen-dependent can be rescued in ovariectomized rodents with epidermal growth factor (EGF), but this rescue is entirely dependent on the presence of ER (Curtis *et al*, 1996; Ignar-Trowbridge *et al*, 1992).

Sequencing studies of metastatic BC identify acquisition of genetic alterations in the *ESR1* gene (~20%), MAPK pathway (~20%), and other transcription factors (~10%) , which means that no genetic alterations have been identified in the remaining 50% of cases (Razavi *et al*, 2018). This may be because additional genetic alterations remain undefined, for example, because only targeted gene panels or exomes have been sequenced and genetic alterations in the non-coding genome have so far escaped identification in hormone therapy resistant BC (Li *et al*, 2020c; Rheinbay *et al*, 2020). Alternatively, epigenetic alterations may play important roles in contributing to the development of hormone resistance (Achinger-Kawecka *et al*, 2020; Bi *et al*, 2020; Nguyen *et al*, 2015). For example, a population of hormone therapy-resistant

persistent cells has been found that contain distinguishable epigenetic modifications maintained by the histone demethylase KDM5A (Sharma *et al*, 2010).

While hormone therapies have revolutionized the treatment of ER+ BC, resistance to these therapies poses an important clinical challenge. The diverse resistance mechanisms and heterogeneous cancer genomes further complicates identification of potential therapeutic liabilities. However, there have been important recent developments in the identification of drugs targeting key kinase signalling and cell cycle drivers in BC, which are described in the sections below.

1.2 The cell cycle and cyclin-dependent kinases in the cell cycle

1.2.1 The cell cycle and CDK background

BC is driven by the de-regulation of the cell cycle resulting in unregulated cell growth and division. Cell division consists of three main events, growth, DNA replication and mitosis, which can be summarised by the four main phases of the cell cycle: the gap 1 (G1), DNA synthesis (S phase), gap 2 (G2), and mitosis (M phase) (**Figure 1.3**) (Schafer, 1998). Immediately following mitosis, cells can enter a quiescent state known as G0 where they do not proceed through the cell cycle. Cells in G0 represent a significant proportion of non-growing and non-proliferating cells that continue to perform normal cellular functions. Quiescence can be triggered by environmental cues, such as a lack of nutrients, and occurs when cells exit the cell cycle but are still capable of renewed division upon stimulation by mitogens (Marescal & Cheeseman, 2020). Entry into quiescence can be advantageous to cancer cells as it provides a means of protecting certain cell populations from cancer therapies (Nik Nabil *et al*, 2021). For example, entry into G0 can prevent cell starvation due to nutrient depletion, avoid DNA damage from exogenous agents, and minimize competition from selective pressure of the tumour microenvironment (Pack *et al*, 2019; Terzi *et al*, 2016). As a result, quiescent cancer cells often avoid death from cancer therapies and persist as recurrent or metastatic tumours after re-entering cell cycling.

After mitosis or quiescence, but before DNA synthesis, is a gap phase known as G1, during which cells grow and prepare for DNA synthesis (Bertoli *et al*, 2013; Schafer, 1998). Entry into G1 is triggered by mitogenic signalling. In G1, cells grow and increase their metabolic activity, including increased expression of cell cycle genes. A critical stage of G1 is the

restriction point, which represents a "point of no return" where cells will continue to cycle independently of mitogenic signalling. The restriction point is characterized by sufficient accumulation of CDK4, CDK6, their cognate cyclin D, and high retinoblastoma protein phosphorylation.

Before entry into the S phase, genome integrity is evaluated and if cells pass the cell cycle checkpoint, they will continue through S phase and replicate the genome (Segurado & Tercero, 2009). Following S phase is another cell cycle checkpoint where genome integrity is evaluated, then another gap phase called G2 where the cell prepares for mitosis. Lastly, cells enter mitosis where cellular division takes place. Cell cycle progression is tightly regulated by various cellular proteins including key TFs, several checkpoint and surveillance factors, and cyclin-dependent kinases (CDKs) and their partner cyclins (Vermeulen *et al*, 2003).

1.2.2 E2F transcription factors regulate cell cycle progression via transcription programs

The E2F family of transcription factors are key cell cycle regulators that act as either transcriptional activators or repressors. There are four E2F subgroups based on their mechanism of action. First, E2Fs 1 – 3 promote the transcription of genes involved in several cell cycle processes including cell cycle regulators (*e.g.*, cyclin A and E, CDK1 and CDK2), DNA replication and repair genes (*e.g.*, DNA polymerase α , BRCA1), and apoptosis regulators (*e.g.*, p14ARF and p73) (Cam & Dynlacht, 2003; Trimarchi & Lees, 2002; Wu *et al*, 2001b). E2F2 and the E2F3a isoform share many target genes with E2F1 and promote cell cycle progression from G1 to S phase (Cam & Dynlacht, 2003; Trimarchi & Lees, 2002). This family of E2F transcriptional activators is typically bound and inhibited by hypophosphorylated RB in early G1 (Attwooll *et al*, 2004; Burkhardt & Sage, 2008; Dick & Rubin, 2013). Phosphorylation of RB by CDKs 4/6 and CDK2 releases these E2Fs, enabling them to activate gene transcription (Akiyama *et al*, 1992; Burkhardt & Sage, 2008; Dick & Rubin, 2013). Further, repressors compete with activator E2Fs for binding to target gene promoters, thereby modulating gene expression. Second, E2F4 and E2F5 are transcriptional repressors, primarily active in G0 and early G1 phases to prevent entry into the S phase (Muller *et al*, 1997; Verona *et al*, 1997). E2F4 and E2F5 form complexes with RB and the RB-like proteins p107 and p130 to repress target genes (Burkhardt & Sage, 2008; Dick & Rubin, 2013). Lastly, E2F6, E2F7, and E2F8 lack the classic transactivation domains found in other E2F members, act as transcriptional repressors, and can function independently of the RB family proteins to initiate

stable epigenetic silencing via the polycomb transcriptional repressor complex (Dahlet *et al*, 2021; de Bruin *et al*, 2003; Di Stefano *et al*, 2003; Trimarchi *et al*, 1998).

The E2F family of transcription factors regulates gene expression through the cell cycle by binding to specific gene promoters (Attwooll *et al*, 2004). E2F target binding specificity is enhanced through interactions with dimerization partners, which stabilize the E2F-DNA complex (Magae *et al*, 1996). Additionally, E2Fs often interact with other cofactors and accessory proteins, such as p300/CBP (CREB-binding protein) and PCAF (P300/CBP-associated factor), to form transcriptional complexes (Lang *et al*, 2001). Such cofactors are histone acetyltransferases, which facilitate chromatin accessibility and Pol-II assembly at gene promoters. E2F target genes have diverse roles in cell physiology. For example, E2Fs regulate genes involved in DNA replication (*e.g.*, MCM2, MCM3, and ORC1), cell cycle progression (*e.g.*, cyclin E, cyclin A, and CDK2), DNA repair (*e.g.*, BRCA1 and RAD51), and apoptosis, (*e.g.*, p73 and APAF1), thereby integrating cell cycle regulation with cell survival mechanisms (Ishida *et al*, 2001; Polager *et al*, 2002; Ren *et al*, 2002).

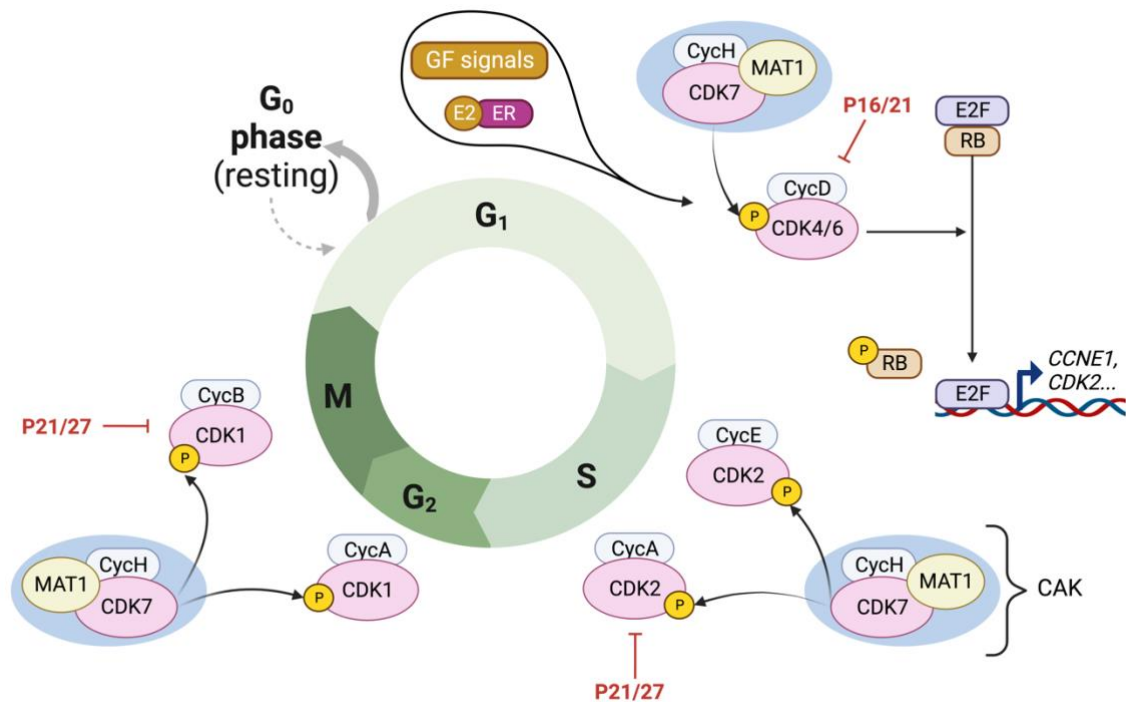


Figure 1.3 The mammalian cell cycle is driven by expression of cyclins and activation of cyclin-dependent kinases.

Growth factor (GF) and mitogenic signalling, such as through the estrogen receptor (ER), stimulates quiescent cells to enter G1 through transcription of early cell cycle genes. The CDK activating kinase (CAK), comprised of the CDK7/cyclin H/MAT1 complex phosphorylates and activates CDK4/CDK6. CDK4/6-cyclin D phosphorylates the retinoblastoma (RB) protein, facilitating RB release from E2F, to allow for E2F-mediated transcription. Among the E2F target genes are cyclin E, which bind to CDK2 for its activation. CAK activates CDK2-cyclinE/A via phosphorylation. The CDK2-cyclin E complex further phosphorylates RB resulting in expression of cyclin A and CDK1. Cyclin A replaces cyclin E as CDK2's binding partner and is also the cyclin binding partner for CDK1. Following CAK activation, CDK1-cyclin A drives the S phase to G2 transition where cyclin B is expressed. CDK1-cyclin B drives mitosis and entry into G1. CIP/KIP proteins, including p21 / 27, and the INK4 protein p16 can inhibit CDKs. CCNE1, cyclin E1; RB, retinoblastoma protein; Cyc, cyclin; CDK, cyclin-dependent kinase; CAK, CDK-activating kinase. Figure was generated using BioRender.

1.2.3 Cyclin-dependent kinases

CDKs are serine/threonine protein kinases that require binding to their cognate partner cyclins for full activation and function (Malumbres, 2014). There are 21 CDKs and five additional CDK-like kinases in the human genome (Malumbres *et al*, 2009). Among these CDKs, five play pivotal roles through the different stages of the cell cycle, including G1 (CDK4 and CDK6), S (CDK2), G2, and M (CDK1), as well as CDK7 (described below). Upon activation, CDKs initiate downstream processes by phosphorylating specific target proteins. CDKs were first discovered in yeast with the identification of *cdc28* in *Saccharomyces cerevisiae* and *cdc2* in *Schizosaccharomyces pombe* (Hartwell *et al*, 1974; Hartwell *et al*, 1973; Nurse *et al*, 1976). Compared to yeast and other unicellular organisms, complex multicellular organisms require proliferation control of numerous cell types. This may be the reason for the evolutionary expansion in the numbers of CDK and cyclin genes in metazoans. The metazoan CDK family consists of two groups based on their activities: those involved in cell cycle regulation (CDKs 1-6, 11, 14-18) and those involved in transcriptional regulation (CDKs 7-13, 19 and 20) (Malumbres *et al*, 2009). Interestingly, while several CDKs are required for appropriate cell cycle progression, CDK1 is the only CDK that is absolutely essential for complete cell cycle progression in mammals, as is the case in yeast cells (Enserink & Kolodner, 2010; Huang *et al*, 2007). For example, mice in which the *Cdk2*, *Cdk4* and *Cdk6* genes have been knocked out show evidence for organogenesis and continue into midgestational stages (Santamaria *et al*, 2007). By contrast, there is failure in the blastocyst stage in *Cdk1* knockout mice. These and other findings have led to the proposal that while CDK1 is essential for the cell cycle, the interphase CDKs (CDK2, CDK4, CDK6) are necessary in a tissue-specific manner. However, it has further been proposed that cancer cells may have greater dependency on interphase CDKs than normal cells (Malumbres & Barbacid, 2009), which raises the possibility of selective CDK inhibitors as cancer targets.

1.2.4 CDK structure and substrate recognition

CDKs demonstrate a mostly conserved structural homology, with some CDKs containing additional domains with specialized functions (Wood & Endicott, 2018). In the conserved part of CDKs, the proteins have a two-lobed structure with the active site hidden between a N-terminal beta-sheet and the C-terminal alpha-helices. The N-terminal lobe contains a glycine-rich region termed the G-loop, which negatively regulates CDK activity when phosphorylated, and the P-loop, which is involved in ATP phosphate binding (Bartova *et al*, 2004; Hanks & Quinn, 1991). The N-terminal lobe also contains a major helix called the C-helix, sometimes

known as the PSTAIRE due to the consensus amino acid sequence of Pro-Ser-Thr-Ala-Ile-Arg-Glu, which binds cyclins (Wood & Endicott, 2018). Conformational changes in the C-helix upon cyclin binding reveals the ATP-binding and enzymatic site. The C-terminal lobe contains the activation loop, called the T-loop, and the substrate-binding and catalytic regions. The T-loop must be phosphorylated to fully expose the substrate binding site and so this phosphorylation is important for the CDK kinase activity.

In addition to the CDK kinase motif, CDK-substrate specificity depends on the specific CDK-cyclin complex, timing of CDK-cyclin activation, sub-cellular localization, accessory proteins, and post-translational modifications of the substrate (Bartova *et al.*, 2004; Gu *et al.*, 1992; Jackman *et al.*, 2002; Kornev & Taylor, 2015; Lees *et al.*, 1992; Obaya & Sedivy, 2002; Solomon *et al.*, 1992; Takeda *et al.*, 2001). CDKs phosphorylate specific amino acid residues on substrates that have a motif recognized by the respective CDK (Malumbres, 2014). Cyclins confer specific conformations once they combine with CDKs and form the CDK-cyclin complex (Kornev & Taylor, 2015). The conformational change of a CDK-cyclin complex defines substrate specificity. For example, the complex of CDK2 with cyclin A targets different proteins compared to the complex of CDK2 with cyclin E, even though the CDK component remains the same (Lees *et al.*, 1992). Temporally, cyclin binding activates CDKs to phosphorylate substrates that are proximal to the CDK-cyclin complex. Thus, some CDK substrates may not be targeted under normal cell cycle conditions because they are not expressed when the CDK-cyclin complex is active (Lees *et al.*, 1992). Regionally, cyclins may directly bind the substrate in specific subcellular location, activating the CDK only in that location (Jackman *et al.*, 2002). Various accessory proteins and docking motifs interacting with or comprising the CDK-cyclin complex can influence CDK substrate recognition (Wood & Endicott, 2018). For example, some CDK substrates have docking motifs that enhance their affinity for a particular cyclin-CDK complex, further refining target specificity (Takeda *et al.*, 2001). Lastly, phosphorylation or other modifications of either the CDK, cyclin, or target protein can also affect substrate recognition and binding affinity (Gu *et al.*, 1992).

1.2.5 Activation of CDKs

Activation of a CDK is a two-step process (Malumbres, 2014). First, a cyclin partner binds the CDK resulting in low-level catalytic activity. Second, the CDK is phosphorylated at a conserved threonine site within the T-loop by the CDK-activating kinase (CAK), which facilitates greater kinase activity. However, CAK phosphorylation may be necessary for

formation of a complete CDK-cyclin complex for some cell cycle CDKs (Fisher, 2012). CAK is a complex consisting of CDK7, Cyclin H, and the accessory protein MAT1 (Solomon *et al.*, 1992). CAK is constitutively active in eukaryotic cells throughout the cell cycle to activate CDK-cyclin complexes as they form and ensure proper cell cycle progression (Bartkova *et al.*, 1996). CAK substrate specificity is regulated through MAT1-mediated CAK formation and MAT1-dependent and cyclin H-dependent protein-protein interactions (Shiekhhattar *et al.*, 1995). The variety of mechanisms regulating CDK activation demonstrates the importance of tightly modulating CDK activities for cell cycle progression.

Temporal regulation of CDK activity is controlled through association of CDKs with cyclin partners. In its inactive state, the substrate binding site of the CDK is blocked by the T loop (Johnson & Lewis, 2001). Upon binding to the cyclin partner, conformational changes in the CDK C-helix, containing the conserved PSTAIRE cyclin-binding motif, leads to displacement of the T-loop (Bartova *et al.*, 2004). Displacement of the T-loop enables its phosphorylation via the CAK, further displacing the T-loop, providing rigidity to the CDK-cyclin complex, and activating the kinase. Lastly, T-loop phosphorylation exposes the substrate-binding site, making CDK interactions with substrates possible.

1.2.6 Cyclin expression regulates CDK activation

CDK activity and substrate specificity is dependent on binding to a cyclin partner. In contrast to CDKs, cyclin protein levels exhibit periodic fluctuations during the cell cycle. Cyclin expression is mostly controlled at the transcriptional and post-translational levels (Musgrove, 2006). For example, cyclin D and E levels are regulated at the transcriptional level by growth factor signalling pathways (Klein & Assoian, 2008; Sherr & Roberts, 1999). Post-transcriptional regulation of cyclins via RNA helicases and microRNAs controls the translational rates (Chivukula & Mendell, 2008). For example, cyclin E translation is facilitated by the RNA helicase DDX3 while cyclin D levels are controlled by the microRNA MiR-17/20 in some BCs (Lai *et al.*, 2010; Yu *et al.*, 2008). Cyclins are also rapidly proteolyzed and degraded to ensure their levels oscillate appropriately during the cell cycle. For example, the anaphase-promoting complex/cyclosome (APC/C) targets cyclin B for degradation during exit from mitosis (Clute & Pines, 1999). Lastly, the subcellular location of cyclins also plays a role in cyclin function, such as in the regulated translocation of cyclin B from the cytoplasm to the nucleus at the onset of mitosis to activate CDK1 (Pines & Hunter, 1991).

1.2.7 Cyclin-activating kinase (CAK) is necessary for cell cycle CDK activation

As mentioned above, CAK regulates the activity of cell-cycle CDKs by promoting their activation through T-loop phosphorylation (Lolli & Johnson, 2005). A minimal functional CDK7 kinase consists of CDK7 bound to cyclin H (Adamczewski *et al*, 1996). However, CAK formation and activity is facilitated by the scaffold protein MAT1, which stabilizes the complex and enhances CDK7 kinase activity (Fisher *et al*, 1995; Wu *et al*, 2001a). Phosphorylation and ubiquitination modulate CAK activity. However, the consequences of CDK7 phosphorylation are context dependent with phosphorylation directing CDK7 activity depending on the stage of the cell cycle (Fisher, 2019; Martinez *et al*, 1997). For example, T-loop phosphorylation is possible at two sites in CDK7, with phosphorylation directing CAK activity towards either transcription elongation or CDK activation (Larochelle *et al*, 2001; Martinez *et al*, 1997; Schachter *et al*, 2013). Cyclin H is activated through phosphorylation by CK2 and can also be regulated by binding p53 (Schneider *et al*, 2002; Schneider *et al*, 1998). Consistent CAK activity is important because CAK is a regulator of both cell cycle and transcription, the latter as a part of the general transcription factor IIH (TFIIH) (Bartkova *et al*, 1996; Lolli & Johnson, 2005). In the context of DNA repair, CAK dissociation from TFIIH is essential for proper nucleotide excision repair (NER) (Abdulrahman *et al*, 2013; Coin *et al*, 2008). However, CAK activity is also important for the process of DNA repair as CAK facilitates DNA unwinding by XPD, a DNA helicase subunit of TFIIH (Abdulrahman *et al*, 2013). These multiple roles of CAK establish CDK7 as an essential kinase for activating CDKs (Ganuza *et al*, 2012; Larochelle *et al*, 1998; Rimel & Taatjes, 2018).

1.2.8 Cell cycle checkpoints and phosphatases downregulate CDK activity

The cell cycle is arrested at critical points in response to DNA damage at two major cell-cycle interphase checkpoints that exist before and after DNA synthesis in the G1 and G2 phases, respectively (Pizzul *et al*, 2022). The DNA damage signalling pathway is initiated when Ataxia Telangiectasia and Rad3-related protein (ATR) or Ataxia Telangiectasia Mutated protein (ATM) detects DNA damage. ATR responds to broad-spectrum DNA damage while ATM responds to double-stranded DNA breaks (Marechal & Zou, 2013). Activation of either of these proteins leads to phosphorylation and activation of CHK1 or CHK2. Activated CHK2 plays a role in the activation of the tumour suppressor protein p53, leading to p53-dependent arrest in the early G1 phase (Hirao *et al*, 2000). Activation of p53 results in the induction of the CDK inhibitor (CKI) gene p21^{CIP1}, which impairs CDK1-cyclin B, CDK2-cyclin E, and CDK4/6-cyclin D complex formation (Al Bitar & Gali-Muhtasib, 2019; Zalzal *et al*, 2015) (see also

below). If DNA repair cannot be completed or if cells cannot tolerate extended cell-cycle arrest, they are directed toward apoptosis by p53.

Inactivation of CDKs successively throughout the cell cycle is important for ensuring proper cell cycle progression. First, phosphorylation and dephosphorylation of CDKs at specific residues can inhibit CDK activity (Chow *et al*, 2011). For example, CHK1 activates CDC25C, a CDK phosphatase, which results in cell-cycle arrest in the G2 phase via T-loop dephosphorylation of CDK1 (Liu *et al*, 2019). Similarly, failure to downregulate CDK activity leads to cell cycle arrest, with the CDC14 phosphatase being a key player in mitotic exit and essential for CDK downregulation (Visintin *et al*, 1998). CDKs also have phosphorylation sites that directly regulate their inhibition (Bartova *et al.*, 2004). Inhibitory phosphorylation sites occur at the P-loop of the active site, with such phosphorylation interfering with the catalytic core and ATP binding pocket, sometimes in a CHK1/WEE1-dependent mechanism (Mueller *et al*, 1995). CDK activity can further be suppressed through the phosphorylation of residues in the G-loop (Bartova *et al.*, 2004). Lastly, CDKs may also be inactivated by the loss of their cyclin binding partner, which may occur through various mechanisms, including rapid cyclin degradation (Clute & Pines, 1999).

1.2.9 CIP/KIP inhibitors of CDK-cyclin complexes

Two CDK inhibitor (CDKI) gene families, namely CIP/KIP (p21, p27, and p57) and INK4 (p16, p15, p18 and p19), provide an additional layer of CDK activity regulation (Canepa *et al*, 2007; Cerqueira *et al*, 2014). The CIP/KIP family contains three members: p21^{CIP1} (CDKN1A), p27 (CDKN1B) and p57 (CDKN1C), which contain conserved N-terminal regions responsible for mediating their interaction with cyclin-CDK complexes (Galea *et al*, 2008). Typically, CIP/KIP family members inhibit CDK-cyclin A/B/E complexes; however, at lower concentrations, p21 and p27 have been reported to increase the activity of CDK4 and CDK6, thus providing a proliferative advantage to the cell (Cheng *et al*, 1999; LaBaer *et al*, 1997; Sugimoto *et al*, 2002). p21^{CIP1} (p21) is a p53 target gene (Engeland, 2022). Upon activation of p53, often through activation of DNA damage responses, expression of p21 increases rapidly (Nguyen *et al*, 2018). p21 has been observed to interact with CDK complexes involved in the S phase and mitosis, specifically CDK2-cyclin E and CDK4/6-cyclin D (Xiong *et al*, 1993). Additionally, p21 inhibits DNA synthesis by binding to PCNA, a key regulator of DNA replication and cell cycle regulation (Pan *et al*, 1995; Waga *et al*, 1997).

The primary targets of p27 inhibition are CDK2 and CDK4 (Cerqueira *et al.*, 2014; Guiley *et al.*, 2019). p27 is predominantly active during G0 and early G1 phases of the cell cycle when it inhibits the CDK2–cyclin A complex. However, p27 also binds to cyclin E via its cyclin binding domain to suppress CDK2 activity. By inactivating CDK2–cyclin A, p27 inhibits RB phosphorylation, keeping E2F1 inactive and preventing the G1 – S transition (Wang *et al.*, 2005). Without the inhibition by p27, CDK2 phosphorylates RB, promoting its dissociation from the E2F1 transcription factor, which leads to E2F1 activation and expression of genes that promote progression to the S phase. E2F1 itself can bind to the p27 promoter, activating p27 transcription and protein expression (Wang *et al.*, 2005). Thus, p27 acts in a negative feedback mechanism that regulates E2F1 activity.

While *in vivo* studies have established that p27 predominantly inhibits CDK2 complexes, it is also capable of inhibiting CDK4, CDK6, and CDK1 *in vitro* (Guiley *et al.*, 2019). However, in certain classes of proliferating cells such as keratinocytes and some epithelial and lymphoid cells, p27 does not inhibit CDK4-cyclin D (Malumbres & Barbacid, 2009). Instead, p27 can promote CDK4 and CDK6 activity (Cheng *et al.*, 1999; Guiley *et al.*, 2019). The stoichiometry of p21 changes between activating CDK4 and CDK6 to inactivating cyclin-CDK complexes, depending on the amounts of p21; active complexes contain a single p21 molecule, while the presence of two molecules is inhibitory for CDKs. Expression of p27 increases in response to transforming growth factor β (TGF- β), contributing to the induction of growth arrest in specific tissue environments (Hannon & Beach, 1994; Reynisdottir *et al.*, 1995).

p57 targets CDK4 and CDK6-cyclin D and sustains the activity of CDK2-cyclin E, promoting the completion of the G1 phase and facilitating entry into the S phase (Gomez Lahoz *et al.*, 1999). p57 also maintains the activity of CDK2-cyclin A and increases cyclin A levels, thus aiding progression through the S phase (Malumbres & Barbacid, 2009). p21 and p57 can further modulate cell cycle progression through interactions with and inhibition of PCNA (Luo *et al.*, 1995). p57 also serves as an additional barrier against uncontrolled proliferation by indirectly influencing telomere length maintenance (Nijjar *et al.*, 1999). p21 and p57 are also crucial in cellular stress responses, balancing cell cycle arrest, apoptosis, and senescence (Joaquin *et al.*, 2012). p21 is unique in its role as a mediator of cell cycle arrest downstream of the tumour suppressor p53 (el-Deiry *et al.*, 1993). Cellular stresses like DNA damage and oncogene activation stimulate p53 expression, which then activates its targets including pro-

apoptotic genes (Bax, PUMA, Noxa) and p21 (El-Deiry, 2003; Mandinova & Lee, 2011; Vousden & Prives, 2009).

1.2.10 INK4 family inhibits CDKs independently of cyclin binding

The INK4 family of inhibitors specifically inhibit CDK4 and CDK6. While the CIP/KIP family of proteins primarily interacts with assembled cyclin-CDK complexes, the INK4 family, consisting of p15^{INK4b} (CDKN2B), p16^{INK4a} (CDKN2A), p18^{INK4C} (CDKN2C), and p19^{INK4D} (CDKN2D) proteins mainly interact with CDK4 and CDK6 independently of cyclin binding (Serrano *et al*, 1993). p15 is an effector of TGF β signalling, and p16 inhibits the activity of CDK4 and CDK6 to arrest the cell cycle at the G1 phase in response to reactive oxygen species, DNA damage, or cell senescent signals (Hannon & Beach, 1994; Rayess *et al*, 2012; Reynisdottir *et al.*, 1995). By directly interacting with CDK4 and CDK6, p15 and p16 prevents CDK4 and CDK6 from forming functional complexes with cyclin D, therefore, halting cell cycle progression to the S phase. Similarly, p18 and p19 specifically inhibit CDK4 and CDK6 (Hirai *et al*, 1995). However, p18 and p19 inhibition is not competitive with cyclin D while p16 appears to compete with cyclin D for CDK4 binding.

INK4 proteins also regulate cell cycling through more conventional tumour-suppressor activities. The expression of p16 maintains RB family proteins in a hypophosphorylated state, facilitating their binding to E2F1 and leading to G1 cell cycle arrest (Serrano *et al*, 1997). The expression of p16 significantly increases with age in various tissues (Ressler *et al*, 2006; Zindy *et al*, 1997), emphasizing the importance of this tumour suppressor in the processes of aging and senescence (Zhu *et al*, 2002). Further, p16 overexpression has been observed in senescent fibroblasts and in response to oxidative stress, DNA damage, and alterations in chromatin structure (Canepa *et al.*, 2007; Quereda *et al*, 2007; Wu *et al*, 2007). Collectively, the INK4 proteins provide an important mechanism for preventing uncontrolled cell growth and proliferation, as well as ensuring proper DNA replication and cell division, the dysregulation of which is a hallmark of cancer (Hanahan & Weinberg, 2011).

1.2.11 CDKs and cyclins in the cell cycle

The most critical CDKs involved in cell cycle regulation are CDK4 and CDK6, CDK2, and CDK1 (Malumbres & Barbacid, 2009). CDK4 and CDK6 are predominantly active in the G1 phase, binding cyclin D and facilitating cell cycle transition into the S phase via the inactivation of RB, which leads to the subsequent expression and activation of CDK2 via genes regulated

by E2F transcription factors. CDK2, which binds cyclin E and A, is responsible for progression through the S phase via a CDK2-RB positive feedback mechanism, initiation of DNA replication, and continuation through S phase checkpoints. CDK1 binds mostly cyclin B and is essential for initiating and ensuring continued progression through mitosis.

1.2.12 CDK4/6 and its role in G1 progression

CDK4 and CDK6 (CDK4/6) have exceptionally high sequence conservation and appear to have redundant functions in driving the cell cycle in response to mitogenic signalling (Malumbres *et al*, 2004; Sherr & Roberts, 1999). While non-transformed mammalian cells can proliferate without CDK4/6, the kinases are tightly regulated because of their key role in regulating cell cycle progression from G1 to S phase (Kozar *et al*, 2004; Malumbres *et al.*, 2004). CDK4/6 associates with D-type cyclins (cyclin D1, D2, and D3). Cyclin D1 is the canonical driver of CDK4/6 activity and cell cycle progression (Sherr & Roberts, 2004). Consequently, high cyclin D transcription is considered the rate-limiting step for CDK4/6 activation.

Activation of mitogen signalling pathways via exogenous signalling or soluble mitogenic factors triggers the transcription of cyclin D (Assoian & Klein, 2008). Specifically, RTK signalling through the Ras-Raf/MAPK kinase (MEK)-extracellular signal-regulated kinase (ERK) pathway enhances cyclin D1 transcription via the ETS transcription factor (Albanese *et al*, 1995; Lavoie *et al*, 1996; Weber *et al*, 1997; Winston *et al*, 1996) (Weber *et al.*, 1997). Several other transcription factors regulate cyclin D expression including downstream effectors of RAS-activated PI3K /RAL and CREB/ATF2 (D'Amico *et al*, 2000; Gille & Downward, 1999; Musgrove, 2006). Cyclin D has a very fast turnover rate in the cell (Alao, 2007). Post-translationally, cyclin D levels are regulated by glycogen synthase kinase 3 β (GSK-3 β) (Musgrove, 2006). Akt activation, via PI3K, inactivates GSK-3 β thus stabilizing cyclin D.

Expression of cyclin D genes in G1 enables the formation of the active CDK4/6-cyclin D complex (Lavoie *et al.*, 1996; Weber *et al.*, 1997) (Grossel & Hinds, 2006). Once activated by CAK phosphorylation of CDK4/6, the CDK4/6-cyclin D complex initiates phosphorylation of the tumour suppressor protein RB as well as its closely related homologues p107 (RBL1) and p130 (RBL2) (Burkhart & Sage, 2008; Dick & Rubin, 2013). RB serves as a master regulator of the cell cycle through various mechanisms. First, in its hypo-phosphorylated state, RB binds to members of the E2F family of transcription factors to prevent the transcription of genes necessary for cell cycle progression. Second, RB creates a chromatin-repressive state to reduce

expression of cell cycle-related genes through the recruitment of chromatin remodelling and modifying complexes, such as histone deacetylases, DNA methyl transferases, and helicases (Luo *et al*, 1998; Robertson *et al*, 2000; Singh *et al*, 1995; Talluri & Dick, 2012). Alleviating RB-induced repression initiates the G1-S transition by leading to the transcription of G1-S target genes, including cyclins E, A, and B, which are essential for the G1-S transition and overall cell-cycle progression (Polager *et al.*, 2002).

1.2.13 CDK2 and progression from S phase to G2 and mitosis

CDK2 activity is mostly restricted to the late G1 and S phases, playing important roles in DNA replication initiation and repair (Akiyama *et al.*, 1992; Neganova *et al*, 2011). CDK2 binds to cyclin E in G1 / early S phase and, to a lesser extent, cyclin A later in the S phase. Entry into S phase is regulated by CDK2 directed phosphorylation of components of the pre-replication complex, such as CDC6 and ORC, and phosphorylation of NPAT, which activates the transcription of histone genes (Petersen *et al*, 1999; Zhao *et al*, 2000). CDK2 activity is driven by increased cyclin E expression and E2F transcription of CDK2 via CDK4/6 activation of E2Fs (Patel *et al*, 2018b).

Cyclin E (E1 and E2) levels are controlled through regulation of gene expression and protein degradation (Fagundes & Teixeira, 2021). Activation of E2F transcription factors stimulates cyclin E expression (Geng *et al*, 1996; Ohtani *et al*, 1995). Cyclin E transcription is inhibited as cells continue through S phase by repressive E2F transcription factors, including E2F6 and E2F7 (Giangrande *et al*, 2004; Westendorp *et al*, 2012). Degradation of cyclin E is driven by the SCFFBW7 ubiquitin ligase complex following cyclin E phosphorylation by GSK3 or by CDK2 (Clurman *et al*, 1996; Welcker *et al*, 2003; Won & Reed, 1996; Ye *et al*, 2004).

Once activated by association with cyclin E, CDK2 phosphorylates RB more extensively than CDK4/6 to further drive cell cycle progression (Akiyama *et al.*, 1992). CDK2-induced RB phosphorylation releases more E2Fs, to promote S phase progression by transcription of cell cycle factors, including *CDC6*, *CDT1*, factors of the MCM complex, and components of the pre-replication complex (pre-RC) (Fagundes & Teixeira, 2021). Further, CDK2-cyclin E directly regulates these DNA replication factors to control progression through S phase. First, CDK2-cyclin E directly phosphorylates the pre-RC subunit CDC6, stabilizing CDC6 and enabling formation of the pre-RC (Mailand & Diffley, 2005; Petersen *et al.*, 1999). Second, CDK2-cyclin E is essential for MCM loading at replication initiation sites by directly engaging

with pre-RC components and complexing with ORC1 and CDC6 (Furstenthal *et al*, 2001; Hossain *et al*, 2021; Hossain & Stillman, 2016). Third, CDK2 phosphorylates Treslin, which is a protein that facilitates S to G2 transition following DNA replication, enhancing Treslin interaction with TOPBP1 and aiding the recruitment of CDC45, GINS, and DNA polymerases to chromatin (Boos *et al*, 2011; Kumagai *et al*, 2010, 2011; Sansam *et al*, 2015).

Lastly, CDK2 also participates in DNA repair pathways such as nucleotide excision repair (NER) and homologous recombination via phosphorylation of replication protein A, BRCA1, BRCA2 and RAD51 (Neganova *et al.*, 2011). During the G1/S DNA damage checkpoint arrest, activation of p53 through ATM/ATR pathways leads to the activation of p21 (el-Deiry *et al.*, 1993; Engeland, 2022; Xiong *et al.*, 1993). p21 then binds to and inhibits CDK2-cyclin E complexes, halting cell progression at the G1/S transition (Cerqueira *et al.*, 2014; Reynisdottir *et al.*, 1995). Further, CDK2-cyclin A can phosphorylate *BRCA2*, which is necessary for RAD51 binding and DNA repair (Davies *et al*, 2001; Esashi *et al*, 2005). Similarly, CDK2 may also phosphorylate CHK1, accelerating the activation of DNA repair proteins (Chung & Bunz, 2010; Xu *et al*, 2012).

RB phosphorylation by CDK2 causes a positive feedback loop whereby E2F-driven transcription leads to a greater expression of cyclin A (Xu *et al*, 1994). As cyclin E expression is reduced and cyclin A expression increases in an E2F-dependent manner, CDK2 binds cyclin A more than cyclin E. CDK2 binding cyclin A leads to greater CDK2 activity and further increases in the transcriptional activities of E2Fs by RB hyperphosphorylation. CDK2-cyclin A promotes DNA replication and is also important for ensuring only a single round of DNA replication occurs (Coverley *et al*, 2002). Increased cyclin A expression results in inhibition of E2F-mediated transcription, thus creating a negative feedback loop (Krek *et al*, 1995; Wang *et al*, 2012).

1.2.14 CDK1

CDK1 is the only mammalian CDK that is essential for cell cycle progression (Enserink & Kolodner, 2010; Santamaria *et al.*, 2007). In mice where CDK2 and CDK4/6 are knocked out, CDK1 can replace their function and bind several cyclins to substitute for the loss of other cell cycle CDKs (Aleem *et al*, 2005; Satyanarayana *et al*, 2008; Satyanarayana & Kaldis, 2009). Under normal physiological conditions, CDK1 activity begins slowly increasing at late G1 phase with the expression of cyclin A (Satyanarayana & Kaldis, 2009). Cyclin A expression

increases at the onset of S phase through an E2F-dependent mechanism (Schulze *et al*, 1995). CDK1 binds cyclin A in the S and G2 phases and cyclin B in G2 and M phases (Malumbres & Barbacid, 2005; Strauss *et al*, 2018). Cyclin B expression is controlled by the FOXM1 transcription factor and begins at the onset of G2 (Ito, 2000; Leung *et al*, 2001). Cyclin B expression increases in late S phase following activation of E2F transcription factors and replaces cyclin A as the primary binding partner of CDK1 (Lukas *et al*, 1999). Full activation of CDK1 following cyclin A/B binding requires CAK-mediated phosphorylation at Thr161 (Ali *et al*, 2009).

CDK1 activity is tightly regulated throughout all phases of the cell cycle and is involved in the control of transcription, DNA replication, and chromosome segregation in mitosis (Enserink & Kolodner, 2010). CDK1 coordinates late cell cycle gene expression by phosphorylating a network of transcription factors and regulating the transcription of histone genes, crucial for mitotic progression and genome stability (Landry *et al*, 2014). In the S phase, several components of the origin recognition complex (ORC), CDC6, and the MCM2-7 complex are phosphorylated by CDK1 to prevent DNA re-replication (Diril *et al*, 2012; Nguyen *et al*, 2001; Piatti *et al*, 1996). CDK1 becomes highly active in G2, following binding with cyclin B and activation by the CAK, driving cells into mitosis and remaining active until anaphase, the third phase of mitosis (Crasta *et al*, 2006; Mendenhall & Hodge, 1998; Piatti *et al*, 1996; Schwob *et al*, 1994; Teh *et al*, 2009). In late G2 and the onset of mitosis, CDK1-cyclin complexes phosphorylate over 70 substrates, leading to centrosome separation, nuclear envelope breakdown and chromosome condensation (Malumbres & Barbacid, 2005). Proper chromosome cohesion, which is the attachment of sister chromatids, is dependent on CDK1 phosphorylation of PDS1, inhibiting cohesion dissolution during the metaphase to anaphase transition (Holt *et al*, 2008). In anaphase, the APC/C targets cyclin B for degradation, effectively reducing CDK1 activity and promoting exit from mitosis and the cell cycle (Mendenhall & Hodge, 1998; Schwob *et al*, 1994). Reduced CDK1 activity is essential for cytokinesis and the subsequent exiting from mitosis (Potapova *et al*, 2006).

1.3 Cyclin-dependent kinases in transcription

1.3.1 The roles of CDKs in transcription

In addition to their importance for the cell cycle, several CDKs play critical roles in transcriptional control. In particular, CDK7, CDK9, CDK12/13, and CDK8/19, have been

implicated in the direct regulation of RNA polymerase II (Pol-II)-directed gene expression through modulating the activities of transcription factors and transcriptional co-regulator complexes (Malumbres, 2014). Similarly to the cell cycle, transcription may be envisaged as a cycle that occurs in phases: initiation, elongation, and termination (**Figure 1.4**) (Parua & Fisher, 2020). Transcriptional CDKs typically regulate transcription initiation and processivity by Pol-II through phosphorylation of the C-terminal domain (CTD) of the largest Pol-II subunit, Rpb1. The Pol-II CTD is comprised of 52 repeats of a seven amino acid motif having the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (YSPTSPS) (Hsin & Manley, 2012). As with cell cycle CDKs, temporal phosphorylation of different transcriptional CDK substrates, including multiple amino acids in the heptad motifs of the Pol-II CTD, drives the orderly progression of the transcription cycle. CDK8, similarly to its paralog CDK19, comprises the enzymatic component of the Mediator complex. The Mediator complex is a multi-protein complex that bridges transcription factors bound to DNA with Pol-II machinery. Thus, CDK8/19 may function as repressors or activators of different genes, depending on the stage of the transcription cycle, cell type or in response to specific stimuli (Bourbon *et al*, 2004; Chen *et al*, 2023; Fant & Taatjes, 2019; Galbraith *et al*, 2010). CDK9, and its subunit cyclin T, is part of the positive transcription elongation factor (P-TEFb) complex (Peng *et al*, 1998). Similarly, CDK12/13 are required for transcription elongation by Pol-II (Blazek *et al*, 2011; Marques *et al*, 2000). Further, CDK12 is involved in the DNA damage response while CDK13 is involved in pre-mRNA splicing (Blazek *et al*, 2011; Marques *et al*, 2000). In addition to being the kinase subunit of CAK, CDK7 is a subunit of transcription factor TFIIH and is involved in phosphorylation of the CTD of Pol-II for transcription initiation and elongation and in DNA repair (Adamczewski *et al*, 1996; Bartkova *et al*, 1996; Chou *et al*, 2020; Clopper & Taatjes, 2022). Collectively, CDKs play several important roles in regulating transcription. Further description of the action of the transcriptional CDKs follows below.

1.3.2 The process of transcription

Transcription is a tightly regulated process of converting DNA into effector RNA. Transcription begins with the binding of transcription activators to DNA enhancer elements, which is highly dependent on chromatin accessibility (Thomas & Chiang, 2006). Thus, transcription initiation depends on chromatin remodelling and distant enhancer activation. Enhancers often act as regulatory hubs for several genes with long-range interactions capable of activating several promoters simultaneously (Li *et al*, 2016). Enhancer function depends on local chromatin architecture, which affects transcription factor recruitment to the DNA

enhancer elements, and general chromatin structure, which in turn impacts enhancer association with often distant gene promoters (Hu & Tee, 2017). Transcription factor recruitment at gene enhancers can also recruit chromatin-modifying enzymes that prevent chromatin repression (Bose *et al*, 2017).

Next, general transcription factors (GTFs) are recruited to the core promoter elements proximal to the transcription start site. Chromatin looping enables co-localization of enhancers with gene promoters to facilitate recruitment of GTFs at the core promoter elements (Barral & Dejardin, 2023). These GTFs are part of the pre-initiation complex (PIC), which leads to the recruitment of an RNA polymerase for initiation of transcription (Petrenko *et al*, 2019). Transcription of ribosomal RNA (rRNA) and transfer RNA (tRNA) genes is carried out by RNA polymerase I and III, respectively (White, 2008; Woychik & Hampsey, 2002). Transcription of protein coding genes, as well as non-coding genes such as those encoding long non-coding RNAs and miRNAs, is undertaken by Pol-II.

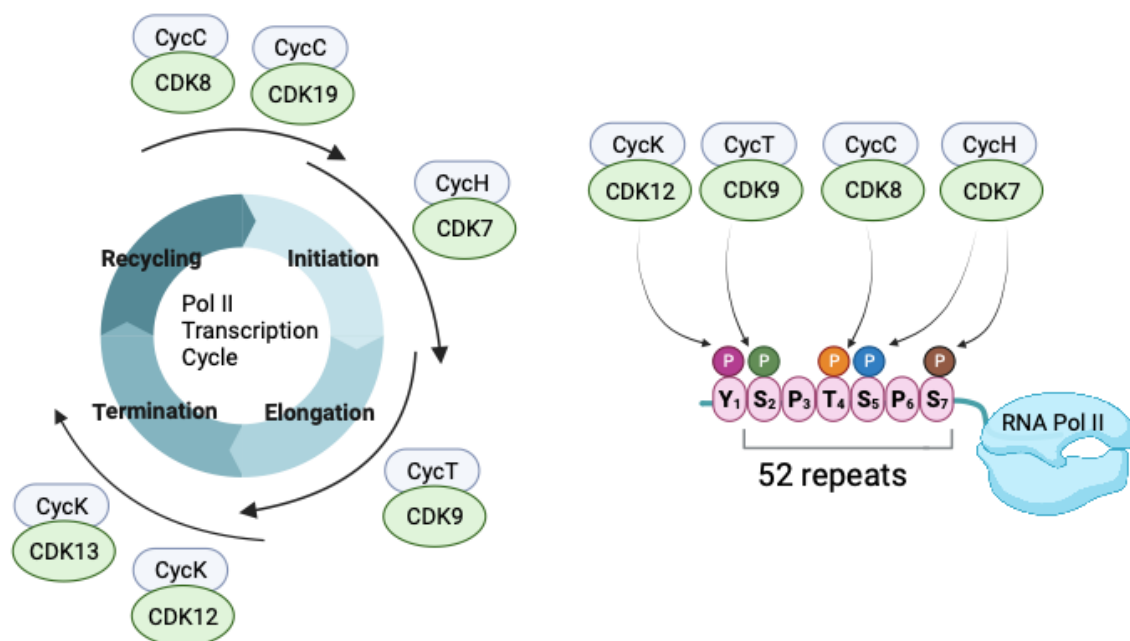


Figure 1.4 The roles of CDK7, CDK8/19, CDK9, and CDK12/13 in regulating the transcription cycle.

(Right) The transcription cycle is regulated by several CDKs, which rely on phosphorylation by CDK7 within the CDK activating kinase (CAK) for proper activation. CDK8 and CDK19 are involved in transcription initiation as CDK8 is a key component of the Mediator complex, which is an interface between general transcription factors and RNA polymerase II (RNA Pol-II). CDK7, a component of the CAK complex and TFIIH, is important for activating other transcription-regulating CDKs and regulating Pol-II promoter escape. CDK9 forms part of the positive transcription elongation factor, p-TEFB, and promotes transcription elongation and mRNA maturation. CDK12/13 are also involved in transcription elongation and mRNA maturation, specifically through splicing. (Left) Regulation of Pol-II promoter escape is influenced by CDK7, 8, 9, and 12 phosphorylation of the heptapeptide repeat region of the Pol-II C-terminal domain. Figure was generated using BioRender, based on Parua & Fisher (Parua & Fisher, 2020).

In the case of Pol-II genes, core promoter elements proximal to the transcription start site of genes recruit the transcription machinery, including the GTFs that comprise the PIC (**Figure 1.5**) (Juven-Gershon *et al*, 2008). GTFs are recruited sequentially to the gene promoter and include TFIIA, TFIIB, TFIID, TFIIIE, TFIIF, TFIIH, and Pol-II (Juven-Gershon *et al*, 2008). First, the TATA box, which is a very common feature of core promoter elements, is bound by the TFIID complex (Kuras & Struhl, 1999). Second, TFIIA is recruited via interactions with TFIID (Kraemer *et al*, 2001). Third, TFIIB is recruited to promoter elements flanking the TATA box, which stabilizes the TFIID complex binding to DNA (Deng & Roberts, 2007). Fourth, TFIIH and TFIIIE are recruited to the PIC. TFIIIE recruits TFIIH to the PIC and regulates its enzymatic functions (Okamoto *et al*, 1998). In addition to TFIIH and TFIIIE, the mediator complex, which is responsible for transducing regulatory signals from DNA-bound transcription factors to Pol-II, is recruited to the PIC (Allen & Taatjes, 2015). TFIIH recruitment also requires CDK8 and the Mediator complex (Soutourina, 2018). Generally, TFIIH controls the transition from the closed to open PIC to enable transcription initiation (Laine & Egly, 2006). Specifically, TFIIH, which contains the CDK7/Cyclin H/MAT1 complex, has a dual role (Laine & Egly, 2006). TFIIH unwinds the DNA at the promoter region using DNA helicase activity of the XPB and XPD subunits. TFIIH also phosphorylates Pol-II at the Ser5 and Ser7 residues of the C-terminal domain (CTD) to initiate transcription by expediting Pol-II release from the gene promoter. Lastly, Pol-II and TFIIF (which is a stimulator of transcription elongation and facilitator of Mediator-Pol-II interactions) are recruited to the core promoter (Bernecky *et al*, 2011; Price *et al*, 1989).

The major regulator of transcription elongation following initiation is phosphorylation of the Pol-II CTD at Ser5 and Ser7 in the heptad repeat. Ser5 and Ser7 are phosphorylated by CDK7-TFIIH. Recently, CDK12 has also been found to stimulate phosphorylation of Ser2 and Ser5 in the CTD via interactions with the elongation factors LEO1 and CDC73 within the PAF1 complex (Tellier *et al*, 2020). This suggests a novel role for CDK12 in addition to the known roles of CDK12/13 in splicing (Panzeri *et al*, 2023). Following transcription initiation, Pol-II synthesizes a short stretch of RNA before pausing. Transcriptional pausing is ensured by the phosphorylation of Ser5 and Ser7 of the Pol-II CTD hepta-peptide repeat region. Transcriptional pausing is also regulated by the DRB sensitivity inducing factor (DSIF), the negative elongation factor (NELF), and Gdown1 (Palangat *et al*, 2005; Wada *et al*, 1998; Yamaguchi *et al*, 1999). Productive transcription initiation is achieved if the transcript length reaches at least ~20 nucleotides downstream of the transcription start site (TSS), leading to the

beginning of transcription elongation (Saunders *et al*, 2006). To resume elongation, the Pol-II CTD is de-phosphorylated at the CAK-phosphorylation sites and phosphorylated at other sites by the positive transcription elongation factor (P-TEFb, *i.e.*, CDK9 and cyclin T1) (Peng *et al.*, 1998). Dephosphorylation of Ser5 at the Pol-II CTD hepta-peptide repeats is important for productive transcription elongation (Harlen & Churchman, 2017). Specifically, P-TEFb phosphorylates the Ser2 residues of the Pol-II CTD heptad repeat region to enable transcription elongation (Jonkers & Lis, 2015). P-TEFb also phosphorylates the DSIF and NELF factors, triggering their release from the paused Pol-II (Larochelle *et al*, 2012).

Pol-II transcription continues until it encounters a polyadenylation signal and a transcription termination signal. Cleavage and polyadenylation specificity factor (CPSF) and cleavage stimulation factor (CstF) are recruited at the Poly-A signal, leading to the cleavage of the pre-mature mRNA and termination of Pol-II activity (Mandel *et al*, 2008). Alternative methods of transcription in eukaryotes involve R-loop-dependent transcriptional pausing and heterochromatin-dependent pausing (Proudfoot, 2016).

1.3.3 CDK8

CDK8, and its paralog CDK19, directly influences several oncogenic processes such as gene expression, signal transduction, and cellular differentiation and DNA repair (Galbraith *et al.*, 2010). CDK8, and its cyclin partner cyclin C, is a component of the Mediator complex, which serves as an interface between transcriptional activators, Pol-II, and general transcription factors to regulate gene expression by influencing the initiation and elongation stages of transcription (Bourbon *et al.*, 2004). Within the Mediator complex, CDK8 phosphorylates the C-terminal domain of Pol-II to either promote or inhibit transcription, depending on its association with the Mediator complex (Galbraith *et al.*, 2010; Taatjes, 2010). CDK8 also functions in signal transduction as a kinase for β -catenin in the WNT/ β -catenin pathway, with high levels of CDK8 associating with nuclear β -catenin activity (Firestein *et al*, 2010; Rosenbluh *et al*, 2014). This suggests a role for CDK8 in cell proliferation, migration, and fate specification (Komiya & Habas, 2008). Some CDK8-associated signal transduction pathways are involved in cell differentiation. For example, CDK8 has been implicated as essential for the self-renewal capacity of cancer stem cells (Fukasawa *et al*, 2021). CDK8 also regulates DNA damage response through the phosphorylation of p53 (Donner *et al*, 2007).

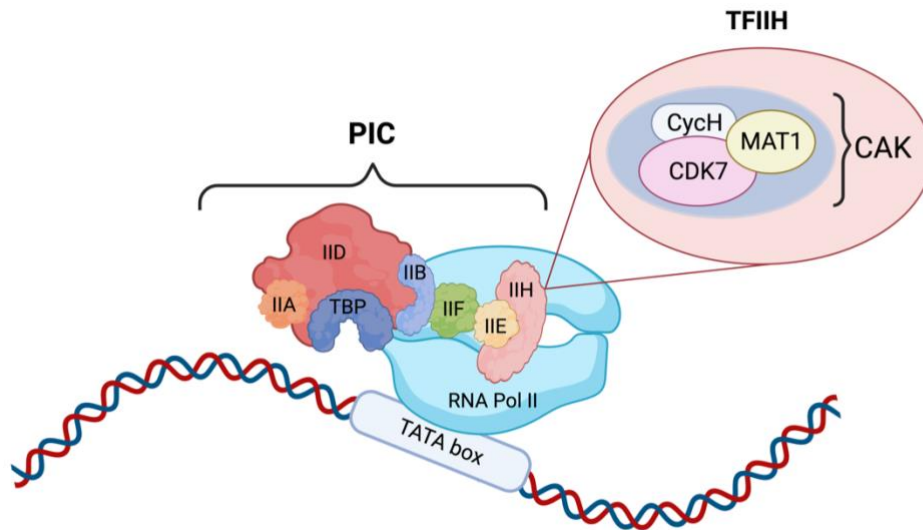


Figure 1.5 The transcription pre-initiation complex (PIC) initiates transcription and is comprised of several general transcription factors.

Transcription begins with the recruitment of the PIC to the promoter region. First, TFIID binds the TATA box. Second, TFIIA is recruited via interactions with TFIID. Third, TFIIB is recruited to promoter elements flanking the TATA box, which stabilizes the TFIID complex binding to DNA. Fourth, TFIIH, which requires CDK8 and the Mediator complex, and TFIIE are recruited to the PIC. TFIIH contains the CDK activating enzyme (CAK), which is comprised of CDK7, MAT1, and cyclin H. Figure was generated using BioRender.

1.3.4 CDK9

CDK9 is a positive regulator of transcription elongation, chromatin accessibility, and mRNA maturation (Anshabo *et al.*, 2021). Cyclins T1, T2, and K are the binding partners of CDK9 (Malumbres, 2014). As a factor of the P-TEFb complex, CDK9 phosphorylates the Pol-II CTD to enable productive transcriptional elongation (Peng *et al.*, 1998). CDK9-mediated phosphorylation of negative transcription elongation factors, such as NELF and DSIF, relieve the transcriptional inhibition imposed by these factors (Bacon & D'Orso, 2019; Mandal *et al.*, 2021). NELF and DSIF are negative regulators of transcription elongation by inducing Pol-II transcriptional pausing (Wu *et al.*, 2003). Transcriptional elongation is further facilitated by CDK9 phosphorylation of histone proteins, such as H2B, to alter chromatin structure (Pirngruber *et al.*, 2009). Recently, CDK9 has been found to participate in mRNA polyadenylation and alternative splicing, however, the mechanism of its involvement remains undefined (Hu *et al.*, 2021a).

1.3.5 CDK12 and CDK13

In addition to CDK9, CDK12, and its paralog CDK13, are also known to be involved in transcription elongation and mRNA maturation (Malumbres, 2014). CDK12/13 share a high degree of sequence and functional similarity (Blazek *et al.*, 2011). Like CDK9, CDK12/13 phosphorylate the CTD of Pol-II during the transcription elongation phase (Bartkowiak *et al.*, 2010). Unlike CDK9, CDK12/13 are especially important in the expression of DNA repair genes, such as *BRCA1* and *FANCI*, and play important roles in RNA processing of DNA repair genes, such as *DNAJB6* (Liang *et al.*, 2015; Tien *et al.*, 2017). Further, genetic analysis of mammalian tumours has identified many CDK12 mutations that are associated with therapy response and cancer subtype classification (Naidoo *et al.*, 2018; Popova *et al.*, 2016; Wu *et al.*, 2018). Due to the important roles of CDK12 in transcription elongation and splicing, CDK12 inhibitors are being explored for their therapeutic potential (Naidoo *et al.*, 2018; Wu *et al.*, 2018).

1.3.6 Cell-cycle CDK inhibitors as cancer therapeutics

CDKs, cyclin genes and CDKI genes are often deregulated in BC, such that CDK activities are upregulated and drive cell proliferation and oncogenic processes (Fassl *et al.*, 2022; Tate *et al.*, 2019; Werooha *et al.*, 2010). For example, CDK1, CDK2, CDK4, and CDK7 are among the most overexpressed CDKs in cancer tissues compared to normal tissues (Cancer Genome Atlas Research *et al.*, 2013). Further, CDK4/6 pathway dysregulation is present in over half of all

cancers (Ortega *et al*, 2002). Particularly, cyclin D1 (*CCND1*) is overexpressed in approximately 50% of ER+ BC, partly due to *CCND1* gene amplification, and is associated with tamoxifen resistance (Arnold & Papanikolaou, 2005; Sutherland & Musgrove, 2004). Similarly, Cyclin E1 is overexpressed/amplified in ER+ BC (Sutherland & Musgrove, 2004). Finally, CDK4 is amplified in nearly 20% of ER+ BC, and CDKI of CDK4/6 are often lost in this BC subtype (Basudan *et al*, 2019). Transcriptional CDKs are also aberrantly regulated in cancer. For example, CDK8 is a putative biomarker in BC and CDK8 overexpression associates with tumour-promoting activities (Broude *et al*, 2015; Porter *et al*, 2012). Due to their importance in regulating the cell cycle and transcription, CDKs are enticing targets for BC therapy.

CDK inhibitors can be generally classified according to specificity (*i.e.*, pan-CDK vs. CDK-selective) and mechanism of action (*i.e.*, ATP-competitive vs. ATP non-competitive) (Peyressatre *et al*, 2015). Pan-CDK inhibitors have been under investigation since the 1990s and comprise the first generation of CDK inhibitors that were developed (Zhang *et al*, 2021). Early pan-CDK inhibitors, including Flavopiridol and Roscovitine, had high toxicity leading to failures in clinical trials (Cicenas *et al*, 2015; Senderowicz, 1999), presumed to be due to their broad, pan-CDK activities. These inhibitors typically targeted the ATP-binding pocket resulting in a general affinity for diverse kinases. Second generation pan-CDK inhibitors have been developed with the assistance of crystallographic structures for providing CDK selectivity and improved pharmacokinetic profiles. These inhibitors target the CDK ATP-binding pocket but with greater CDK selectivity and protein-protein interfaces between CDKs and cyclins and impair CDK-cyclin conformational changes and substrate binding (Peyressatre *et al*, 2015). Second generation inhibitors, including Dinaciclib, P276-00, AT7519, Roniciclib, and RGB-286638, are more selective for CDKs and show fewer side effects in patients (Joshi *et al*, 2007; Kumar *et al*, 2015; Mahadevan *et al*, 2011; Reck *et al*, 2019; van der Biessen *et al*, 2014). For instance, Dinaciclib, a Phase III clinical trial inhibitor with selective activity for CDK1, CDK2, CDK5, and CDK9, has shown significant anti-tumour effects in the treatment of BC. However, in addition to its CDK kinase activity inhibition, Dinaciclib interacts with the acetyl-lysine recognition site of bromodomain testis-specific (Brdt) protein, a member of the BET family of bromodomain proteins, indicating multiple possible mechanisms of action (Martin *et al*, 2013). Despite these advancements, the relatively low binding affinities and variable efficacy of pan-CDK inhibitors in clinical trials have led to the development of yet more selective inhibitors of specific CDKs.

1.3.7 CDK4/6 inhibitors

The evident deregulation of CDK4/6 in BC and potential role in resistance to hormone therapies provided special impetus for the development of selective CDK4/6 inhibitors (CDK4/6i). Pre-clinical studies demonstrated clear efficacy for these drugs and consequent progression to clinical trials in patients with advanced ER+ BC in combination with AI or fulvestrant. The clear improvement in progression-free survival when combined with hormone therapy has led to FDA approval for several CDK4/6i for ER+ BC, even in the first-line adjuvant setting (Fassl *et al.*, 2022). It has proved rather difficult to identify biomarkers of likely response to the CDK4/6i, other than ER positivity and RB absence, which makes it difficult to understand which patients are likely to respond and which will be resistant. This is despite *in vitro* evidence for alterations in several genes in responsiveness to CDK4/6i, none of which have been fully confirmed in clinical studies. To date, three CDK4/6i, palbociclib, ribociclib, and abemaciclib, have received FDA approval for the treatment of ER+ BC, when used in combination with hormone therapies (O'leary *et al.*, 2016; Spring *et al.*, 2020). Abemaciclib is the only one of these drugs to have shown single agent efficacy in metastatic ER+ BC.

Palbociclib is an ATP-competitive CDK4/6i which gained approval for its use in combination with an aromatase inhibitor as the first-line treatment for HR+/HER2- BC in postmenopausal women (Serra *et al.*, 2019). Palbociclib is also approved for use in conjunction with fulvestrant as a second-line treatment for HR+ BC, following resistance to standard HR+ therapies. In the landmark palbociclib clinical trial PALOMA-1/TRIO-18, 165 postmenopausal patients treated with palbociclib and letrozole exhibited a median progression-free survival (PFS) of 20.2 months, while those on letrozole alone had a median PFS of just 10.2 months (Finn *et al.*, 2015). These results led to the accelerated FDA approval of palbociclib in February 2015. Notably, the median overall survival (OS) was not significantly different at 37.5 months with palbociclib and letrozole versus 33.3 months with letrozole alone (Finn *et al.*, 2020). However, the subsequent phase III clinical trials, PALOMA-2 and PALOMA-3 further supported the use of palbociclib in combination with letrozole and fulvestrant, respectively (Rugo *et al.*, 2018; Turner *et al.*, 2015). Importantly, PALOMA-3 revealed patients with endocrine-sensitive BC had a greater PFS if treated with palbociclib compared to endocrine-resistant patients (Turner *et al.*, 2015). Details regarding the major CDK4/6 inhibitor clinical trials in patients with advanced ER+, HER2- BC are summarised in **Table 1.1**.

Ribociclib, also an ATP-competitive CDK4/6i, has received FDA approval for use in combination with an aromatase inhibitor for the first-line treatment of HR+/HER2- advanced BC in pre-, peri- and post-menopausal women, and in combination with fulvestrant for second-line treatment in postmenopausal women (Hortobagyi *et al*, 2016; Im *et al*, 2019; Slamon *et al*, 2020). The success of the MONALEESA trials played a pivotal role in driving the clinical approval of ribociclib. MONALEESA-2 (phase III) explored the efficacy of ribociclib in combination with letrozole as a first-line therapy in 668 postmenopausal women with advanced ER+/HER2- BC. The trial revealed a significantly improved median PFS when ribociclib was combined with letrozole compared to letrozole with placebo (25.3 versus 16 months) (Hortobagyi *et al*, 2018). Furthermore, the phase III MONALEESA-3 trial investigated ribociclib in combination with fulvestrant in endocrine-resistant patients with metastatic HR+/HER2- BC. This trial demonstrated the efficacy of ribociclib in improving PFS to 20.5 months compared to 12.8 months without ribociclib, and unlike palbociclib, ribociclib demonstrated a significant improvement in OS as well (53.7 vs. 41.5 months with placebo) (Slamon *et al*, 2021). Finally, the phase III MONALEESA-7 trial compared ribociclib or placebo in combination with anastrozole/letrozole in pre- and peri-menopausal women with advanced ER+/HER2- BC. Once again, ribociclib in combination with endocrine therapy improved the median PFS compared to placebo (23.8 versus 13.0 months) and demonstrated a benefit in OS compared to placebo (58.7 versus 48.0 months) (Lu *et al*, 2022; Tripathy *et al*, 2018).

Abemaciclib, the third ATP-competitive CDK4/6i approved by the FDA is also used to treat metastatic HR+/HER2- BC (Sledge *et al*, 2017). While abemaciclib is typically used in combination with an endocrine therapy, such as letrozole, to improve therapeutic efficacy, abemaciclib is the only CDK4/6 selective inhibitor to receive FDA approval as a monotherapy in advanced ER+ BC, even after progression on endocrine therapy or chemotherapy (Corona & Generali, 2018; Dickler *et al*, 2017). Unlike palbociclib and ribociclib, abemaciclib has a distinct binding mechanism, as it positions two fluorine atoms against the back wall of the ATP-binding pocket of CDK4/6 (Chen *et al*, 2016). This leads to a greater affinity for the ATP-binding pocket and facilitates its systemic use in metastatic BC. This higher affinity for CDK4/6 sits alongside the slightly lower selectivity for CDK4/6 with abemaciclib also inhibiting CDK1, CDK2, CDK7, and CDK9. This reduced selectivity may in fact promote abemaciclib's use as a monotherapy, as the exact mechanism of action for abemaciclib across tumours remains unknown (Chen *et al*, 2016; Dickler *et al*, 2017). Abemaciclib also inhibits

non-CDKs, including glycogen synthase kinase 3 (GSK3) and calmodulin-dependent protein kinase II (CAMKII), further demonstrating the broad specificity and unclear mechanism of action of this drug (Cousins *et al*, 2018).

The phase II MONARCH I clinical trial led to the approval of abemaciclib as a BC monotherapy in patients with advanced ER+/HER2- BC, who previously progressed on endocrine therapy or chemotherapy (Dickler *et al*, 2017). Patients treated with abemaciclib exhibited an objective response rate (ORR) of 19.7%, a clinical benefit rate (CBR) of 42.4%, a median PFS of 6 months, and a median overall survival of 17.7 months. In the subsequent phase III MONARCH-2 trial, which interrogated the combination of abemaciclib with fulvestrant in patients with advanced BC who had progressed on prior endocrine therapy, the median PFS was significantly improved in the abemaciclib plus fulvestrant combination (16.4 months) compared to fulvestrant alone (9.3 months) (Sledge *et al*, 2017). The combination treatment also significantly prolonged overall survival (46.7 months versus 37.3) (Sledge *et al*, 2020). The phase III MONARCH-3 trial explored the use of abemaciclib plus AI in women with advanced ER+/HER2- BC who had no previous treatments for advanced disease (Goetz *et al*, 2017; Johnston *et al*, 2019). The combination therapy resulted in a significantly longer median PFS of 28.2 months compared with AI alone (14.8 months). Abemaciclib monotherapy has also been investigated for use in TNBC. Initial *in vitro* results for the use of abemaciclib in TNBC showed poor efficacy (Ozman *et al*, 2021). However, recent studies have identified a subset of TNBC with higher levels of RB and lower levels of p16 protein respond to abemaciclib-induced CDK4/6 inhibition in cell lines and mouse xenograft models (Hu *et al*, 2021b). Nonetheless, clinical trials evaluating abemaciclib as a single agent in metastatic RB-positive TNBC did not show clinical success (ClinicalTrials.gov identifier: NCT03130439) (Goel *et al*, 2024).

This remarkable success of CDK4/6i in ER+ BC, with a general lack of efficacy in other cancer types is puzzling. Possible explanations include the fact that CDK4/6i are used in combination with hormone therapies, raising the possibility that their utility in other cancer types requires identification of appropriate combination opportunities. Abemaciclib has activity in ER+ BC patients as a single agent and demonstrable efficacy in early-stage clinical trials in lung and other solid cancers (Patnaik *et al*, 2016), indicative of the potential importance of targeting CDK4/6i in other cancer types. However, this must be tempered by the fact that abemaciclib is less CDK4/6 selective than the other CDK4/6i and inhibits other CDKs and additional kinases.

As CDK4/6i use is accompanied by widespread toxicity, in particular neutropenia (Becherini *et al*, 2023; Stanciu *et al*, 2023), it may be that identification of drugs that can be used in combination with CDK4/6i is especially important to obtain sufficient efficacy at the concentrations that can be tolerated by patients. Finally, it is possible that the activity of CDK4/6i in BC is reflective of the proposed tissue-selective dependencies of cell cycle CDKs other than CDK1 (Malumbres & Barbacid, 2009).

Table 1.1 Major clinical trials with CDK4/6 inhibitors in ER+/HER2- breast cancer.

	PALOMA-1	PALOMA-2	PALOMA-3	MONARCH-1	MONARCH-2	MONARCH-3	MONALEESA-2	MONALEESA-3	MONALEESA-7
Clinical trial ID	NCT00721409	NCT01740427	NCT01942135	NCT02102490	NCT02107703	NCT02246621	NCT01958021	NCT02422615	NCT02278120
Phase	II	III	III	II	III	III	III	III	III
Treatment groups	Palbociclib + letrozole <i>versus</i> letrozole	Palbociclib + letrozole <i>versus</i> placebo + letrozole	Palbociclib + fulvestrant <i>versus</i> placebo + fulvestrant	Abemaciclib monotherapy	Abemaciclib + fulvestrant <i>versus</i> placebo + fulvestrant	Abemaciclib + anastrozole/letrozole <i>versus</i> placebo + anastrozole/letrozole	Ribociclib + letrozole <i>versus</i> placebo + letrozole	Ribociclib + fulvestrant <i>versus</i> placebo + fulvestrant	Ribociclib + goserelin/AI/tamoxifen <i>versus</i> goserelin/AI/tamoxifen
Patient criteria	Postmenopausal women with ER+/HER2-MBC; no prior CT for MBC; prior ET permitted	Postmenopausal women with ER+/HER2-MBC; no prior CT for MBC; prior ET permitted	Pre- or postmenopausal women with ER+/HER2-MBC; ≤1 prior CT for MBC; any line of previous ET in MBC required	Progression on or after prior endocrine therapy; 1–2 lines prior CT for MBC	Pre- and postmenopausal women with ER+/HER2-MBC; ≤1 prior CT for MBC; one line of previous ET in MBC required	Postmenopausal women with ER+/HER2-MBC; no prior CT for MBC; prior ET permitted	Postmenopausal women with ER+/HER2-MBC; no prior CT for MBC; prior ET permitted	Postmenopausal women with ER+/HER2-MBC; no prior CT for MBC; any line of previous ET in MBC required	Pre- or perimenopausal women with ER+/HER2-MBC; no prior CT for MBC; prior ET permitted
Patients, n	165	666	521	132	669	493	668	726	
Median PFS (months)	20.2 <i>versus</i> 10.2	27.6 <i>versus</i> 14.5	11.2 <i>versus</i> 4.6	6	16.4 <i>versus</i> 9.3	28.2 <i>versus</i> 14.8	25.3 <i>versus</i> 16	20.5 <i>versus</i> 12.8	23.8 <i>versus</i> 13.0
Median OS (%)	37.5 <i>versus</i> 34.5	53.9 <i>versus</i> 51.2	34.9 <i>versus</i> 28.0	17.7	46.7 <i>versus</i> 37.3	66.8 <i>versus</i> 53.7	63.9 <i>versus</i> 51.4	53.7 <i>versus</i> 41.5	58.7 <i>versus</i> 48.0
References	(Finn <i>et al.</i> , 2015)	(Finn <i>et al.</i> , 2022)	(Turner <i>et al.</i> , 2018)	(Dickler <i>et al.</i> , 2017)	(Sledge <i>et al.</i> , 2020)	(Goetz <i>et al.</i> , 2024; Johnston <i>et al.</i> , 2019)	(Hortobagyi <i>et al.</i> , 2021; Hortobagyi <i>et al.</i> , 2018)	(Slamon <i>et al.</i> , 2021)	(Lu <i>et al.</i> , 2022; Tripathy <i>et al.</i> , 2018)

CT, chemotherapy; ET, endocrine therapy; MBC, metastatic breast cancer; OS, overall survival; PFS, progression-free survival

1.3.8 Resistance to CDK4/6i

Despite the efficacy of CDK4/6 inhibitors and their use as first-line treatment, BCs display several mechanisms of resistance, both intrinsic and acquired. Intrinsic resistance to CDK4/6 inhibitors occurs at the start or immediately after the start of treatment and results in no treatment response, often due to the presence of pre-existing genetic alterations that circumvent the effects of CDK4/6 inhibition (Kong *et al*, 2019; Xu *et al*, 2021b). Approximately 20% of BC patients have intrinsic resistance to CDK4/6i, with, despite extensive research, the best predictive biomarker for initial response being ER positivity (Shah *et al*, 2018). Acquired resistance to CDK4/6 inhibitors occurs later in treatment and appears to be due to the selection of cell populations that are resistant to CDK4/6 inhibitors (Knudsen & Witkiewicz, 2017; Spring *et al.*, 2020; Zhou *et al*, 2023a).

Specific mechanisms of intrinsic and acquired resistance can perturb the CDK4/6-RB pathway or activate compensatory mechanisms. First, resistance to CDK4/6 inhibition can occur due to loss of the RB gene (RB1) or RB function (Scheidemann & Shajahan-Haq, 2021). Loss of RB function alleviates RB inhibition of E2F transcription factors, promoting uncontrolled G1 to S phase transition independently of CDK4/6-cyclin D function (Singh *et al.*, 1995). RB1 loss-of-function through point mutations or gene loss is a driver mutation in many cancer types (Beroukhi *et al*, 2010; Condorelli *et al*, 2018). Second, overexpression or hyperactivity of E2F transcription factors can confer resistance to CDK4/6 inhibitors (Kent & Leone, 2019). Hyperactive E2F can promote transcription of cell cycle genes and drive cell cycle progression independent of RB status (Chen *et al*, 2018). Finally, resistance to CDK4/6 inhibitors can occur by overexpression of CDK4, CDK6 or cyclin D (Cai *et al*, 2023; Li *et al*, 2020d; Yang *et al*, 2017). Overexpression of CDK4/6 or cyclin D can compensate for loss of activity in the presence of CDK4/6 inhibitors.

Intriguingly, upregulation of CDK2 or cyclin-E can overcome CDK4/6 inhibition and drive cell cycle progression by phosphorylating RB and alleviating inhibition of E2Fs in a mechanism independent of CDK4/6 (Akiyama *et al.*, 1992; Malumbres *et al.*, 2004; Min *et al*, 2018; Spencer *et al*, 2013). Thus, CDK4/6 inhibition can be compensated for by increased CDK2 activity. Similarly, targeting CDK2 on its own may be compensated for by CDK4/6 activity (Arora *et al*, 2023). However, co-targeting CDK2 and CDK4/6 offers an intriguing solution. Indeed, targeting CDK2 together with CDK4/6 has been shown to overcome resistance to aromatase and CDK4/6 inhibitors in pre-clinical models of ER+ BC (Al-Qasem

et al, 2022). To this effect, Pfizer has developed a CDK2/4/6 inhibitor (Freeman-Cook *et al*, 2021), now in phase II clinical trials (ClinicalTrials.gov: NCT03519178).

In addition to the several molecular mechanisms of CDK4/6 inhibitor resistance, tumours can acquire resistance to CDK4/6 inhibitors by activating mitogenic pathways. First, aberrant fibroblast growth factor receptor (FGFR) signalling has been shown to participate in CDK4/6 resistance (Formisano *et al*, 2019). FGFR activates ERK and mTOR, resulting in increased expression of CDK6 and cyclins D and E. Second, MAPK signalling may provide an alternative mechanism of resistance as MAPK is often activated in CDK4/6 inhibitor-resistant tumours (de Leeuw *et al*, 2018). Further supporting this, treating CDK4/6 inhibitor-resistant tumours in combination with MEK inhibitors often confers re-sensitization to CDK4/6 inhibitors, in pre-clinical studies. Third, loss of function of the FAT atypical cadherin 1 (FAT1) gene is prevalent in CDK4/6 inhibitor-resistant tumours, increasing expression of CDK6 and reducing sensitivity to CDK4/6 inhibitors (Li *et al*, 2018). In addition to the various proposed mechanisms of resistance, CDK4/6i treatment often induces adverse side effects, including neutropenia and liver toxicity (Silvestri *et al*, 2021; Thill & Schmidt, 2018), which can limit their clinical utility. As a result, there is a need to develop novel BC therapies following progression on CDK4/6 inhibitors.

1.3.9 CDK9 inhibitors

CDK9 is an appealing BC therapeutic target due to its important role in the regulation of transcriptional elongation and mRNA maturation (Bourbon *et al.*, 2004; Peng *et al.*, 1998). The development of several selective CDK9 inhibitors has been reported, demonstrating impressive anti-tumour efficacy in preclinical investigations with examples including fadraciclib, AZD-4573, CDKI-73, and MC180295 (Cidado *et al*, 2020; Poon *et al*, 2020; Rahaman *et al*, 2019; Staff, 2021; Zhang *et al*, 2018). Fadraciclib, when combined with the DNA alkylating agent temozolomide, effectively suppresses MYCN-amplified neuroblastoma growth *in vivo* (Poon *et al.*, 2020). Fadraciclib also has some affinity for CDK2 (Staff, 2021). AZD-4573, a highly selective CDK9 inhibitor, causes rapid downregulation of the expression of oncogenic genes such as MCL-1, exhibiting significant anti-cancer efficacy in hematologic malignancies (Cidado *et al.*, 2020). AZD-4573 has been pre-clinically tested in palbociclib-resistant BC (Soosainathan *et al*, 2024). Of note, AZD-4573 has reached a first-in-human study for relapsed hematological malignancies (NCT03263637) as well as in combination with the BTK inhibitor, acalabrutinib, for treatment of non-Hodgkin lymphoma (NCT04630756). CDKI-73, in

combination with the PARP inhibitor Olaparib, synergistically enhances therapeutic outcomes in BRCA1-mutated proficient ovarian cancer (Li *et al*, 2020b). MC180295 facilitates gene activation by dephosphorylating SWI/SNF protein *BRG1*, leading to the restoration of tumour suppressor gene expression (Zhang *et al*, 2018). Furthermore, CDK9 inhibition sensitizes tumours to immune checkpoint inhibitors like anti-PD-1, positioning it as an attractive target for immunotherapy. Such clinical and preclinical studies have propelled the development of CDK9 inhibitors for clinical application in BC therapy.

Sixteen CDK9 inhibitors are at various stages of clinical development for cancer therapy (Borowczak *et al*, 2022). P276-00, ZK-304709, BAY-1000394, and SNS-032 have been terminated due to their limited selectivity and substantial toxicity. However, several other inhibitors are currently undergoing evaluation in multiple clinical trials. It is worth noting that many of these compounds lack specificity as they also inhibit other CDKs. For example, Dinaciclib also targets CDK1, CDK2, and CDK5, TG-02 also targets CDK1, CDK2, CDK3, and CDK5 and AT7519 also targets CDK1, CDK2, CDK4, CDK5, CDK6, as well as several other kinases to a lower extent (Goh *et al*, 2012; Gregory *et al*, 2015; Sallam *et al*, 2013).

1.3.10 CDK12/13 inhibitors

CDK12 and CDK13 have been implicated in the end points of transcription by Pol-II, including transcription termination, and have also been implicated in splicing. Recent reports indicate that CDK12/13 inhibitors may be useful for impairing oncogene transcription and signal transduction in BC. CDK12/13 inhibitors are currently being explored for their potential to induce a state of "BRCAness", making the cancer cells more susceptible to chemotherapy, especially in TNBC (Hopkins & Zou, 2019). Typically, CDK12 phosphorylates the C-terminal domain of Pol-II to enable transcription elongation, when in complex with cyclin K (Bartkowiak *et al*, 2010). However, recent studies have unveiled several novel functions of CDK12 in BC encompassing the regulation of diverse biological activities, including the control of c-MYC expression, modulation of the Wnt/ β -catenin signalling pathway, regulation of alternative NA splicing, participation in ERBB-PI3K-Akt and MAPK signalling, regulation of expression of the noncanonical NF- κ B pathway, and orchestration of DNA damage response signalling (Choi *et al*, 2019; Henry *et al*, 2018; Houles *et al*, 2022; Krajewska *et al*, 2019; Li *et al*, 2021a; Liang *et al*, 2015; Peng *et al*, 2020). CDK12 inhibitors, including SR-4835 and THZ531, have exhibited anti-tumour activity in preclinical studies of TNBC (Quereda *et al*, 2019; Zhang *et al*, 2016). SR-4835 targets both CDK12 and CDK13 and

suppresses the expression of core DNA damage response genes. This, in turn, can induce a "BRCAness" phenotype characterized by impaired DNA damage repair, thereby enhancing the synergistic effects of DNA damage chemotherapy and PARP inhibitors that are important therapies in TNBC (Quereda *et al.*, 2019). THZ531, a covalent inhibitor of CDK12 and CDK13, also downregulates the expression of DNA damage response genes, as well as key transcription factors associated with super-enhancers, for example at the c-MYC gene (Zhang *et al.*, 2016). Recent investigations have highlighted the synergistic effect of THZ531 when combined with sorafenib, a protein serine/threonine/tyrosine kinase inhibitor, for the treatment of hepatocellular carcinoma (Wang *et al.*, 2020). Lastly, non-covalent CDK12/13 dual inhibitors have been developed that are based on the proteolysis-targeting chimera (PROTAC) method (Niu *et al.*, 2022). PROTAC drugs target client proteins to the ubiquitin-proteasome system to promote their degradation (Bekes *et al.*, 2022). Thus, the combination of PROTACs, degrading CDK12 and/or its binding partner cyclin K, with PARP inhibitors induces antiproliferative effects in TNBC.

1.4 The cellular functions of CDK7

The focus of the work presented in my thesis has been to investigate the mechanisms by which the selective CDK7 inhibitor ICEC0942, also known as CT7001 or samuraciclib, inhibits growth of ER+ BC cells. Therefore, below I present a detailed description of CDK7 and its modes of action. I also describe ICEC0942 and other CDK7i that have been developed, together with information on their action in cancer cells.

1.4.1 CDK7 structure and function

CDK7 is a 346 amino acid polypeptide serine/threonine kinase. Unlike other CDKs, CDK7 also interacts with the accessory protein MAT1 (Martinez *et al.*, 1997). The association of CDK7 with MAT1 enhances CDK7-cyclin H complex stability. The CDK7/cyclin H/MAT1 complex is known as the Cdk-activating kinase (CAK), which is the enzymatic activity responsible for phosphorylation of cell cycle CDKs (as described in section 1.2.5). Notably, the presence of MAT1 can obviate the need for the activating phosphorylation of CDK7 on Thr170 and increases Pol-II CTD phosphorylation rates by a factor of four compared to Thr170-unphosphorylated CDK7-cyclin H alone (Larochelle *et al.*, 2001). Cyclin H phosphorylation by the protein kinase CK2 is important for CAK formation (Schneider *et al.*, 2002). Remarkably, in addition to being required for regulating cell cycle CDK activities,

CDK7 is implicated in transcription control as part of the general transcription factor TFIID complex (Larochelle *et al.*, 2012). Cyclin H and MAT1 also regulate CDK7 substrate specificity (Yankulov & Bentley, 1997). As part of TFIID, CDK7 is responsible for phosphorylation of Pol-II, a critical event necessary for transcription initiation by Pol-II.

In addition to positive regulation of CDK7 via cyclin H and MAT1, CDK7's kinase activity is modulated through phosphorylation at amino acid residues on its T-loop: Ser164, Thr170, and, to a lesser extent, Thr176 (Garrett *et al.*, 2001). T-loop phosphorylation at Ser164 enhances the CDK7 kinase activity and increases binding affinity for cyclin H (Larochelle *et al.*, 2001; Lolli *et al.*, 2004). Further, phosphorylation at Thr170 has been observed to prioritize the enzyme's activity towards Pol-II over CDK2 (Larochelle *et al.*, 2001). While CDK2 and CDK1 have been shown to phosphorylate CDK7 *in vitro*, the kinase responsible for activating CDK7 *in vivo* remains elusive, with current theories postulating CDK7 auto-phosphorylation (Garrett *et al.*, 2001). CAK activity is negatively regulated by p53, which has been shown to directly interact with cyclin H (Schneider *et al.*, 1998). In cycling cells, CAK complex activity and levels are stable and the levels of CDK7, cyclin H and MAT1 are not controlled by transcription or selective proteolysis (Lolli & Johnson, 2005).

1.4.2 CDK7 in regulating cell-cycle CDK activity

As part of the CAK complex, CDK7 is responsible for T-loop phosphorylation of cell cycle and transcriptional CDKs, thus ensuring the timely and orderly progression of the cell cycle (Lolli & Johnson, 2005). During the G1 phase, CAK phosphorylation of CDK4/6 at Thr172 and Thr177, respectively, is required for CDK4/6-cyclin D complex activation and the transition through G1 (Sherr & Roberts, 2004) (Bisteau *et al.*, 2013; Bockstaele *et al.*, 2009). Progression at the restriction point, which is when cell cycle progression becomes mitogen-independent, depends on CAK activity and phosphorylation of CDK4/6 (Pardee, 1974; Pennycook & Barr, 2020). At the G1/S phase transition, CAK activates CDK2-cyclin E via phosphorylation of CDK2 at Thr160 for its activity in facilitating DNA replication and the expression of cell cycle regulated genes (Jeffrey *et al.*, 1995; Petersen *et al.*, 1999; Zhao *et al.*, 2000). In G2, CAK activates CDK1-cyclin B by phosphorylating Thr161 and so helps to initiate entry into M phase (Bockstaele *et al.*, 2009; Larochelle *et al.*, 2007). CAK also phosphorylates non-CDK cell-cycle genes. For example, CAK phosphorylation of cell-cycle transcription factors, such as E2F1, is required for their degradation during S-phase and proper

cell cycling (Vandel & Kouzarides, 1999). CAK requires CDKs to be in complex with their cyclin for productive phosphorylation (Lolli & Johnson, 2005).

1.4.3 CDK7 in regulating gene expression

CDK7 activity is essential for several steps of the transcription process. First, as a component of the TFIID transcription factor complex, the CAK complex is recruited to transcription start sites where CDK7 phosphorylates the Pol-II CTD to initiate transcription (Akhtar *et al*, 2009; Bataille *et al*, 2012; Tietjen *et al*, 2010). TFIID is a 10-subunit general transcription factor complex that contains the dissociable proteins that form CAK and seven other subunits that form its core, including xeroderma pigmentosum type D (XPD) and six other structural and regulatory proteins (Bernecky *et al.*, 2011; Okamoto *et al.*, 1998). The ARCH domain in XPD is critical for the recruitment of CAK to TFIID via the latch domain of MAT1 (Abdulrahman *et al.*, 2013). CDK7 serves an important role in regulating promoter escape and Pol-II transcriptional elongation (Larochelle *et al.*, 2012; Thomas & Chiang, 2006) by directly phosphorylating the hepta-peptide repeating sequence of the Pol-II CTD at Ser5 and Ser7, initiating Pol-II transcription pausing, and phosphorylation and activating the negative transcription elongation factors DSIF and NELF, respectively (Glover-Cutter *et al*, 2009; Kelso *et al*, 2014; Larochelle *et al.*, 2012; Ohkuma & Roeder, 1994; Sanso *et al*, 2016).

Second, transcription elongation and post-transcriptional mRNA processing is regulated by several CDKs whose activities are dependent on CDK7 phosphorylation. CDK7 promotes Pol-II transcriptional elongation through phosphorylation of CDK9, promoting its activity (Booth *et al*, 2018; Peterlin & Price, 2006). Phosphorylation of the Pol-II CTD at Ser2 in the hepta-peptide repeats, which promotes transcription elongation, is performed by CDK9 (Jonkers & Lis, 2015). Further, CDK8, a subunit of the Mediator complex, acts as both a positive and negative regulator of transcription, depending on its cyclin partner and the target protein (Galbraith *et al.*, 2010; Taatjes, 2010). Lastly, CDK12/13, a CAK target, is involved in the transcription processes of elongation via Pol-II CTD phosphorylation and the post-transcriptional processes of transcript alternative splicing (Bosken *et al*, 2014; Ko *et al*, 2001; Kohoutek & Blazek, 2012).

Third, CDK7 can act to regulate the activities of several transcription factors and thereby promote the expression of their target genes. Examples include the Yap/Taz transcription regulators of the Hippo pathway, where CDK7 phosphorylation helps to prevent their

degradation (Cho *et al*, 2020). In the absence of CDK7-mediated phosphorylation, Yap/Taz binds DCAF12, which recruits an E3 ubiquitin ligase complex, leading to ubiquitination and proteasomal degradation of Yap/Taz (Cho *et al.*, 2020). Yap/Taz proteins are transcriptional coactivators that are essential for organ regeneration after tissue damage, implicating CDK7 in tissue growth regulation and regenerative processes (Moya & Halder, 2019). Several nuclear receptor transcription factors including the retinoic acid receptor, ER, and androgen receptor (AR) are phosphorylated by CDK7, either as free CAK or when in complex with TFIIH (Anbalagan & Rowan, 2015; Chymkowitch *et al*, 2011; Gaillard *et al*, 2006). Thus, CDK7-TFIIH phosphorylation of retinoic acid receptors and ER promotes their activities and turnover (Bastien *et al*, 2000; Chen *et al*, 2000). Further, there is evidence for CDK7 activity increasing the sensitivity of BC cells to low estrogen, suggesting a role in driving HR+ BC (Chen *et al.*, 2000). CDK7 phosphorylation of the AR directs its ubiquitin-dependent degradation (Chymkowitch *et al.*, 2011).

TFIIH is involved in transcription-coupled DNA repair. As a component of TFIIH, CAK participates in repair of single-stranded DNA damage induced by ultraviolet light (Rimel & Taatjes, 2018). MAT1 negatively regulates the helicase activity of the nucleotide excision protein XPD (Abdulrahman *et al.*, 2013). Thus, removal of CAK from TFIIH is required to enable nucleotide excision repair. Lastly, CAK phosphorylates p53 (Ko *et al*, 1997), however, the implications of this phosphorylation for p53 activity in the DNA damage response and cell cycle arrest/cell death, remain unknown.

1.4.4 Evidence for the potential for targeting CDK7 in cancer

CDK7 is overexpressed in a range of cancer types, with elevated expression of all components of the CAK complex found in BC, compared to normal tissue (Patel *et al*, 2016). siRNA-mediated knockdown of any CAK component decreased the mRNA levels of the remaining CAK subunits, indicating transcriptional co-regulation of CAK subunit expression. Importantly, CDK7 is differentially expressed between BC subtypes with evidence suggesting that CDK7 is a useful prognostic biomarker for the ER+ and TNBC subtypes (Li *et al*, 2017; Patel *et al.*, 2016; Tang *et al*, 2021b). While expression of CDK7, cyclin H and MAT1 in ER+ BC has been positively correlated with ER expression and improved patient outcomes (Patel *et al.*, 2016), in TNBC, CAK expression is associated with poor prognosis (Li *et al.*, 2017). Similarly, in other cancers such as gastric cancer, ovarian cancer, hepatocellular carcinoma, and glioblastoma, elevated CDK7 expression has been associated with reduced survival (Meng

et al, 2018; Tsang *et al*, 2019; Wang *et al*, 2016; Zhang *et al*, 2017). Consequently, cancers with elevated CDK7 expression may be particularly sensitive to inhibition of the kinase.

Dysregulation of transcriptional programs, leading to aberrant gene expression, is a hallmark of cancer. Many cancer types are dependent on the transcription of a key set of genes and therefore, are highly sensitive to transcription inhibition. This process has been termed “transcriptional addiction”, and is common in highly aggressive cancers, such as TNBC (Bradner *et al*, 2017). TNBC has been shown to be exceptionally dependent on CDK7-driven transcription, whereby CDK7 inhibition targets an “Achilles cluster” of TNBC-specific genes, resulting in apoptotic cell death (Wang *et al*, 2015). CDK7 inhibition plays an important role in impairing the transcription of critical oncogenes that drive cancer progression, such as *MYC* and *BRCA1* (Chipumuro *et al*, 2014; Tee *et al*, 2020; Yao *et al*, 2023), sensitizing tumours to DNA-damaging therapies such as radiation and chemotherapy (Tang *et al*, 2021b; Zhong *et al*, 2019). Beyond its role in transcription, CDK7 exerts influence over cell cycle progression by phosphorylating CDK1, CDK2, and CDK4/6 (Lolli & Johnson, 2005). Dysregulation of cell cycle CDKs and their cyclin binding partners is also a common hallmark of cancer (Alao, 2007; Cai *et al*, 2023; Ortega *et al*, 2002). Consequently, cancer cells may exhibit heightened sensitivity to the inhibition of cell cycle CDK activities compared to their normal counterparts.

1.4.5 Development of CDK7 inhibitors

Due to its common overexpression and central role in mediating aberrant transcriptional networks and the cell cycle, CDK7 emerges as an attractive target for new anti-cancer drug development (Fisher, 2005). Several pan-CDK inhibitors that also target CDK7, such as seliciclib and alvocidib, failed to show compelling results in most clinical trials, largely owing to their lack of CDK selectivity (Asghar *et al*, 2015; Benson *et al*, 2007). As a result, there has been a greater focus on the development of CDK7-specific inhibitors. CDK7-specific inhibitors can generally be classified by their mechanism as reversible or irreversible CDK7 inhibitors (Sava *et al*, 2020). Covalent (irreversible) CDK7 inhibitors include pyrimidine based THZ1, SY-1365, and the pyrrolidinopyridine-based YKL-5-124 (Hu *et al*, 2019; Kwiatkowski *et al*, 2014; Olson *et al*, 2019).

THZ1 is an adenine analog and is the first-in-class covalent CDK7 inhibitor, which targets a cysteine residue (Cys312) outside the CDK7 kinase domain with affinity for CDK7 in the low nanomolar range (Kwiatkowski *et al*, 2014). THZ1 has shown anti-proliferative and apoptotic

effects in several cancer types including MYCN-amplified neuroblastoma, lung cancer, and BC (Chipumuro *et al.*, 2014; Christensen *et al.*, 2014; Wang *et al.*, 2015). Importantly, BC cells resistant to palbociclib displayed increased sensitivity to THZ1 (Dowsett, 2017), which highlights the use of CDK7 inhibitors in the context of resistance to drugs targeting other CDKs. Considering that co-crystal structures revealed CDK7 Cys312 occupies similar spatial positions as CDK12 Cys1039 and CDK13 Cys1017 (Bosken *et al.*, 2014; Greifenberg *et al.*, 2016; Lolli *et al.*, 2004), it is unsurprising that THZ1 proved to also inhibit CDK12/13, albeit at higher concentrations (Zeng *et al.*, 2018). Since its discovery, a THZ1 analog known as THZ2 has been developed with improved pharmacokinetics and more promising therapeutic potential *in vitro* (Wang *et al.*, 2015). Interestingly, both THZ1 and THZ2 are more effective at impairing cell proliferation in TNBC than in HR+ BC, possibly due to the dependence of TNBC on CDK7 and high rates of transcription. Due to the short half-life of THZ1 *in vivo* from metabolic instability, no clinical trials in BC were attempted (Li *et al.*, 2019).

SY-1365 was developed following attempts to improve THZ1 potency, selectivity, and metabolic stability (Hu *et al.*, 2019). Select molecular replacements improved SY-1365 affinity and specificity for CDK7 compared to THZ1 while also increasing the anti-cancer potency of SY-1365 *in vitro*. Several cancer types were highly susceptible to SY-1365 *in vitro* and in xenograft models including leukemia, neuroblastoma, and, to a lesser extent, BC (Hu *et al.*, 2019). However, while SY-1365 was highly selective for CDK7, clinical trials with SY-1365 have thus far produced disappointing results resulting in its discontinuation in clinical trials (Hu *et al.*, 2019; Kovalova *et al.*, 2023; Syros Pharmaceuticals, 2019).

YKL-5-124 was also initially derived from THZ1 and covalently targets CDK7 (Olson *et al.*, 2019). Unlike THZ1, YKL-5-124 specifically inhibits CDK7 and has no significant affinity for CDK12/13. Interestingly, YKL-5-124 has been reported to block CAK function to disrupt cell cycling while having no discernable effects on Pol-II CTD phosphorylation. YKL-5-124 testing in pre-clinical xenograft models found significant, albeit moderately, reduced cell proliferation and improved survival in lung cancer models (Zhang *et al.*, 2020).

SY-5609 was developed as a non-covalent analogue of SY-1365 to target CDK7 (Marineau *et al.*, 2022). However, SY-5609 shows some affinity for other CDKs including CDK16. SY-5609 induces G2/M cell cycle arrest and minimal apoptosis in cancer cell lines and mouse

xenografts, including in TNBC cells (Marineau *et al.*, 2022). SY-5609 is currently being tested in clinical trials for TNBC and HR+ BC (Sava *et al.*, 2020).

BS-181 was the very first selective CDK7 inhibitor to be reported (Ali *et al.*, 2009). BS-181 is an ATP-competitive, reversible non-covalent CDK7 inhibitor, which caused inhibition of cancer cell growth, accompanied by reduced Pol-II and cell cycle CDK phosphorylation. However, the poor pharmacological properties of BS-181 led to the development of BS-181 analogues. Although *in vivo* activity was demonstrated in human tumour (MCF7) xenografts, poor bioavailability and cell permeability prevented BS-181 from progressing to clinical trials. An effort to develop BS-181 with improved drug properties led to the discovery of ICEC0942 (CT7001/samuraciclib), the first orally bioavailable CDK7 inhibitor (Patel *et al.*, 2018a). ICEC0942 showed efficacy in a panel of cancer cell lines, and in ER+ BC *in vivo*.

1.4.6 Samuraciclib inhibition of CDK7

ICEC0942 (hereafter referred to as samuraciclib) was developed in our lab in collaboration with the chemistry department at Imperial College London (Hazel *et al.*, 2017; Patel *et al.*, 2018a).

Samuraciclib was screened against the NCI-60 cancer cell line panel, revealing effective growth inhibition with a mean GI₅₀ of 250 nM (Patel *et al.*, 2018a). The growth of BC cell lines, specifically, was effectively inhibited with GI₅₀ concentrations of 200-300 nM. Non-tumourigenic breast epithelial cells (*i.e.*, MCF10A) and primary human mammary epithelial cells were notably less sensitive to samuraciclib, indicating a heightened susceptibility of cancer cell lines versus non-cancer cell lines to CDK7 inhibition. At higher concentrations (*i.e.*, 10µM), samuraciclib inhibited other kinases including ERK8, STK33, CHK2, CLK2, PHK, and CDK2. Pol-II CTD, CDK1, CDK2, and RB phosphorylation was inhibited by samuraciclib, suggesting a broad mechanism of CDK7 inhibition that affects both transcription and cell cycle progression (Patel *et al.*, 2018a). Further, ER phosphorylation at a CDK7 target site, which is linked to ER activation (Chen *et al.*, 2000), was also reduced by samuraciclib.

The efficacy of inhibiting CDK7 as means of cancer cell growth inhibition is further exemplified by studies where samuraciclib is co-administered with anti-estrogens. The combination of samuraciclib with tamoxifen or fulvestrant resulted in combinatorial effects in MCF7 ER+ BC cells (Patel *et al.*, 2018a). In mice, the combination of tamoxifen and

samuraciclib showed potent inhibition of MCF7 xenograft tumour growth (Patel *et al.*, 2018a). At higher concentrations, samuraciclib also exhibited inhibitory effects on CHK2, suggesting its involvement in cell cycle arrest via the DNA damage response (Patel *et al.*, 2018a). The primary mechanism of samuraciclib inhibition of BC cell proliferation appears to be through the inhibition of cell cycling (Wilson *et al.*, 2023). Existing evidence suggests that samuraciclib treatment induces mTOR-dependent cellular senescence in ER+ BC cells after treatment *in vitro* (Wilson *et al.*, 2023). However, a more comprehensive understanding of how samuraciclib inhibition of CDK7 results in impaired cell growth is required. Samuraciclib has recently reported on the phase 1 clinical trial where the maximum tolerated dose was established and clinical efficacy was demonstrated in a proportion of ER+ BC patients who had advanced on prior CDK4/6 inhibitor treatment in combination with AI (Coombes *et al.*, 2023). The FDA granted fast track designations to samuraciclib for progression through phase II clinical trials for the treatment CDK4/6 inhibitor resistant, HR+, HER2- advanced BC and locally advanced or metastatic TNBC, in combination with fulvestrant and chemotherapy, respectively (Patel *et al.*, 2018a; Sava *et al.*, 2020).

1.5 Ferroptosis

1.5.1 Ferroptosis overview

Ferroptosis is a type of programmed or regulated cell death (RCD) that was identified in 2012 as a novel mechanism of RCD (Dixon *et al.*, 2012). Ferroptosis exhibits notable distinctions from other forms of RCD such as apoptosis, necroptosis, and autophagic cell death (Dixon & Stockwell, 2019). As opposed to accidental cell death, which is an uncontrolled passive process, RCD is mediated by a series of molecular mechanisms and signalling pathways (**Table 1.2**) (Tang *et al.*, 2019). One of the most widely studied forms of RCD is apoptosis, which involves activation of caspases that initiate the breakdown of cell components and eventual death of the cell, followed by uptake of remaining cellular components by other cells (Elmore, 2007). Evidence generated with genome-wide CRISPR-Cas9 screening prior to the start of my project implicated ferroptosis as a mechanism for cell death by samuraciclib. This section therefore describes ferroptosis in some detail.

Ferroptosis is distinct from other forms of RCD both functionally and morphologically. Ferroptotic cell death does not result in condensation of chromatin, a compromised plasma membrane, or the development of autophagic vacuoles (Dixon & Stockwell, 2019). Rather,

ferroptosis is characterized by peroxidation of the phospholipids of cellular membranes and the accumulation of intracellular iron (Stockwell *et al*, 2017; Zilka *et al*, 2017). Ferroptosis is morphologically distinguished by the presence of smaller mitochondria with condensed membrane densities, reduced or vanishing cristae, and rupturing of outer mitochondrial membranes (Yang & Stockwell, 2008). Ferroptotic cell death is iron-dependent and typically the result of an imbalance in cellular antioxidant capacity and accumulation of lethal lipid reactive oxygen species (ROS) derived from iron metabolism (*e.g.*, superoxides, hydrogen peroxide, and peroxy radicals) (Dixon *et al.*, 2012). Recently, non-apoptotic RCD has garnered attention as a novel approach to cancer therapy especially because of intrinsic resistance to apoptosis frequently observed in cancer (Hanahan & Weinberg, 2011). Therefore, eliciting distinct non-apoptotic RCD, such as ferroptosis, may provide novel BC therapeutic options.

Regulation of ferroptotic cell death involves control of phospholipids including polyunsaturated fatty acids, mechanisms that drive phospholipid peroxidation, and antioxidant systems that facilitate the clearance of lipid peroxides. Endogenously, the major cellular antioxidant systems are glutathione peroxidase (GPX4) and the nuclear factor erythroid 2-related factor 2 (NRF2), which limit ROS production and regulate free intracellular iron levels, respectively (Forcina & Dixon, 2019; Kerins & Ooi, 2018). The detailed antioxidant mechanisms of GPX4 and NRF2 are explained below.

Table 1.2 Distinguishing features of ferroptosis, apoptosis, necroptosis, autophagy, and pyroptosis.

Regulated Cell Death (RCD)					
	Ferroptosis	Apoptosis	Necroptosis	Autophagy	Pyroptosis
Morphology	Reduced mitochondrial size and increased mitochondrial membrane density. Vanishing Crista, outer mitochondrial membrane rupture	Cell/nuclear shrinkage, membrane blebbing chromatin condensation, nuclear fragmentation, cytoskeletal collapse, formation of apoptotic bodies. No changes in mitochondria	Breakdown of plasma membrane, cell/ organelle swelling, modest chromatin condensation, spillage of intracellular constituents into microenvironment	Double-membraned autophagosome formation	Inflammasome formation, nuclear condensation and DNA fragmentation, cell swelling and membrane rupture
Biochemical features	Lipid peroxidation, iron accumulation	DNA fragmentation	Lower levels of ATP	Lysosomal activity	Inflammatory caspases
Regulatory pathways	GPX4, System Xc-, NRF2-Keap1, p53-SLC7A11, ATG5-ATG7-NCOA4, HSPB1-TRF1, FSP1-CoQ10-NAD(P)H	Death receptor, caspase, mitochondrial/ ER pathway, Caspase, Bcl-2, p53	PKC-MAPK-AP-1, TNF-R1 and RIP1/RIP3-MLKL, ROS-related pathways	mTOR, p53, Beclin-1	Caspase 4/5/11 - gasdermin
Gene signature	GPX4, SLC7A11, NRF2, NCOA4, p53, FSP1, BH4, ACSL4, HSPB1, TFR1	Caspase, Bcl-2, p53, Fas, Bax	RIP1, RIP3	LC3, ATG5, ATG7, Beclin-1, TFEB, DRAM3	NLRP1, NLRP3, NLRC4, AIM2, CASP1, GSDMD, and PYCARD, GSDME, CASP3, CASP4, CASP5, CASP11, CASP8, TNF, GSDMC, GZMB, GSDMB, GZMA.

Table adapted from Li *et al* (Li *et al*, 2020a).

1.5.2 Hallmarks of ferroptosis: lipid peroxidation

Ferroptosis is distinguished from other forms of RCD by several “hallmarks of ferroptosis”. The first hallmark of ferroptosis is membrane lipid peroxidation (Dixon & Stockwell, 2019; Stockwell *et al.*, 2017). During lipid peroxidation, peroxy groups oxidize weak C-H bonds in polyunsaturated fatty acids (PUFAs) in both enzymatic and non-enzymatic reactions (Iuchi *et al.*, 2019). Non-enzymatic oxidation can involve an attack by free radicals, such as ROS and reactive nitrogen species. Enzymatic oxidative reactions, such as lipoxygenases, cyclooxygenases, and cytochrome P450s, can also oxidise PUFAs to produce fatty acid peroxides. In ferroptosis, high concentrations of free, intracellular iron facilitate the production of free-radicals and ROS which results in non-enzymatic lipid peroxidation (Dixon *et al.*, 2012; Dixon & Stockwell, 2019). Thus, ferroptotic lipid peroxidation is an iron-dependent process. Peroxidised fatty acids alone can trigger intracellular inflammatory responses and cascades that result in cell death (Clay *et al.*, 2002; Miller & Shyy, 2017).

For ferroptosis to proceed and RCD to occur, PUFAs must be incorporated into membranes (Dixon & Stockwell, 2019). Two enzymes are responsible for incorporating PUFAs into membrane lipids and, therefore, enabling ferroptosis, ACSL4 and LPCAT3 (Dixon *et al.*, 2015). The pivotal role of these enzymes in ferroptosis is evidenced by the fact that ACSL4 inactivation suppresses ferroptosis (Doll *et al.*, 2017). Further, inactivation of ACSL4 and LPCAT3 instilled resistance in cells to two ferroptosis-inducing drugs, the Ras-selective lethal small molecule 3 (RSL3) and ML162 (Dixon *et al.*, 2015). While not all PUFA-containing membrane lipids lead to ferroptosis, it has been shown that the oxidation of phosphatidylethanolamines containing arachidonoyl or adrenoyl fatty acyl chains in their head groups are particularly tightly linked to the induction of ferroptosis in various cell types (Dixon & Stockwell, 2019; Kagan *et al.*, 2017; Wang *et al.*, 2019b). Moreover, both lipids and glycerol-derived lipids containing PUFA tails have also been shown to drive ferroptosis. However, once the oxidized PUFA tail is cleaved from a peroxidised lipid, the oxidized PUFA tail no longer contributes to ferroptosis (Wang *et al.*, 2019b). Thus, peroxidised PUFA tails must remain in the membrane to enable ferroptotic cell death (Chen *et al.*, 2021b). In summary, the generation of oxidized PUFAs and the incorporation of oxidized PUFAs in membranes is a hallmark of ferroptosis.

1.5.3 Hallmarks of ferroptosis: availability of intracellular iron

The second hallmark of ferroptosis is the accumulation of intracellular and bioavailable iron (Dixon & Stockwell, 2019). Ferroptosis is characterized by free iron catalyzing the oxidation of membrane localized PUFAs to generate lipid ROS and cell death (Dixon *et al.*, 2012). Both the abundance of intracellular free iron and the presence of membrane PL-PUFAs are essential for the initiation of ferroptosis. However, excessive free iron alone is potentially toxic because it catalyzes the generation of reactive radicals that can damage cellular processes and create ROS via the Fenton reaction ($\text{Fe}^{2+} + \text{HOOH} \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}\cdot$) (Emerit *et al.*, 2001; Wardman & Candeias, 1996). Thus, the stringent control of intracellular iron levels and iron-dependent biological processes defines ferroptosis as a regulated form of cell death.

Iron is essential for the transport of oxygen and the function of many enzymes (Torti & Torti, 2002). Iron has two oxidation states, Fe^{2+} (ferrous) and Fe^{3+} (ferric). Ferric iron is the most common oxidized state of iron in food and must be reduced to ferrous iron to become biologically available (Dev & Babitt, 2017). To enter a cell, ferrous iron binds to transferrin in the serum and is imported into the cell as iron-transferrin complexes, a process that is dysregulated in several cancers, including BC (**Figure 1.6**) (Basuli *et al.*, 2017; Pantopoulos *et al.*, 2012; Torti & Torti, 2013). As a complex, iron-transferrin is internalized to endosomes. The iron-binding properties of transferrin are dependent on changes in pH. As endosomes mature, lower pH levels within endosomes trigger conformational changes in transferrin, releasing ferrous iron within the endosome. Free ferrous iron is reduced to ferric iron via ferrireductases, such as STEAP3, and exported into the cytosol across the endosomal membrane via DMT1 (Katsarou & Pantopoulos, 2020). Intracellular iron is transferred to mitochondria for heme and Fe-S cluster biosynthesis, while excess is exported via ferroportin (FPN) or stored in the iron-storage protein ferritin (Pantopoulos *et al.*, 2012).

Iron homeostasis is managed by ferritin (Torti & Torti, 2002). The ferritin protein complex contains 24 subunits of ferritin light and heavy chains (FTL, FTH1) and each ferritin molecule can sequester up to ~4,500 iron atoms (Harrison & Arosio, 1996). Ferritin is typically intracellular and controls intracellular iron levels through its regulated expression and degradation. However, at times of excessive iron and inflammation, such as in cancer, extracellular (serum) ferritin levels may increase drastically (Torti & Torti, 1994). A key regulator of ferritin expression is the post-transcriptional binding of iron regulatory proteins 1

and 2 (IRP1/2) to the mature mRNA of ferritin at stem-loop structures, which is dependent on intracellular iron levels (Rogers & Munro, 1987). Ferrous iron is incorporated into ferritin via ferritin iron pores, and is oxidised to ferric iron by FTH1, storing iron for later use (Honarmand Ebrahimi *et al*, 2015).

Cells control intracellular iron levels through several mechanisms including ferritinophagy and iron export (Katsarou & Pantopoulos, 2020). The cargo receptor nuclear receptor coactivator 4 (NCOA4) mediates ferritinophagy, which is the process of autophagic degradation of ferritin and increases intracellular iron levels, therefore dictating cellular sensitivity to ferroptosis (Liu *et al*, 2022b). Reduced NCOA4 activity inhibited ferroptosis in HT-1080 fibrosarcoma, pancreatic cancer (Panc-1) and other cell types likely by reducing free intracellular iron pools (Gao *et al*, 2016; Hou *et al*, 2016). Other mechanisms that contribute to intracellular iron levels also influence the sensitivity of cells to ferroptosis, such as through iron export via ferroportin and prominin-2-mediated multi-vesicular bodies (Brown *et al*, 2019; Ward & Kaplan, 2012). The several redundant mechanisms of intracellular iron homeostasis emphasize the importance of controlling of free iron concentrations intracellularly to tightly regulate ferroptosis.

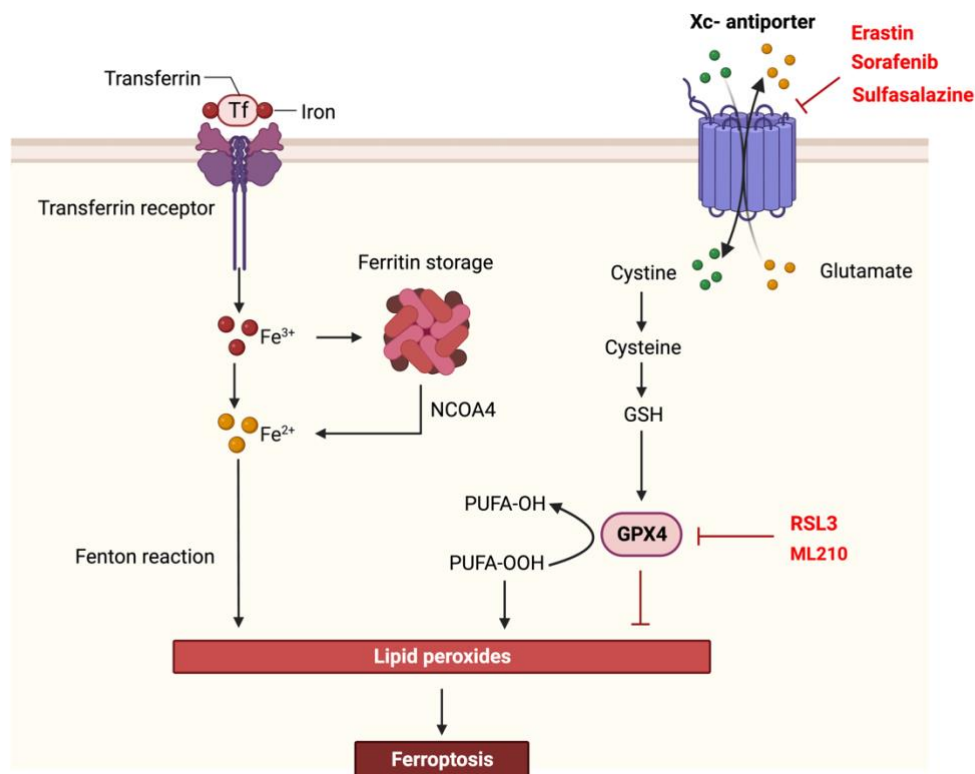


Figure 1.6 Methods of promoting and inhibiting ferroptosis.

(Left) The transferrin receptor binds extracellular transferrin, which chelates ferric iron (Fe³⁺). Following endocytosis and maturation of the endosome, ferric iron is reduced to ferrous iron (Fe²⁺) and becomes biologically available. Ferrous iron can participate in non-enzymatic lipid peroxidation via the Fenton reaction, creating lipid peroxides that promote ferroptosis. (Right) System Xc⁻ is a cystine-glutamate transporter that imports cystine for synthesis of the metabolite GSH. GSH is used by GPX4 to reduce lipid peroxides (PUFA-OOH) into non-reactive lipid alcohols (PUFA-OH), inhibiting ferroptosis. Tf, transferrin; Fe²⁺, ferrous iron; Fe³⁺, ferric iron; Ras-selective lethal small molecule 3, RSL3; NCOA4, nuclear receptor coactivator 4. Figure was generated using BioRender.

1.5.4 Hallmarks of ferroptosis: loss of GPX4 lipid peroxide repair capacity

The third hallmark of ferroptosis is the loss of lipid peroxide repair via GPX4 inhibition (Dixon & Stockwell, 2019). The lipid hydroperoxidase glutathione peroxidase 4 (GPX4), a selenoenzyme, plays a central role in ferroptosis inhibition by reducing lipid peroxides to lipid alcohols and preventing membrane lipid peroxidation (**Figure 1.6**) (Forcina & Dixon, 2019; Ingold *et al*, 2018). There are a total of 25 selenoproteins in humans. However, GPX4 is the only essential one for normal mammalian development (Ingold *et al.*, 2018). GPX4 uses glutathione (GSH) to reduce reactive phospholipid hydroperoxides (PUFA-PL-OOH) to non-reactive phospholipid alcohols (PUFA-PL-OH) (Ursini *et al*, 1982). System Xc⁻, the glutamate-cystine antiporter located in the phospholipid bilayer, plays an important role in the GPX4-dependent reduction of PUFA-PL-OOH by transporting cystine in and glutamate out of the cell at a 1:1 ratio (Shih *et al*, 2006). Cystine gets metabolized into the main intracellular antioxidant, GSH (**Figure 1.6**). Due to the important role of System Xc⁻ in regulating GPX4 activity and thus ferroptosis, inhibition of System Xc⁻ can facilitate ferroptosis. System Xc⁻ is composed of two targetable subunits: SLC7A11 (xCT) and SLC3A2 (4F2hc, CD98). Erastin is a classic small molecule ferroptosis inducer that inactivates system Xc⁻ (Dixon *et al*, 2014). Other ferroptosis inducers include the two similar compounds, RSL3 and ML210, which covalently bind GPX4 and trigger ferroptosis (Liu *et al*, 2022a; Sun *et al*, 2021). Similarly, FIN56 induces ferroptosis by promoting GPX4 protein degradation, although the exact mechanisms of ferroptosis induction by FIN56 remain to be fully understood *in vivo* (Liu *et al.*, 2022a; Sun *et al.*, 2021). Deletion of GPX4 in mice is lethal, while its inactivation is sufficient to cause ferroptosis in numerous cell types (Ingold *et al.*, 2018; Seiler *et al*, 2008; Yang *et al*, 2014). However, even after GPX4 deletion, some cancer cell lines have been shown to continue proliferating, which may be a result of compensatory antioxidant pathways, undefined proteins that protect against lipid peroxidation, or the presence of cancer cells with low levels of oxidisable membrane phospholipids (Viswanathan *et al*, 2017). Importantly, this suggests that not all cancer cells respond to GPX4 inhibition, which indicates the presence of alternative antioxidant mechanisms.

1.5.5 Molecular modulators of ferroptosis sensitivity

Indeed, while GPX4 is the major cellular defence system against ferroptosis, there are others, which can work independently or in unison to prevent ferroptotic cell death independently of GPX4 (Doll *et al*, 2019; Viswanathan *et al.*, 2017). One such pathway involves the electron-transport chain protein coenzyme (CoQ10) and ferroptosis suppressor protein (FSP1)

(Bersuker *et al*, 2019). In this alternative ferroptosis-protecting pathway, FSP1, a NADP-dependent coenzyme (CoQ) oxidoreductase localized in the cell membrane, catalyses the regeneration of CoQ10. CoQ10 in its reduced (ubiquinol) form traps lipid peroxyl radicals, preventing lipid peroxidation (Bersuker *et al*., 2019).

Another GPX4-independent ferroptosis suppressing pathway involves the antioxidant GTP cyclohydrolase-1 (GCH1)-tetrahydrobiopterin (BH4) (Kraft *et al*, 2020). BH4 is an essential cofactor for nitric oxide synthases and prevents the formation of superoxides (Goncalves *et al*, 2021). GCH1 synthesizes BH4, and overexpression of GCH1 renders cancer cells resistant to ferroptosis through upregulation of BH4 (Kraft *et al*., 2020). While GCH1-BH4 is an important GPX4-independent endogenous antioxidant system, the contribution of GCH1-BH4 and FSP1-CoQ10 protection varies across cancer and tissue types, indicating cellular anti-ferroptotic mechanisms may be highly context-dependent (Nehring *et al*, 2020).

1.5.6 KEAP1-NRF2 signalling in ferroptosis

The KEAP1-NRF2 pathway (**Figure 1.7**) is a major regulator of antioxidant response to ROS and electrophiles (Yamamoto *et al*, 2018). KEAP1-NRF2 is comprised of two key proteins 1) nuclear factor erythroid 2-related factor 2 (NRF2), a transcription factor that binds to antioxidant response elements, and 2) Kelch ECH associating protein 1 (KEAP1), which is a cysteine-rich protein that binds to and represses NRF2 by promoting ubiquitin-dependent degradation. The cysteine residues on KEAP1 can be modified by various oxidants and electrophiles (Kobayashi *et al*, 2004; Zhang *et al*, 2004a). Modifications in KEAP1 cysteine residues result in structural changes in KEAP1 that inhibit its ubiquitin ligase activity, preventing NRF2 ubiquitination and proteasomal degradation. In turn, NRF2 is stabilized and translocates to the nucleus where it induces its target gene expression (Horie *et al*, 2021; Takaya *et al*, 2012).

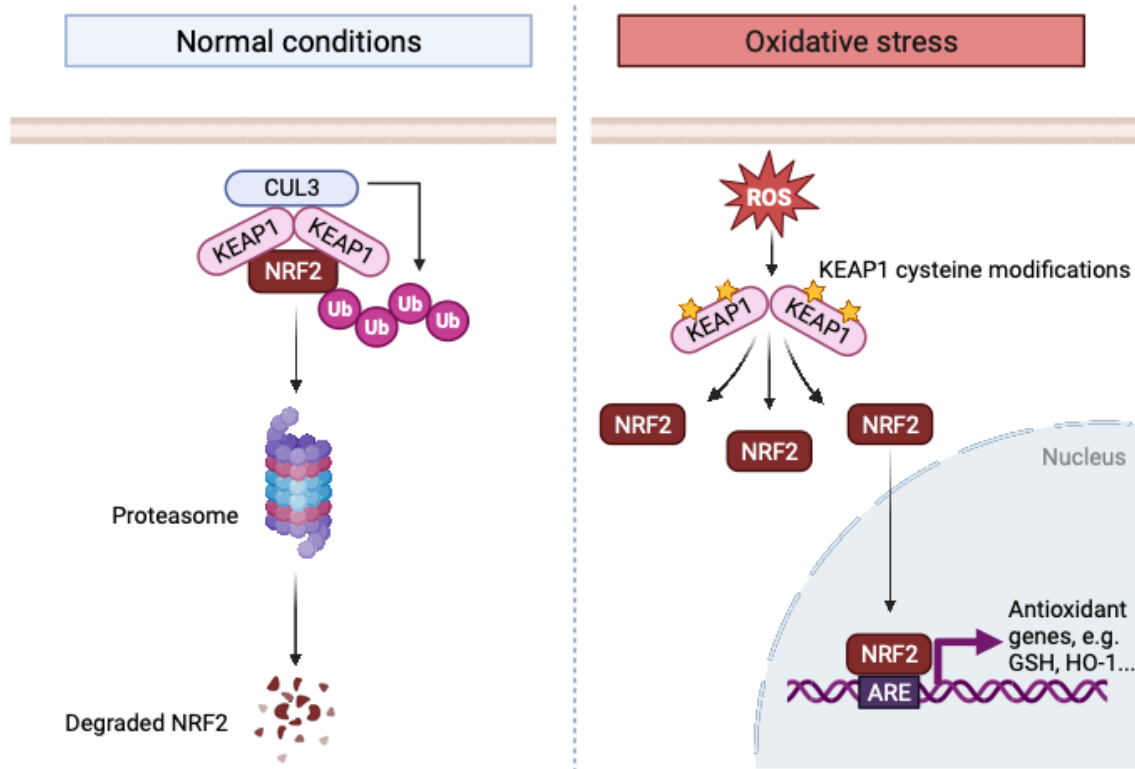


Figure 1.7 The NRF2-KEAP1 signalling pathway is a major regulator of intracellular antioxidant response.

(Left) Under normal conditions, KEAP1-bound NRF2 is maintained at low levels via the ubiquitin-proteasomal degradation pathway. (Right) In oxidative stress conditions, such as exposure to reactive oxygen species, cysteine modifications on KEAP1 prevent binding to NRF2, stabilizing NRF2. Stable NRF2 is translocated to the nucleus where it acts as a positive transcription factor for the expression of antioxidant genes, such as GSH and HMOX-1 (HO-1). NRF2, nuclear factor erythroid 2-related factor 2; KEAP1, Kelch ECH associating protein 1; ARE, antioxidant response element; CUL3, cullin 3. Figure was generated using BioRender.

NRF2 has two KEAP1-binding motifs located in the Neh2 domain in the N-terminus, namely the DLGex motif and the ETGE motif (Horie *et al.*, 2021; Itoh *et al.*, 1999). The DLGex motif is composed of three α -helices and has low affinity for KEAP1. In contrast, the ETGE motif comprises of a single β -hairpin that binds with high affinity to KEAP1 (Canning *et al.*, 2013). KEAP1 is made of four domains the Broad complex, Tramtrack and Bric-à-Brac (BTB) domain, the intervening region (IVR), the double glycine repeat (DGR) and the C-terminal region (CTR). The BTB domain mediates KEAP1 dimerization and the IVR domain contains cysteine residues involved in NRF2 repression (Canning *et al.*, 2015). The DGR and CTR, known as the DC domain of KEAP1, interact with the Neh2 domain of NRF2 (Ogura *et al.*, 2010). To repress NRF2 activity, KEAP1 forms a dimer and binds NRF2 in a 2:1 ratio. KEAP1 binding NRF2 at two sites (DLG and ETGE) is essential for efficient ubiquitination of NRF2 (Tong *et al.*, 2006). Abrogation of KEAP1-NRF2 binding, especially at the low-affinity DLG site, prevents NRF2 degradation, highlighting its function as a stress sensor (Horie *et al.*, 2021; Ogura *et al.*, 2010).

Under normal conditions, the NRF2 transcription factor is maintained at relatively low concentrations by constant ubiquitination mediated by KEAP1, a component of the Cul3-based ubiquitin E3 ligase complex (Cullinan *et al.*, 2004; Kobayashi *et al.*, 2004; Zhang *et al.*, 2004a). Following stress, such as exposure to electrophiles or ROS, NRF2 is not ubiquitinated via KEAP1 and is instead stabilized to translocate into the nucleus where it induces its target gene expression (Yamamoto *et al.*, 2018). NRF2 target genes are involved in glutathione synthesis, ROS elimination, and other cytoprotective processes (Taguchi *et al.*, 2011). For example, several NRF2 target genes prevent lipid peroxidation and the accumulation of free iron, thereby reducing the risk of ferroptotic death (Chen *et al.*, 2009). Similarly, knockdown of NRF2 was shown to be sufficient to sensitize cancer cells to ferroptosis (Anandhan *et al.*, 2023). Overall, the NRF2-KEAP1 pathway functions to prevent cancer initiation and progression in normal conditions. However, in malignant cells and stress conditions, NRF2-KEAP1 function can be overactive and increase chemoresistance and enhance tumour cell growth.

1.5.7 The RAS/MAPK pathway in ferroptosis

The RAS/MAPK pathway may sensitize cancer cells to ferroptosis by regulating the expression of transferrin and intracellular iron levels (Yang & Stockwell, 2008). For example, KRAS silencing in KRAS-mutant Calu-1 cells reduced the efficacy of the ferroptosis inducer Erastin (Yagoda *et al.*, 2007). However, these observations have been contradicted by recent evidence

that increased RAS expression increases resistance to ferroptosis in a different cancer cell line (Schott *et al*, 2015). Thus, current theories suggest lineage- and mutation-specific sensitivity of cancer cells to RAS/MAPK-induced resistance or sensitivity to ferroptosis (Dixon & Stockwell, 2019; Poursaitidis *et al*, 2017; Tsoi *et al*, 2018).

1.5.8 Ferroptosis inducers and inhibitors

Methods of regulating ferroptosis exogenously generally impact intracellular iron concentrations or lipid peroxide repair capacity, which, in turn leads to lipid peroxidation (Dixon & Stockwell, 2019). Various compounds induce ferroptosis by facilitating phospholipid peroxidation or inhibiting ferroptosis suppression (Ursini & Maiorino, 2020; Yang *et al.*, 2014). For example, Erastin, RSL3, and buthionine sulfoximine (BSO) induce ferroptosis through inhibition of system Xc⁻, inhibition of *GPX4*, and depletion of GSH, respectively (Dixon *et al.*, 2014; Sui *et al*, 2018; Yang *et al.*, 2014). Additionally, Sorafenib and Sulfasalazine, which are FDA approved for the treatment of various cancers and rheumatoid arthritis, respectively, induce ferroptosis in cancer and normal cells (Dixon *et al.*, 2014; Lachaier *et al*, 2014). Sorafenib and Sulfasalazine can induce ferroptosis in BC cells via the depletion of intracellular GSH and inhibition of cystine uptake, respectively (Dixon *et al.*, 2014; Lachaier *et al.*, 2014).

While reduced enzymatic function of *GPX4*, or the dysregulation of cystine intake and GSH synthesis can induce ferroptosis in cancer cells (Demierre *et al*, 2005; Viswanathan *et al.*, 2017; Warner *et al*, 2000), intracellular iron levels also play a major role in promoting ferroptosis. Synthetic intracellular iron loading can promote ferroptosis by introducing exogenous iron using nanoparticles (Kim *et al*, 2016). Regulating the expression of iron endocytosis pathway components can also induce or protect cells from ferroptosis (Gao *et al.*, 2016; Gao *et al*, 2015; Torii *et al*, 2016). However, merely introducing iron exogenously does not trigger ferroptosis (Dixon *et al.*, 2012).

In contrast, introducing iron chelators modulates intracellular iron availability and functions to protect cells against ferroptosis (Dixon & Stockwell, 2019). Several iron chelators, such as deferoxamine and desferrioxamine mesylate, inhibit ferroptosis by depleting levels of free intracellular iron (Stockwell *et al.*, 2017; Yang & Stockwell, 2008). Furthermore, ferroptosis can be prevented by administering exogenous antioxidants (Dixon & Stockwell, 2019).

Synthetic lipophilic antioxidants, such as ferrostatin-1 and liproxstatin-1, can be introduced to effectively inhibit oxidative damage within cell membranes (Zilka *et al.*, 2017).

1.6 mTOR

1.6.1 mTOR overview

The mammalian target of rapamycin (mTOR) complex is a key signal transducer of cell proliferation and apoptosis pathways. mTOR forms two distinct multi-protein complexes: mTORC1, which is involved in cell growth and metabolism (**Figure 1.8**), and mTORC2, which is involved in cell proliferation and survival (Unni & Arteaga, 2019). The mTORC1 complex is composed of mTOR protein, proline rich Akt substrate 40 (PRAS40), mammalian LST8 (mLST8), and RAPTOR, while the mTORC2 complex consists of mTOR, mLST8, mSIN1, protor and RICTOR (McAuliffe *et al.*, 2010). mTORC2 phosphorylates Akt, which, for the most part, inhibits negative regulators of the cell cycle, transcription, metabolism, and cell survival. For instance, Akt phosphorylates forkhead box 01 (FoxO), which is a forkhead transcription factor, p27 and p21, which are cell cycle regulators, and GSK3, which is a metabolism regulator (Jacinto *et al.*, 2006). Thus, the mTORC2 downstream target, Akt, serves an important role in the activation of several cellular processes. The other mTOR complex, mTORC1, phosphorylates the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) as well as the S6 kinase 1 (S6K1). 4E-BP1 prohibits translation initiation by binding to the eukaryotic initiation factor 4E (eIF4E), preventing recruitment of translation machinery to mRNA (Gingras *et al.*, 2001). When hyperphosphorylated, 4E-BP1 can no longer bind to eIF4E, therefore activating translation of genes such as cyclin D1, Bcl-2, Bcl-xL and vascular endothelial growth factor (VEGF), promoting cell survival, proliferation and angiogenesis (De Benedetti & Graff, 2004; Soni *et al.*, 2008). In response to cellular energy status, nutrients, and mitogens, mTORC1 phosphorylates S6K1, which in turn phosphorylates the S6 ribosomal protein, the insulin receptor substrate 1 (IRS-1), and the eukaryotic initiation factor 4B (eIF4B), driving oncogenic transformation (De Benedetti & Graff, 2004; Jastrzebski *et al.*, 2007). Importantly, mTORC1 regulates cellular responses to nutrients and growth factors primarily as part of the PI3K/Akt/mTOR pathway, which is associated with tumorigenesis and is often dysregulated in BC (Fresno Vara *et al.*, 2004; Ippen *et al.*, 2019; Paplomata & O'Regan, 2014; Steelman *et al.*, 2016).

The PI3K/Akt/mTOR pathway promotes cell survival, senescence and regulates autophagy (Carroll *et al.*, 2017; Deleyto-Seldas & Efeyan, 2021; Fresno Vara *et al.*, 2004; Hung *et al.*, 2012). Notably, the PI3K/Akt/mTOR pathway has also been implicated in endocrine resistance with Akt activating ER independent of estrogen (Fresno Vara *et al.*, 2004). Similarly, the PI3K/Akt/mTOR pathway is implicated in trastuzumab resistance in HER2+ BC and mutations in the catalytic subunit (PIK3CA) of the PI3K enzyme is associated with ER expression (Saal *et al.*, 2005). The following section discusses the PI3K pathway in more detail.

1.6.2 PI3K/Akt/mTOR pathway

The PI3K/Akt/mTOR pathway transduces growth factor signals influencing cell proliferation and migration (Unni & Arteaga, 2019). First, insulin or insulin-like growth factor (IGF) binds the insulin-like growth factor receptor (IGFR). In turn, this leads to activation of intracellular, membrane-bound Phosphatidylinositol 3-kinase (PI3K) (McAuliffe *et al.*, 2010). Activated PI3K phosphorylates phosphatidylinositols converting PI(3,4)P₂ to PI(3,4,5)P₃ at the plasma membrane. This leads to the recruitment of phosphoinositide-dependent kinase 1 (PDK1) and Akt to the cell membrane, and activation of downstream effectors, including the mTOR complexes, described above.

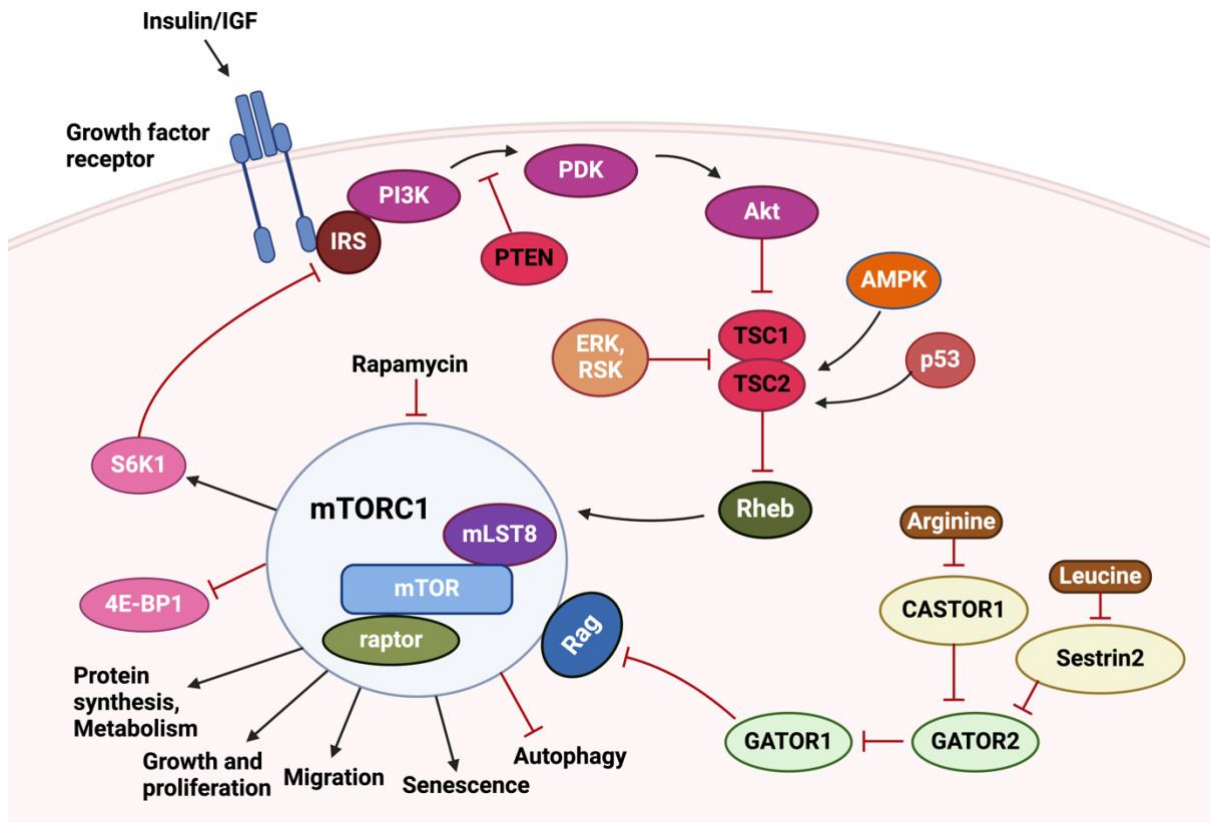


Figure 1.8 The mTORC1 pathway in mammalian cells is a key signalling hub for several biological processes.

The PI3K/Akt/mTOR regulates cellular responses to nutrients and growth factors. mTORC1 activation regulates metabolism and migration and promotes cell survival and senescence while inhibiting autophagy. For a detailed description of the pathway, refer to section 1.6. Figure was generated using BioRender.

The PI3K/Akt/mTOR pathway has many positive and negative regulators. A central negative regulator of PI3K often deleted in between 6 and 20% of BC (Nik-Zainal *et al.*, 2016) is the tumour suppressor phosphatase and tensin homolog (PTEN), which downregulates Akt signalling by dephosphorylating PIP₃, converting it back to PIP₂ (Georgescu, 2010; McAuliffe *et al.*, 2010; Miller *et al.*, 2009). PIP₃ is required for direct activation of AKT, as well as indirect activation via PDK1. Tuberosclerosis complex 1 and 2 (TSC1/2) is also a critical negative regulator of mTOR which integrates signals from the LKB1-adenosine monophosphate kinase (AMPK) pathway, involved in monitoring cellular energy levels (McAuliffe *et al.*, 2010), with signals from PI3K, PTEN, and Akt (Huang & Manning, 2008; Shackelford & Shaw, 2009). Additionally, in response to DNA damage, p53 can inhibit mTORC1 via TSC1/2 and Rheb (Feng *et al.*, 2005). The positive mTORC1 regulator GATOR2 interacts with GATOR1, a negative regulator of mTORC1, at the lysosomal membrane (Bar-Peled & Sabatini, 2014). Sestrin2 and CASTOR1 are cytosolic leucine and arginine sensors, respectively (Chantranupong *et al.*, 2016). GATOR2 canonically interacts with GATOR1, a negative regulator of mTORC1, at the lysosomal membrane to form the GATOR complex (Bar-Peled & Sabatini, 2014; Sahu & Ben-Sahra, 2023). When unbound by their amino acids, Sestrin2 and CASTOR1 inhibit GATOR2. This prevents GATOR2 from inhibiting GATOR1, allowing for mTORC1 inhibition by GATOR1 (Chantranupong *et al.*, 2016; Parmigiani *et al.*, 2014). In the presence of leucine and arginine, sestrin1 and CASTOR1 are inhibited, which leads to downstream activation of mTORC1 where the Rag GTPase mediates the amino acid-induced activation of mTORC1 via a Rheb-dependent mechanism (Bar-Peled & Sabatini, 2014; Sancak & Sabatini, 2009). Due to the central importance of mTOR in tumourigenesis and endocrine resistance, several mTOR precision therapies have been developed.

1.6.3 mTOR inhibitors

Several therapies have been developed to directly target mTOR and the PI3K/Akt/mTOR pathway. Sapanisertib and torin1 are specific inhibitors of both mTOR complexes (mTORC1 and mTORC2) that have been shown to inhibit cell growth (Hsieh *et al.*, 2010; Thoreen *et al.*, 2009). One clinical trial showed sapanisertib in combination with fulvestrant can improve patient survival in ER+ HER2- BC (Garcia-Saenz *et al.*, 2022). However, sapanisertib therapy showed adverse side-effects leading to early termination of the trial. Rapamycin analogs (rapalogs), including torin1, have been shown to selectively inhibit mTOR and impair cancer cell growth (Meng & Zheng, 2015). In BC, everolimus, which selectively targets mTORC1, has received FDA approval for use in HR+, endocrine-therapy resistant patients (Steelman *et*

al., 2016). Samotolisib is a dual kinase inhibitor of PI3K and mTORC1/2 that has been shown to promote cell cycle arrest in cancer cells (Fiebig *et al.*, 2004; Smith *et al.*, 2016). Samotolisib has been tested in combination therapies with CHK1 inhibitors in BC to exploit homologous recombination repair vulnerabilities in TNBC (Anglian Breast Cancer Study, 2000). Alpelisib was developed to target the PI3K p110 α mutant isoform, which is a frequent mutation in HR+/HER2- BC with approximately 35% of tumours containing activating mutations in the PIK3CA gene that encodes the p110 α subunit of PI3K (Cancer Genome Atlas, 2012; Fritsch *et al.*, 2014; Martinez-Saez *et al.*, 2020). Clinically, the FDA-approved PIK3CA inhibitor alpelisib improved survival in patients after receiving endocrine therapy (Andre *et al.*, 2019). Altogether, the clinical progress of various mTOR pathway inhibitors presents the need for understanding their implementation, especially in the context of combination therapies.

1.7 Project aims and hypothesis

Samuraciclib is the most clinically advanced, orally bioavailable selective CDK7 inhibitor in clinical development. It has been shown to be effective in inhibiting many cancer cell types (Patel *et al.*, 2018a), and has recently completed dose finding in a phase 1 clinical trial (Coombes *et al.*, 2023). Moreover, samuraciclib demonstrated clinical efficacy in combination with fulvestrant in a proportion of patients with advanced ER+ BC who had progressed on prior CDK4/6i + AI treatment. These exciting findings have led to FDA fast track designation for phase 2 studies for samuraciclib use in combination with fulvestrant for CDK4/6 inhibitor resistant HR+, HER2- advanced BC. Additionally, samuraciclib treatment efficacy was demonstrated in some TNBC patients, which has also led to FDA fast track designation for samuraciclib in combination with chemotherapy for the treatment of locally advanced or metastatic TNBC. Considering the rapid clinical development of this, and other, CDK7 inhibitors, it is imperative to profile the molecular and clinical features associated with resistance and susceptibility to samuraciclib, while further evaluating the molecular mechanisms behind the response to samuraciclib. The aims of the work presented in thesis were the following:

1. Profile the association between samuraciclib-induced cell growth arrest and the PI3K/Akt/mTOR pathway.

Genes associated with sensitivity and resistance to samuraciclib-induced growth arrest have been previously profiled in my lab (G Sava *et al.*, unpublished) and by others (Wilson *et al.*, 2023) using genome-wide CRISPR-Cas9 knockout screens. Results from these studies have found that mTOR and PI3K/Akt/mTOR pathway genes are required for samuraciclib-induced cell growth arrest. However, the correlation between PI3K/Akt/mTOR pathway activity and cell responsiveness to samuraciclib has not been fully delineated. Similarly, samuraciclib-response has not been profiled in cells with a hyper-active PI3K/Akt/mTOR pathway.

I hypothesized that, since the PI3K/Akt/mTOR pathway is important for a response to samuraciclib, a hyper-activate PI3K/Akt/mTOR pathway would enhance the samuraciclib-induced growth-arrest. My objectives for this aim were to confirm the PI3K/Akt/mTOR pathway is essential for the samuraciclib-induced cell-growth arrest, to understand how two activating PIK3CA mutations, namely E545K and H1047R, influence the growth-arrest

phenotype, and to evaluate changes in protein and protein phosphorylation levels of key cell cycle CDKs following combination treatment with samuraciclib and PI3K/Akt/mTOR pathway inhibitors and in PIK3CA-mutant and -WT cell lines.

2. Profile the proteomic response associated with samuraciclib treatment in BC cells.

Previously, the transcriptomic (RNA-sequencing) changes to samuraciclib and genes involved in samuraciclib response (genome-wide CRISPR-Cas9 dropout screen) have been investigated in the lab. Other work from the lab investigated changes in gene expression following treatment with samuraciclib (V. Nguyen *et al*, unpublished). As expected from the transcriptional role of CDK7, samuraciclib reduced expression of most genes. However, the downstream impact of samuraciclib on levels of proteins has not been investigated. The goal of this aim was to determine if samuraciclib, by inhibiting gene expression, would cause reduction levels of all proteins, or whether it would be possible to identify changes in levels of specific proteins, which would clarify mechanisms of samuraciclib action, including the identification of potential biomarkers of response to samuraciclib. My objectives were to identify changes in protein levels using quantitative mass-spectrometric analysis of BC cells treated with samuraciclib.

3. Investigate the role of ferroptosis in the cellular response to samuraciclib.

CRISPR-Cas9 gene essentiality screens in MCF7 and NALM-6 cell lines identified the ferroptosis suppressor *GPX4* as an important gene associated with samuraciclib resistance. Given the importance of GPX4 in ferroptosis, I hypothesized that BC cells are sensitized to or undergo ferroptosis by samuraciclib. My objectives were to determine if samuraciclib alters ferroptotic response.

The results of these investigations are presented in this thesis. The conclusions of this work have improved our understanding of the molecular and cellular response to samuraciclib treatment and will help guide BC patient therapeutic strategies.

2. Materials and Methods

2.1 Materials

2.1.1 Reagents

The PureLink genomic DNA (gDNA) isolation kit was obtained from Invitrogen, UK. Reverse transcriptase cDNA synthesis kits, polymerase chain reaction (PCR) kits, and DNA molecular weight ladders were obtained from Thermo Fisher Scientific (Life Technologies, UK). PCR purification kits and ExoSAP were obtained from Thermo Fisher Technology, UK. Synthesized oligonucleotides were purchased from Integrated DNA Technologies. SYBRTM Green real-time quantitative PCR (RT-qPCR) reagents were obtained from Applied Biosystems (Life Technologies, UK). PierceTM protease inhibitor was obtained from Thermo Fisher Scientific, UK. BCA quantification kit, SuperSignal West Pico, and Femto Western blotting reagents were obtained from Thermo Scientific, UK. Protein molecular weight markers and 10% Tween-20 solution was obtained from Bio-Rad Laboratories Ltd, UK (Bio-Rad, UK).

2.1.2 Sundries

Microcentrifuge tubes (1.5 mL and 2 mL) and pipette tips were obtained from STARLAB Ltd, UK. 0.2 mL Thermo-PCR tubes were obtained from Thermo Fisher Scientific (Life Technologies, UK). Serological pipettes, tissue culture, and microbiology grade plastics were obtained from Corning®, UK. Nitrocellulose membrane (0.45 µm) was obtained from Bio-Rad, UK. MicroAmp® Fast 96-well reaction plates and MicroAmp® Optical 384-well reaction plates were obtained from Applied Biosystems (Life Technologies, UK).

2.1.3 General laboratory equipment

For tissue culture work, BIOMAT 2 Microbiological Safety Cabinets (Contained Air Solutions Ltd, UK) were used. Cells were maintained at 37°C in a humidified air atmosphere supplied (5% CO₂) in RS Biotech Galaxy R+ CO₂ incubators. Benchtop microcentrifuges from Sorvall PicoTM (Sorvall, UK) or HeraeusTM Biofuge PicoTM (Thermo Fisher Scientific, UK) were used to spin down samples with volumes under 2 mL. Incubations were performed in incubator ovens (LEEC, UK), shaking incubators (New Brunswick Scientific Company Incorporated, USA), and block heaters (Grant-bio, Wolf Laboratories, UK).

DNA and RNA quantification and quality evaluation was performed using spectrophotometry in a NanodropTMND-1000 (Labtech International, UK). PCR reactions were performed in

Veriti™ 96-well thermal cyclers (Life Technologies, UK). RT-qPCR reactions were performed using StepOne™ Plus Real Time PCR system (Life Technology, UK) or the QuantStudio™ 7 Flex Real Time PCR system (Life Technology, UK). Where applicable, samples were mixed using a Vortex-Genie 2 (Scientific Industries, UK).

Agarose gel electrophoresis was performed in horizontal gel electrophoresis tanks from Thistle Scientific, UK. Vertical gel electrophoresis and transfer tanks used for Western blotting were obtained from Bio-Rad, UK. Chemiluminescence imaging was performed using iBright™ CL 1500 imaging system (Thermo Fisher Scientific, UK) and Fusion Solo system (Vilber Lourmat, France). Photometric absorbance was measured using a Tecan Sunrise™ absorbance microplate reader (Tecan UK Ltd, UK). Long term cell growth was monitored, and cell images were obtained using Incucyte Zoom and IncuCyte S3 live cell imaging systems (Sartorius, UK).

2.1.4 Cell culture reagents

L-Glutamine-Penicillin-Streptomycin (200 mM L-glutamine, 10,000 units / mL penicillin and 10mg/mL streptomycin; PSG), Penicillin-Streptomycin (10,000 units / mL penicillin and 10mg/mL streptomycin; PS), ethylenediaminetetraacetic acid (0.02%; EDTA), and 10X trypsin-EDTA solution were obtained from Sigma-Aldrich, UK. Accutase® Cell Detachment Solution (Accutase® enzymes in Dulbecco's PBS containing 0.5mM EDTA and phenol red) was obtained from BioLegend UK Ltd, UK.

Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Sigma-Aldrich, UK and Lonza, UK. Phenol red-free DMEM and Roswell Park Memorial Institute-1640 (RPMI-1640) lacking phenol-red were obtained from Gibco™ (Life Technologies, UK). DMEM/F-12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) medium was supplied by Thermo Fisher Scientific, UK. Fetal calf serum (FCS) and heat-inactivated horse serum were obtained from First Link Ltd, UK and Thermo Fisher Scientific, UK, respectively. Media supplements, such as hydrocortisone (0.5 mg / mL), insulin from bovine pancreas (10 ug / mL), and cholera toxin (100 ng / mL) were all purchased from Sigma-Aldrich, UK. Human epidermal growth factor (hEGF) (20 ng / mL) was obtained from Peprotech, UK.

2.1.5 Chemicals

Dimethyl sulfoxide (DMSO), ML210, Ferrostatin-1 (F1), Sorafenib, Buthionine Sulfoximine (BSO), cumene hydroperoxide, β -naphthoflavone and 70% Tert-Butyl hydroperoxide solution

in H₂O (TBHP) were obtained from Sigma-Aldrich, UK. MK2206 2HCl and RAS-selective lethal (RSL3) were obtained from Selleck Chemicals, UK. Sapanisertib (INK128) and Samotolisib (LY3023414) were obtained from APExBIO, UK. Samuraciclib was kindly provided by Carrick Therapeutics Ltd., Ireland. Torin1 was supplied by Tocris Bioscience, UK. Sulfasalazine was obtained from Cayman Chemical, UK. Z-VAD(OMe)-FMK was supplied by Abcam, UK or MedChem Express, UK. Alpelisib (BYL719) and SY-1365 were supplied by MedChem Express, UK. For details regarding the relevant compounds, see **Table 2.1**.

Table 2.1. List of inhibitors.

Inhibitor	Inhibitor target	Suitability	Other targets	References
Samuraciclib	CDK7	In <i>vivo</i>		(Patel <i>et al.</i> , 2018a)
SY-1365	CDK7	In <i>vivo</i>		(Hu <i>et al.</i> , 2019)
RSL3	GPX4	In <i>vitro</i> (poor systemic bioavailability)		(Hangauer <i>et al.</i> , 2017; Yang & Stockwell, 2016)
ML210	GPX4	In <i>vitro</i> (poor systemic bioavailability)		(Hangauer <i>et al.</i> , 2017)
Sorafenib	SLC7A11	In <i>vivo</i>	Raf kinases, VEGFR, PDGF- β , c-kit, FLT-3	(Flaherty, 2007)
Sulfasalazine	SLC7A11	In <i>vivo</i>	NF- κ B, TNF- α	(Shanahan <i>et al.</i> , 1990; Wahl <i>et al.</i> , 1998)
Ferrostatin-1	Lipid peroxides (ferroptosis inhibitor)	In <i>vivo</i>		(Miotto <i>et al.</i> , 2020)
Buthionine Sulfoximine (BSO)	γ -glutamylcysteine synthetase	In <i>vivo</i>		(Yang <i>et al.</i> , 2014)
Torin1	mTORC1/2	In <i>vivo</i>		(Thoreen <i>et al.</i> , 2009)
Everolimus	mTORC1	In <i>vivo</i>		(Houghton, 2010)
Sapanisertib (MLN0128)	mTORC1/2	In <i>vivo</i>		(Gokmen-Polar <i>et al.</i> , 2012; Lim <i>et al.</i> , 2021)
Samotolisib	mTORC1/2 and PI3K (α , β , γ , and δ)	In <i>vivo</i>		(Sweeney <i>et al.</i> , 2022)
Alpelisib (BYL719)	PI3K α	In <i>vivo</i>		(André <i>et al.</i> , 2019; Markham, 2019)
MK2206 2HCl	AKT	In <i>vivo</i>		(Hirai <i>et al.</i> , 2010)

All compounds were re-constituted in DMSO to working stock concentrations. Geneticin® solution (G418; 0.8 mg / mL) from Gibco, UK was used to select MCF7-derived NF-E2 p45-related factor 2 (NRF2) reporter cells (AREc32). Once prepared, compound stock solutions were stored at -20°C.

2.1.6 General solutions

Unless otherwise stated, all general solutions, were prepared in double distilled deionized water (ddH₂O), autoclaved, and stored at room temperature.

2.2 Tissue culture

2.2.1 Cell lines

MCF7 cells were obtained from ATCC. The MCF7-derived AREc32 cells, containing eight copies of a luciferase gene construct, the expression of which is driven by the antioxidant response element (ARE) (Wang *et al*, 2006), were obtained from Professor Roland Wolf, Cancer Research UK. The T47D cell line was kindly provided by Dr. Steffi Oesterreich (University of Pittsburgh, USA). The triple negative breast cancer (TNBC) cell lines MDA-MB-231, MDA-MB-453 and MDA-MB-468 were obtained from ATCC. Cell lines were maintained in DMEM (10% FCS, 1% PGS). MCF10A parental, and PIK3CA mutant (E545K and H1047R) cell lines were obtained from Dr George Poulogiannis (Institute of Cancer Research (ICR), UK) and cultured in DMEM/F-12 media (5% horse serum, 1% PSG, 100 ng/mL cholera toxin, 20 ng / mL hEGF, 10 µg / mL insulin, 0.5 mg / mL hydrocortisone). Cell lines that were stably transduced with lentivirus encoding nuclear-localized GFP were generated by Dr. Chun-Fui Lai and Prof. Laki Buluwela.

All cell lines were cultured in flasks (25 cm², 75 cm² or 150 cm², Corning®, UK) and incubated at 37°C with 5% CO₂ and humidified conditions. Cells were routinely passaged once cultures reached 70 – 80% confluency. For each passage, medium was removed, and cells washed twice with 0.02% EDTA solution. To detach cells, 0.5 – 3 mL of 1% trypsin was added, suspensions were incubated at 37°C for three to five minutes, then 5 – 7 mL of fresh medium was added. Cell suspensions were then serially passaged in new culture flasks. All cell lines were routinely tested for mycoplasma every three to four months using the MycoAlert™ Mycoplasma Detection kit (Lonza, UK).

Cells were prepared for long term storage using the same method described above. Following resuspension of trypsinized cells in media, cells were pelleted by centrifugation at 1,200 rpm for five minutes before re-suspending at a concentration of $1 \times 10^6 - 2 \times 10^6$ cells per mL of filtered FCS (0.45 μ M syringe filter, Sartorius, UK) containing 10% DMSO. Re-suspended cells were aliquoted into 1mL cryovials and stored at -80°C for 24 hours before long-term storage in liquid nitrogen.

2.2.2 Cell counting

To determine the number of cells, homogenized cell suspensions were combined with an equal volume of 0.04% Trypan blue solution (Thermo Fisher Scientific, UK) in 1.5 mL microcentrifuge tubes. 10 μ L of the cell suspension-Trypan blue mixture was added to each chamber of a CountessTM cell counting chamber slide (Thermo Fisher Scientific Fisher, UK) or CountessTM II reusable slides (Thermo Fisher Scientific, UK). Slides were loaded into the CountessTM II FL Automated Cell Counter (Thermo Fisher Scientific, UK) to determine cell viability and total cell number per mL of mixture. The total number of live cells was calculated and diluted to desired experimental seeding concentrations.

2.3 Complimentary DNA (cDNA) preparation for analysis and sequencing

2.3.1 Total RNA extraction

Cell cultures with less than 5×10^6 cells were prepared for RNA extractions. First, growth medium was removed, and cells were washed twice with ice-cold PBS. Next, 350 μ L of RLT buffer (QIAGEN, UK) with 1% β -mercaptoethanol (Sigma-Aldrich, UK) was added and cells were harvested using a cell scraper. RNA was extracted from harvested cells using the RNeasy Plus Kit (QIAGEN, UK), according to manufacturer instructions. RNA was eluted in 40 μ L of RNase-free water, and concentration and purity estimates were obtained from 260/280 and 260/240 absorbance ratios using a NanoDropTM ND-1000 Spectrometer.

2.3.2 Complementary DNA (cDNA) synthesis by reverse transcription

Reverse transcription PCR (RT-PCR) reactions to convert RNA to complementary DNA (cDNA) were performed in thin-wall PCR tubes using the RevertAid cDNA synthesis kit (Thermo Fisher Scientific, UK). Following manufacturer instructions, PCR tubes contained 4 μ L of 5X Reaction buffer (250 mM Tris HCl, 200 mM KCl, 30 mM MgCl₂, 50 mM DTT) for reverse transcription, 2 μ L of 10 mM dNTP mix (Life Technologies, UK), 1 μ L of random

hexamer primer (0.2 µg / µl; Life Technologies, UK), 1 µl of RevertAid Reverse Transcriptase (200U / µl; Life Technologies, UK), and 2 µg of RNA. RNase free water was added to each tube to a final volume of 20 µL. Tubes were incubated in a Veriti™ 96 well thermal cycler at 42°C for one hour, then heated to 95°C for five minutes to inactivate the reverse transcriptase enzyme. The final cDNAs were diluted 1:20 with RNase-free water. 2 µL of each diluted cDNA mixture was used for end-point PCR. Primer sequences are shown in **Table 2.2**.

2.3.3 End-point polymerase chain reaction (PCR)

PCR of cDNA followed by Sanger sequencing was used to confirm correct integration of the appropriate mutation in the PIK3CA gene locus (E545K or H1047R). PCR was performed using the 2X RedTaq ReadyMix PCR reaction mix (Sigma-Aldrich, UK). Reaction mixtures were prepared in thin wall PCR tubes (Thermo Fisher Scientific, UK) and consisted of 10 µL RedTaq ReadyMix, 1 µL 10µM Forward primer, 1 µL 10 µM Reverse primer, and 100 – 200 ng cDNA adjusted to a final volume of 8 µL using ddH₂O. Primer sequences are listed in **Table 2.2**. PCR reactions were performed in the Veriti™ 96-well thermal cycler according to **Table 2.3**.

Table 2.2 Oligonucleotide primers used in RT-PCR, PCR and Sanger sequencing.

Oligonucleotide name	Sequence	PCR reaction
SA5339PIK3H1047Rf1	AAAGGAGCCCAAGAATGCAC	RT-PCR
SA5340PIK3H1047Rr1	TCAGTTCAATGCATGCTGTTTAA	RT-PCR
SA5341PIK3H1047Rf2	GCCCAAGAATGCACAAAGAC	PCR
SA5342PIK3H1047Rr2	TGCATGCTGTTTAATTGTGTGG	PCR
SA5343PIK3E545Kf1	GGTCTGTATCCCGAGAAGCA	RT-PCR
SA5344PIK3E545Kr1	ACAGACAGAAGCAATTTGGGT	RT-PCR
SA5345PIK3E545Kf2	CAGTGATTGAAGAGCATGCCA	PCR
SA5346PIK3E545Kr2	CAGACAGAAGCAATTTGGGTAGA	PCR

Table 2.3 PCR cycle steps for amplification of cDNA.

Temperature (°C)	Time	Number of cycles	Stage
95	2 minutes	1	Denature
95	30 seconds	30	PCR
55	30 seconds	30	
72	30 seconds	30	
72	5 minutes	1	Hold
4	hold	1	

2.3.4 Agarose gel electrophoresis

cDNA (Section 2.3.3) was separated by size and visualized using agarose gel electrophoresis. Agarose gels were prepared to a final concentration of 1 – 2% (w/v) agarose by dissolving agarose in 100mL of 1X Tris-borate buffer (TBE, Thermo Fisher Scientific, UK; 10X TBE Buffer is a sterile-filtered solution of 1 M Tris, 0.9 M boric acid, and 0.01 M EDTA used to prepare 1X buffer). 10µL of Gel Red™ Nucleic Acid Stain was added to the agarose gel

before the gel set for visualization of nucleic acids. Gel electrophoresis was performed in a solution of 1X TBE buffer in a horizontal gel electrophoresis unit at 100 V. GeneRuler 1 kb DNA ladder, MassRuler™ Low Range DNA ruler, and MassRuler™ High Range ruler (all from Thermo Fisher Scientific, UK) were used as markers to estimate size of the PCR products. Gels were visualised using an UVIpro Platinum GelDoc System ultraviolet transilluminator (UVItec, Cambridge, UK).

2.3.5 Sanger sequencing of PCR products

PCR products were Sanger sequenced after purification using ExoSAP-IT™ (Applied Biosystems, UK), which enzymatically removes excess primers and dNTPs. 2 µL of ExoSAP-IT™ was added per 5 µL of PCR product in thin wall PCR tubes. To ensure inactivation of thermostable DNA synthetases from the PCR reaction, samples were placed in a thermocycler at 37°C for 15 minutes, then at 80°C for 15 minutes to inactivate the ExoSAP-IT™ reagent. DNA samples at 200 ng / µL were prepared and submitted for Sanger sequencing according to the guidelines of the sequencing company, GENEWIZ (UK). DNA sequences and chromatograms were analysed using SnapGene Viewer (GSL Biotech LLC, Chicago, USA).

2.4 Real time reverse transcription quantitative PCR (RT-qPCR)

Relative gene expression analysis was conducted using real-time quantitative PCR (RT-qPCR), following the guidelines for SYBR™ Green reporter and TaqMan™ PCR assays. RT-qPCR primers are described in **Table 2.4**. RT-qPCRs were prepared in MicroAmp® Optical 384-well Reaction Plates with barcode (Applied Biosystems, UK) for the QuantStudio™ 7 Flex Real Time PCR system. Reactions with a total volume of 10 µL were prepared using PowerUP™ SYBR™ Green or TaqMan™ Fast Universal real-time quantitative PCR (RT-qPCR) master mixes (Applied Biosystems, UK), according to manufacturer instructions. The reaction mix set-up for SYBR™ Green and TaqMan™ RT-qPCR reactions is outlined below.

Table 2.4 Oligonucleotide primers used in RT-qPCR.

Oligonucleotide name	Sequence	PCR reaction
SA5841MBOAT1f	GTTTCGCATCTACTTACGTCCTG	RT-qPCR
SA5842MBOAT1r	GCACATTAACACCAGCACAAAA	RT-qPCR
SA5843EMP2f	GTGCTTCTTGCTTTCATCATCG	RT-qPCR
SA5844EMP2r	TGCAATTCGTGTTGTTGGTACA	RT-qPCR

SYBRTM Green reaction mix:

- 5 µL of PowerUpTM SYBRTM Green Master Mix
- 0.5 µL of 10 µM forward primer
- 0.5 µL of 10 µM reverse primer
- 2 µL of nuclease-free water
- 2 µL of 1:20 diluted cDNA

TaqManTM reaction mix:

- 5 µL of TaqManTM Fast Universal Master Mix (2X)
- 0.5 µL of TaqMan primer/probe mix
- 2.5 µL of nuclease-free water
- 2 µL of 1:20 diluted cDNA

Plates were sealed using MicroAmp® Optical adhesive films then placed in a QuantStudioTM 7 Flex Real Time PCR system for thermocycling as describe in **Tables 2.5 and 2.6**. Gene expression was normalised to TBP and GAPDH, and relative gene expression was analysed using the $2^{-\Delta\Delta C_t}$ method.

Table 2.5 RT-qPCR cycle steps for quantitation of mRNA transcripts using SYBR™ Green.

Temperature (°C)	Time	Number of cycles	Stage
50	2 minutes	1	Denature
95	2 minutes	1	
95	1 seconds	40	PCR
60	20 seconds	40	
95	15 seconds	continuous	Melt curve
60	1 minutes	continuous	
95	15 seconds	continuous	

Table 2.6 RT-qPCR cycle steps for quantitation of mRNA transcripts using TaqMan™.

Temperature (°C)	Time	Number of cycles	Stage
95	20 seconds	1	Denature
95	1 second	40	PCR
60	20 seconds	40	

2.5 Protein lysate preparation and western blotting

2.5.1 Protein extraction

Cells were grown in 10 cm plates and upon reaching ~70% confluency, cell lysates were prepared to extract proteins for quantitation and visualization using Western blotting. First, RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, 0.1% SDS (Sigma-Aldrich, UK)) was prepared by combining RIPA buffer with Pierce™ Protease inhibitor cocktail mini tablets. Next, cell growth medium was removed, and cells were washed twice with ice-cold PBS. To lyse the cells, 500 µL of ice-cold RIPA buffer with inhibitors was added per 10 cm dish. Cell lysates were collected by scraping the plate then transferring the RIPA buffer to a 1.5 mL microcentrifuge tube for incubation on ice for 30 minutes with vortexing every five minutes to facilitate cell lysis. To pellet and

remove cell debris, lysates were centrifuged at 13,000 rpm for 10 minutes at 4°C before transferring supernatants to fresh 1.5 mL microcentrifuge tubes.

2.5.2 Protein quantification

Protein concentrations were determined using Pierce™ BCA protein assay kit (Thermo Fisher Scientific, UK), according to manufacturer instructions. A serial dilution of Pierce™ bovine serum albumin (BSA) (Thermo Fisher Scientific, UK) was set up as nine standard controls with concentrations ranging from 0 – 16 µg / µL. 10 µL of each BSA standard was placed in triplicates in a 96-well flat bottom plate (Corning, UK). In the same plate, 2 µL of each protein sample was diluted in 8 µL ddH₂O and loaded in triplicates. 200 µL of BCA solution, in a 50:1 ratio of reagent A to reagent B, was added to each well, then the plate was incubated at 37°C for 30 minutes. Protein concentrations were calculated by measuring sample absorbance at 562 nm using a Tecan Sunrise™ Microplate Reader and extrapolating the standard curve generated for the BSA controls.

2.5.3 Western blotting (SDS-polyacrylamide gel electrophoresis)

Western blots were run to measure relative protein expression. Protein lysates were diluted to 1 – 2 µg / µL in 4X Laemmli buffer supplemented with 10% (v/v) β-mercaptoethanol, to a final concentration of 1X Laemmli buffer (**Table 2.7**). Proteins were denatured by heating samples at 100°C for five minutes, except for lysates blotted for Retinoblastoma (RB), which were heated to 60°C for five minutes. Samples were cooled to room temperature prior to loading on polyacrylamide gels. 10 – 30 µg of each protein sample, along with the Precision Plus Protein Dual colour standards protein ladder (Bio-Rad, UK), was loaded into wells of the gel. The SDS-polyacrylamide gel contained a 5% stacking gel and 8 – 15% resolving gel (**Table 2.8**).

Table 2.7 Western blotting buffers.

Component	Composition
4x Laemmli sample buffer	62.5 mM Tris-HCl, pH 6.8 10% glycerol, 1% SDS, 0.005% Bromophenol Blue
Upper Buffer 10x	1.25 M Tris-HCl pH 6.8, 1% w/v SDS
Lower buffer 4x	1.5 M Tris-HCl pH 8.8, 0.4% w/v SDS
SDS Running buffer 10x	0.25 M Tris, 1.9 M glycine, 1% w/v SDS, pH 8.3
Transfer buffer 50x	0.25 M Tris, 1.9 M glycine, 20% v/v methanol
Tween 20	10% v/v Tween 20 in PBS
PBST	0.1% v/v Tween 20 in PBS
Blocking buffer	5% w/v dry skimmed milk powder in PBST solution

Table 2.8 Polyacrylamide gel recipes for SDS-PAGE electrophoresis.

Percentage	Recipe
5% SDS PAGE	8 mL lower buffer, 5.3 mL Acrylamide solution (Acrylamide/Bis-acrylamide, 30% solution), 62.5 μ L TEMED, 250 μ L 10% APS, 18.7 mL ddH ₂ O
8% SDS PAGE	8 mL lower buffer, 8.5 mL Acrylamide solution (Acrylamide/Bis-acrylamide, 30% solution), 62.5 μ L TEMED, 250 μ L 10% APS, 15.3 mL ddH ₂ O
10% SDS PAGE	8 mL lower buffer, 10.6 mL Acrylamide solution (Acrylamide/Bis-acrylamide, 30% solution), 50 μ L TEMED, 200 μ L 10% APS, 13.4mL ddH ₂ O
12% SDS PAGE	8 mL lower buffer, 12.7 mL Acrylamide solution (Acrylamide/Bis-acrylamide, 30% solution), 41.7 μ L TEMED, 166.7 μ L 10% APS, 11.4 mL ddH ₂ O
15% SDS PAGE	8 mL lower buffer, 15.9 mL Acrylamide solution (Acrylamide/Bis-acrylamide, 30% solution), 34.7 μ L TEMED, 138.8 μ L 10% APS, 8.2 mL ddH ₂ O

Electrophoresis was performed using a Mini-Protean Tetra Vertical Electrophoresis system (Bio-Rad, UK). The gels were electrophoresed at 60 V for 40 minutes followed by 100 V for 1 – 2 hours in 1X running buffer (**Table 2.7**), until the bromophenol blue dye reached the bottom of the gel. After electrophoresis, separated proteins were electroblotted onto nitrocellulose membranes (Bio-Rad, UK) using a Bio-Rad (UK) transfer system at 90 V for 90 minutes in 1X transfer buffer. Nitrocellulose membranes were incubated in blocking buffer at room temperature for 2 – 3 hours followed by incubation for approximately 12 hours at 4°C with primary antibodies (**Table 2.9**) diluted in blocking buffer. Nitrocellulose membranes were washed with PBST (**Table 2.7**) three times and left in PBST for 30 minutes on a shaker to complete the wash. Next, membranes were incubated for 90 minutes at room temperature with HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibody (Dako, Agilent Technologies, UK) diluted in blocking buffer. Following incubation, membranes were washed with PBST, as described earlier. Proteins were visualised by adding SuperSignal West Pico Chemiluminescent Substrate or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, UK) and imaging membranes on a Fusion Solo Chemiluminescence imager (Vilber Lourmat, UK) or an iBright™ CL 1500 imaging system (Thermo Fisher Scientific, UK).

Table 2.9 Primary antibodies for Western blotting.

Antibody name	Dilution	Host species	Supplier	Product code
AIFM/FSP1	1/1000	Rabbit	Proteintech	20886-1-AP
AKT	1/1000	Rabbit	CST	9272
AKT p-S473	1/1000	Rabbit	CST	9271
b-actin	1/100,000	Mouse	Abcam	Ab6276
CDK1	1/1000	Mouse	Abcam	Ab18
CDK1 p-T161	1/1000	Rabbit	CST	9114S
CDK2	1/1000	Rabbit	CST	2546S
CDK2 p-T160	1/1000	Rabbit	CST	2561S
CDK7	1/1000	Mouse	Santa Cruz	7344
COX16	1/1000	Rabbit	Proteintech	19425-1-AP
Cyclin H	1/1000	Mouse	Abcam	Ab54903
ESRP2	1/1000	Rabbit	Abcam	Ab113486

FTH1	1/1000	Rabbit	CST	3998
GCLM	1/1000	Rabbit	Proteintech	14241-1-AP
GPX4	1/1000	Rabbit	Abcam	Ab125066
GSK3b	1/1000	Rabbit	CST	9315
HMOX-1	1/1000	Rabbit	CST	70081
KEAP1	1/1000	Rabbit	CST	8047
LDHA	1/1000	Rabbit	CST	2012
LZTS2	1/1000	Rabbit	Proteintech	15677-1-AP
mTOR	1/1000	Rabbit	CST	2972
NME1/NDKA	1/1000	Rabbit	CST	3345
NRF2	1/1000	Rabbit	CST	12721
p16	1/1000	Mouse	Santa Cruz	Sc-1661
p21	1/1000	Mouse	Santa Cruz	Sc-397
p53	1/200	Mouse	Santa Cruz	Sc-126
p62	1/5000	Rabbit	Proteintech	18420-1-AP
p70S6K	1/1000	Rabbit	CST	9202
p70S6K p-T389	1/1000	Mouse	CST	9206S
PI3Kinase p110 alpha	1/1000	Rabbit	CST	4254
Retinoblastoma (4H1)	1/1000	Mouse	CST	9309
RICTOR	1/1000	Rabbit	CST	2114
RNA Polymerase II	1/1000	Mouse	Abcam	Ab817
RNA Polymerase II (4H8)	1/1000	Mouse	Thermo Fisher	39097
RNA Polymerase II p-S2	1/1000	Rabbit	Abcam	Ab5095
RNA Polymerase II p-S5	1/1000	Mouse	Abcam	Ab5131
RPTOR	1/1000	Rabbit	CST	22805
S100P	1/1000	Rabbit	Abcam	Ab133554
S6	1/1000	Rabbit	CST	2217
Transferrin receptor	1/1000	Mouse	Invitrogen	13-6800
UBE2T	1/1000	Rabbit	CST	12992
UHRF1	1/1000	Rabbit	Abcam	Ab213223
xCT (SLC7A11)	1/1000	Rabbit	Abcam	Ab37185

2.6 Cell growth assays

2.6.1 Determining BC cell GI₅₀ inhibitor concentrations with IncuCyte S3

To determine drug concentrations that impairs BC cell growth by 50% (GI₅₀), 2 x 10³ cells were seeded in 96-well plates (Corning, UK) for 48 hours prior to small molecule inhibitor treatment. Treatments were added in a four-fold dilution series from a maximum concentration of 20 µM for 96 hours with DMSO used as a vehicle control. BC cell growth was monitored by taking images every day for four days on IncuCyte S3 (37°C and 5% CO₂; Sartorius, UK). GI₅₀ concentrations and standard error of the mean (SEM) were calculated from three independent experiments, each with six technical replicates per treatment condition.

2.6.2 Sulforhodamine B (SRB) assay

Following the appropriate treatments, plates were fixed with 100 µL of 40% w/v Trichloroacetic acid (TCA) (Sigma-Aldrich, UK), incubated for 1 hour at 4°C, and washed five times with ddH₂O. To stain the cells, 0.4% w/v SRB dye was added per well. SRB dye (0.04% w/v) was prepared by dissolving SRB salt (Sigma-Aldrich, UK or Alfa Aesar, UK) in 1% acetic acid for 1 hour at room temperature. After staining, plates were washed 5-8 times with 1% v/v acetic acid and left to dry at room temperature. To solubilize the SRB dye, 100 µL of 10 mM Tris base (pH 6.5) was added per well and plates were left to gently shake on a plate shaker for 10 minutes. Absorbance was measured using a Tecan Sunrise™ Microplate Reader at 492 nm. GI₅₀ concentrations and SEM was calculated from three independent experiments, each with six technical replicates per treatment condition.

2.6.3 Cell growth experiments for cells with nuclear GFP

Cells lentivirally transduced with nuclear GFP were grown for up to 35 days with cell growth monitored by IncuCyte Zoome or IncuCyte S3. Cell numbers were calculated for 4 images from each of the six replicate wells per condition. Each experiment was carried out as three independent experiments, unless otherwise stated.

2.7 Proteomics

2.7.1 Protein extraction and preparation

2.7.1.1 Cell lysis and whole protein extract preparation

Whole protein extracts from MCF7 cells treated with samuraciclib were analysed using quantitative mass spectrometry (MS). MCF7 cells (ATCC) were treated for 24 hours with 500 nM or 2 μ M samuraciclib, or DMSO and prepared for whole proteome analysis, as described in the sections below. Each treatment condition was repeated four times ($n = 4$). After treatment, cells were washed twice with ice-cold PBS and scraped in ice-cold PBS supplemented with protease inhibitors (Thermo Fisher Scientific, UK). 20% of each sample was aliquoted, pelleted, and lysed in RIPA buffer (Sigma-Aldrich, UK) for Western blotting, to confirm altered expression of known CDK7 targets. The remaining cells were centrifuged at 5,000 rpm for 5 minutes at 4°C, supernatant removed, and cell pellets stored at -80°C until shipping to Dr Tommaso de Marchi (Lund University, Sweden), for protein preparation and MS analysis.

2.7.1.2 Protein preparation

Protein preparation and MS followed the methods of Mertins et al. (Mertins *et al.*, 2016). Whole protein extracts were prepared from frozen cell pellets for quantitative MS by our collaborators at Lund University. Cells were sonicated for 20 minutes (20 cycles: 30 seconds ON, 30 seconds OFF; Bioruptor, Diagenode), lysates were spun down at 10,000 rpm for 30 minutes at 4°C, and supernatants were collected. Protein concentrations were measured using a bicinchoninic acid assay kit according to manufacturer's instructions (Thermo Fisher Scientific, Sweden). 100 μ g of protein was digested for proteome analyses as described previously (Mertins *et al.*, 2016). Briefly, proteins were precipitated using ice-cold methanol, spun down at 10,000 rpm for 20 minutes then re-suspended in disulphide-bond-reducing and protein-denaturing buffer (100 mM Tris buffer containing 100 mM dithiothreitol (DTT) and 4% w/V sodium-dodecylsulphate (SDS)). The protein solution was heated at 95°C for 30 minutes then urea was added to a final concentration of 8 M to denature the proteins. Samples were loaded on 30 KDa molecular filters (Millipore, Sweden), centrifuged at 10,000 rpm for 20 minutes, then washed twice with 8M urea buffer and incubated in another reducing and denaturing solution (50 mM iodoacetamide in 8 M urea) for 30 minutes. Filters were then washed 4 times: twice in 8 M urea buffer and twice in 50 mM tri-ethyl-ammonium bicarbonate (TEAB) buffer to improve

protein ionization during MS. Proteins were digested with trypsin for 16 hours then centrifuged at 10,000 rpm for 20 minutes to extract tryptic peptides.

2.7.1.3 TMT labelling

In preparation for quantitative mass spectrometry of proteomic samples, each sample was uniquely labelled using Tandem Mass Tags (TMT). Tryptic digests were assigned into groups of six samples for TMT 6-plex labelling (Catalogue #90066, Thermo Fisher Scientific, Sweden). Labelling was performed according to manufacturer's instructions.

2.7.1.4 Peptide fractionation

In preparation for proteome analysis, ~10% of each TMT pool was fractionated for downstream MS analysis using high-pH fractionation spin columns (Catalogue #15362617, Thermo Fisher Scientific, Sweden). Eluates for both whole proteome enrichment were dried and stored at -80°C until mass spectrometry analysis.

2.7.2 Mass spectrometry

2.7.2.1 Data collection

Total proteome mass spectrometry (MS) analysis was performed using a Q-Exactive HF-X (Thermo Fisher Scientific) mass spectrometer. ~1 µg of trypsin-digested peptides from fractionated samples were first trapped on an Acclaim PepMap™ 100 pre-column (Thermo Fisher Scientific, Sweden) and separated on a reverse phase high-performance liquid chromatography (HPLC) EasySpray column (Thermo Fisher Scientific, Sweden) coupled to an Dionex Ultimate 3000 liquid chromatography system (Thermo Fisher Scientific, Sweden). Peptides were eluted over 120-minute gradients. To select abundant peptides for relative quantitation, the 15 most abundant peaks from the initial MS scan were selected and fragmented by higher energy induced collision dissociation using a dynamic exclusion method.

2.7.2.2 Raw data pre-processing and analyses

To identify and quantify peptides, MS RAW files were analysed using MaxQuant 50 (v1.6.3.4). MS spectra were searched using the Andromeda search engine with the Uniprot-Swissprot human proteome database (version download: 2020.04.02). Cys residue modifications were considered as fixed, due to the several reducing steps in sample preparation, while Met and N-terminal residues were considered variable. TMT 6-plex settings were enabled and peptides were filtered by a minimum precursor intensity fraction of 75%. Precursor ion tolerance was

set to 20 and 4.5 ppm for first and main searches, respectively. Match-between-run option was enabled, and the remaining settings were left to default. Identification of peptides resulting from missed cleavages was allowed.

For data imputation, normalization, and batch correction, protein tables were processed in both R (v4.0.5) and Perseus (v2.0.3.1). First, samples were filtered for missing data such that up to 50% missing data was allowed. Missing intensities in each sample were imputed based on the minimum intensity-based normal distributions using Perseus. Protein intensities were then log2-transformed. Limma (v3.46.0) was used to correct for batch effects and to normalize quantiles (Ritchie *et al*, 2015). DEQMS (v1.8.0) and limma were used to perform differential expressed protein analysis (Ritchie *et al.*, 2015; Zhu *et al*, 2020).

2.8 CellROX assay for ROS detection

The CellROX assay was used to measure BC cell line oxidative stress during samuraciclib treatment alone, or in combination with ferroptosis inducers. First, MCF7 cells seeded in T150 flasks (Corning, UK), allowed to adhere for 48 hours, and were then treated every three or four days for seven days with DMSO or samuraciclib (150 nM). Then, cells were seeded into 96-well plates (Corning, UK) with samuraciclib or DMSO already present in the media and left for a further two days such that treated cells reached ~70% confluency by the ninth day. On day nine of treatment, cell media were changed to serum- and glutamine-free media and cells were treated with various drug combinations, including RSL3, ML210, Ferrostatin-1, and samuraciclib, in addition to 2.5 μ M CellROX Green reagent. Immediately after adding the treatments, the plates were transferred to a temperature- and CO₂-controlled IncuCyte S3 (37°C and 5% CO₂; Sartorius, UK). ROS was monitored by recording four images per well on the GFP channel (20x magnification) immediately prior to treatment on day nine, 30 minutes after adding the combination treatments, hourly for the first six hours, and then every four hours until 24 hours after the start of combination treatments. CellROX images were analysed by recording average green area per image. This experiment was repeated two independent times with six technical replicates per treatment condition.

2.9 Fluorescence-activated cell sorting (FACS)

2.9.1 FACS using BODIPY C11 for lipid peroxidation analysis

FACS analysis was performed using the BODIPY 581/591 C11 reagent (Thermo Fisher Scientific, UK) to measure lipid peroxidation of BC cell lines following treatment with samuraciclib alone, or in combination with ferroptosis inducers, namely RSL3 and ML210. First, cells were seeded in T150 flasks (Corning, UK), allowed to adhere for 48 hours, and were then treated every three or four days for seven days with DMSO or samuraciclib (150 nM). At day seven, cells were seeded into 6 cm dishes (Corning, UK), with samuraciclib or DMSO already present in the media, and left for a further two days such that treated cells reached ~70% confluency by the ninth day. After nine days of treatment, cell media were changed to serum- and glutamine-free media and treatment combinations were added for six hours. Cells were then incubated for 20 minutes with 1.5 μ M BODIPY 581/591 C11 and harvested, as previously described (Martinez *et al*, 2020). Briefly, media were removed, cells were washed with 1 mL of EDTA, then cells were treated with 500 mL of 1% trypsin per dish. The cells were incubated at 37°C, 5% CO₂ (RS Biotech Galaxy R+ CO₂ incubators) for 5 minutes and resuspended in 1 mL of serum- and glutamate-free media. Samples were centrifuged at 1,200 rpm for 5 minutes, media was removed, cells were washed with PBS, and samples were centrifuged again. Lastly, supernatant was removed, and cells were resuspended in 1 mL PBS and transferred to round bottom Falcon™ tubes with a cell strainer snap cap (Thermo Fisher Scientific, UK) for analysis using the BD FACSymphony™ A3 Cell Analyzer. At least 10,000 events per condition were collected from the single cell channel (SSC-A versus SSC-H). Cells with lipid ROS were determined based on the shifts in excitation / emission spectra of the oxidised BODIPY probe. This experiment was repeated three independent times.

2.9.2 FACS using EdU incorporation for detection of cells in S-phase

2.9.2.1 Fixing cells for EdU incorporation with FACS

To quantify the number of cells in S-phase following ICE0942 treatment alone, or in combination with mTOR/AKT/PI3K inhibitors, cells were incubated with EdU and analysed using FACS. MCF10A PIK3CA-WT or PIK3CA mutant cells were seeded in 6-well plates (Corning, UK) for 24 hours, then treated with 40 nM torin1, 5 μ M MK2206, 2 μ M alpelisib, or DMSO alone, with and without 75 nM samuraciclib. Cells were treated with the synthetic thymidine analogue, EdU, and harvested according to manufacturer's instructions (BaseClick, EdU Cell Proliferation Kit 488, UK). Briefly, following treatments, cells were incubated with

10 μ M EdU at 37°C and 5% CO₂ (RS Biotech Galaxy R+ CO₂ incubators) for 1 hour. Culture media were removed, and cells were washed twice with 500 mL EDTA. 700 mL of 1% trypsin was added, and cells were incubated for 10 minutes (37°C, 5% CO₂). Cells were resuspended in media and centrifuged at 2,000 rpm for 5 minutes. The cells were washed with 1 mL 1% bovine serum albumin (BSA) (Sigma-Aldrich, UK) in PBS and centrifuged again. Cell pellets were fixed using 4% paraformaldehyde in PBS, pelleted again, fixative removed, and then washed with 1 mL 1% BSA in PBS. Cell pellets were kept at 4°C until staining with EdU.

2.9.2.2 Staining cells with EdU and FACS analysis

Cell pellets were resuspended in 1X saponin-based permeabilization buffer, mixed well, and incubated for 20 minutes at room temperature. EdU assay cocktail (per reaction: 1X PBS, 10 mL proprietary catalyst solution, 50 mM azide dye, 1X proprietary buffer additive) (BaseClick, EdU Cell Proliferation Kit 488, UK) was prepared according to manufacturer's instructions and 500 mL was added onto each sample. For unstained control samples, PBS was added instead. Cells were incubated for 30 minutes at room temperature in the dark, cells were pelleted, and the supernatant was removed. Cells were washed with 1 mL of 1X saponin-based Permeabilization and Wash Reagent according to manufacturer's instructions (BaseClick, EdU Cell Proliferation Kit 488, UK), re-pelleted, and resuspended in 500 mL 1X saponin-based permeabilization and wash reagent. Before FACS analysis, 1:1000 DAPI was added to all samples except for unstained controls, samples were incubated for one minute, and then analyzed by FACS. Actively dividing cells in S-phase were quantified using the BD FACSymphony™ A3 or the BD LSRFortessa™ Cell Analyzers.

2.10 Luciferase assay for NRF2 activity

The antioxidant response element (ARE)-driven reporter gene cell line (Wang *et al.*, 2006), was used to quantify the redox-dependent activation of NRF2 following samuraciclib treatment. This MCF7-derived cell line (AREc32) contains a firefly luciferase gene construct, the expression of which is regulated by eight copies of the ARE cis-element. NRF2 is activated by intracellular oxidation and binds the ARE to drive transcription of antioxidant genes (Kobayashi & Yamamoto, 2005; Lee *et al.*, 2003; McMahon *et al.*, 2001). Cells were seeded in 24-well plates (Corning, UK) and allowed to adhere for 48 hours before treatment for 24 hours with DMSO or samuraciclib at 150 nM, 300 nM, 500 nM, 1 μ M, and 2 μ M concentrations. Two additional treatments were applied as positive controls: 10 μ M tert-butylhydroquinone (t-

BHQ) and 1 μ M β -naphthoflavone. To harvest the cells, culture medium was aspirated from each well and 50 mL of 1X lysis buffer, diluted in ddH₂O from 5X passive lysis buffer (Promega E194A, UK), was added to each well. Plates were placed on a shaking platform for 30 minutes. Lysates were transferred from 24-well plates to a 96-well Opti-plate (Pekin Elmer 6005290, UK) and 50 mL of Dual-Glo reagent was added into each well. The plate was tapped gently to mix and incubated in the dark for 10 minutes. Luminescence was measured on VICTOR Light Luminescence counter (Perkin Elmer, UK).

2.11 Data analysis and statistics

2.11.1 Data analysis, visualization, and statistics

Unless otherwise specified, data was processed and visualized in R (v4.0.5), and growth curves and respective statistics were generated using Prism v10. Scripts and analysis methods were adapted from publications and open-access code databases.

2.11.2 Pathway enrichments

Pathway enrichment was performed using the Gene Set Enrichment Analysis (GSEA)-related method in the R package ActivePathways (Kobayashi & Yamamoto, 2005; Lee *et al.*, 2003; McMahon *et al.*, 2001; Paczkowska *et al.*, 2020) using default parameters. Gene ontology (GO), Reactome, and Hallmarks gene matrix transposed (GMT) files were downloaded from the GSEA web server (4 December 2023).

2.11.3 Protein-protein interaction networks, ferroptosis databases, and GPX4i sensitivity screens

Protein-protein physical interaction networks were downloaded from the BioGRID database (6 December 2023) (Oughtred *et al.*, 2021). Kinase-substrate interaction networks were downloaded from the PhosphoSitePlus database (12 January 2024) (Hornbeck *et al.*, 2015). Ferroptosis drivers, suppressors, hallmarks, and unknown gene sets were acquired from the FerrDB database (20 December 2023) (Zhou *et al.*, 2023b). Gene associations with GPX4-inhibitor (GPX4i) sensitivity screens were downloaded from the Cancer Therapeutics Response Portal (2 January 2024) (Aksoy *et al.*, 2017).

3. Results

3.1 CRISPR knockout screens to identify modulators of response to Samuraciclib

3.1.1 CRISPR overview

This technique relies on the CRISPR-Cas9 system, which is derived from a natural bacterial defense mechanism against bacteriophages and has proven to be a powerful technique for genome editing in eukaryotic cells (Jinek *et al*, 2012). CRISPR-Cas9 for gene editing employs a carefully engineered synthetic guide RNA (sgRNA) that directs the CRISPR-Cas9 complex to a specific target site in the genome (Mali *et al*, 2013). Genome editing mediated by the RNA-guided Cas9 endonuclease recognizes and induces double-strand breaks (DSB) at specific sites in DNA, making use of subsequent cellular DNA repair processes that frequently result in insertion or deletion events specifically at or near the sgRNA targeting sequences (Ma *et al*, 2014). To initiate specific cleavage, the targeted site requires a short protospacer adjacent motif (PAM) sequence (Jinek *et al.*, 2012). After binding the target gene through complementary base-pairing and recognizing the PAM motif, the Cas9 nuclease induces a double-strand break (DSB). This DSB often triggers the error-prone nonhomologous end-joining (NHEJ) DNA repair process, resulting in a mutation that creates a loss-of-function allele (Ma *et al.*, 2014). This method has effectively been employed for developing approaches not simply for knocking out a gene of interest but using libraries of sgRNAs targeting all genes, for functional genetics screens. The method allows for identification of genes that are involved in the action of the drug of interest, with enrichment of gRNAs implying that the gene knockout reduces sensitivity to the drug and depletion of gRNAs identifying genes whose knockout is associated with increased sensitivity to the drug (Shalem *et al*, 2014; Tzelepis *et al*, 2016).

3.1.2 CRISPR-Cas9 sgRNA library screening identifies mTOR and GPX4 as modulators of response to samuraciclib

To identify genes implicated in resistance and sensitivity to samuraciclib, we performed a genome-wide CRISPR-Cas9 knockout screen in the MCF7 ER+ BC cell line (Sava *et al.*, unpublished). Analysis of the screen identified 125 genes whose knockout resulted in reduced sensitivity to samuraciclib. sgRNAs for 11 genes were reduced in number relative to the vehicle (DMSO) group, indicating that knockout of these genes is associated with increased sensitivity to samuraciclib. An example is the DNA-damage-inducible transcript 4 (DDIT4), which is a negative regulator of mTORC1 that is activated by hypoxia and DNA stress (Foltyn *et al*,

2019). DDIT4 sgRNAs were depleted in the pooled screen, implying that DDIT4 knockout increases sensitivity to samuraciclib. Conversely, the mTORC2 subunits RICTOR and MAPKAP1 (mSIN1), as well as the upstream mTOR activators PIK3CA and Akt1 (**Figure 3.1A and C**) gene knockouts were associated with reduced sensitivity to samuraciclib. A similar CRISPR-Cas9 screen undertaken in the samuraciclib-treated NALM6 pre-B-cell leukaemia cell line identified subunits in mTORC1 and mTORC2, as well as regulators of mTORC1 among the most highly enriched or depleted hits (Wilson *et al.*, 2023) (**Figure 3.1B – C**). Collectively, these results indicate an essential, albeit unclear, role of the mTOR pathway in mediating resistance and sensitivity to CDK7 inhibition.

The CRISPR screen in MCF7 cells also identified GPX4 as one of the most significantly depleted genes (**Figure 3.1A and C**). As ferroptosis-promoting drugs are emerging as effective cancer therapeutics, the combination effects of samuraciclib and ferroptosis inducers emerges as an interesting avenue to explore. However, a more detailed molecular characterization of how samuraciclib relates to ferroptotic processes is required.

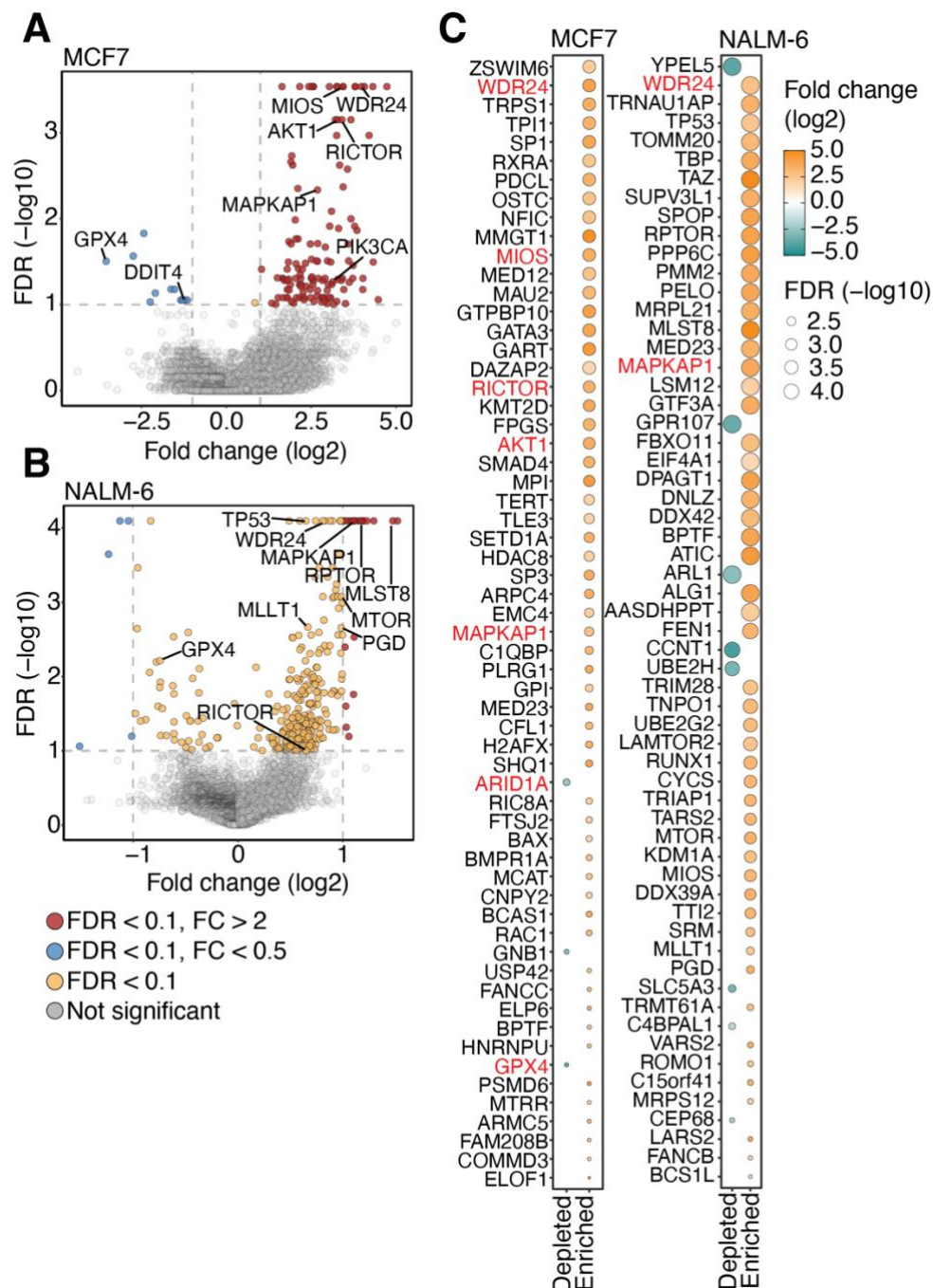


Figure 3.1 Genome-wide CRISPR-Cas9-mediated functional screening in MCF7 cells identifies genes modulating cellular responses to samuraciclib.

(A) Volcano plot shows the genome wide CRISPR-Cas9 screening of MCF7 cells treated with 150 nM samuraciclib. MCF7 cells stably expressing CRISPR-Cas9 were transduced with a CRISPR lentiviral library with 5 – 10 sgRNAs for each protein coding gene, followed by growth of the cells for 30 days in the presence of 150 nM samuraciclib or an equal volume of DMSO. Genomic DNA was collected, and lentiviral barcode regions were PCR amplified and sequenced using an Illumina sequencing platform. The MCF7 CRISPR screen was performed by Dr G Sava (Imperial College London) using a lentiviral CRISPR library provided by Dr S Omarjee and Prof J Carroll (CRUK CRI, Cambridge). A subset of genes with blue colouring denotes a knockout increases sensitivity to samuraciclib and red denotes a knockout reduces sensitivity to samuraciclib. **(B)** The pre-B cell leukaemia cell line NALM-6, lentivirally infected with doxycycline-induced Cas9 and a sgRNA library to a minimum of 500 cells per sgRNA, was treated with doxycycline followed by treatment with 500 nM samuraciclib for 8 days. Illumina sequencing was performed on genomic DNA for identifying sgRNAs. NALM-6 CRISPR screening was undertaken by Prof M Tyers' group (University of Montreal). **(C)** Plots show the top 60 significantly differentially enriched or depleted gRNA target genes, sorted by FDR. Genes labelled in red appear in the top 60 list in both CRISPR screens.

3.2 The PI3K/Akt/mTOR pathway in response to samuraciclib treatment

3.2.1 The PI3K/Akt/mTOR pathway is important for the cellular response to samuraciclib

To identify modulators of the cellular response to CDK7i and specifically to samuraciclib, a CRISPR-Cas9 genome-wide gene dropout screen in the pre-B cell leukaemia cell line NALM-6 was analysed. NALM-6 cells were chosen because they have few gene rearrangements, except for some leukaemia driver mutations such as *NRAS*-A146T, and they have a nearly complete diploid genome (Bertomeu *et al*, 2018). The most prominent genes associated with response to samuraciclib (0.5 μ M) were genes in the PI3K/Akt/mTOR pathway (**Figure 3.1B**). Remarkably, guide RNA (gRNA) sequences for many genes in the mTORC1/2 complexes, including *RICTOR*, *RAPTOR*, *MLST8*, *MAPKAP1*, as well as *mTOR* itself were enriched, implying that loss of PI3K/Akt/mTOR activity leads to resistance to samuraciclib. These findings confirmed those of a prior CRISPR-Cas9 screen using 0.35 μ M samuraciclib, which also identified knockout of PI3K/Akt/mTOR pathway genes reduces samuraciclib-induced growth arrest (Wilson *et al.*, 2023). The authors demonstrated that active cell growth driven by mTOR was required to permit entry of samuraciclib-treated cells into a cellular growth-arrested state.

To determine if mTOR dependency in a leukaemia cell line and in transformed but non-malignant fibroblast cells (Wilson *et al.*, 2023) is also important in BC cells, CRISPR-Cas9 screening of MCF7 cells was undertaken. MCF7 cells stably expressing CRISPR-Cas9 were transduced with a lentiviral library containing 5 – 10 sgRNAs for each protein coding gene (Nagarajan *et al*, 2020), followed by growth of the cells for 30 days in the presence of 150 nM samuraciclib or an equal volume of DMSO. Genomic DNA was collected, and lentiviral barcode regions were PCR amplified and sequenced using an Illumina sequencing platform (G Sava *et al.*, unpublished). The results of the sequencing data identified 125 genes ($P < 0.1$) whose knockout reduced responsiveness to samuraciclib, whereas just 11 genes were associated with greater sensitivity to samuraciclib (**Figure 3.1A**). In agreement with the NALM-6 screens, knockout of genes that promote PI3K/Akt/mTOR pathway activity (*MIOS*, *WDR24*), as well as genes for proteins in the mTORC1/2 complex (*RICTOR*, *MAPKAP1*) led to samuraciclib resistance. Additionally, *PIK3CA* and its downstream effector *AKT*, both of which promote mTOR activity, were also identified in the MCF7 screen with gene knockout reducing sensitivity to samuraciclib.

These screening data suggested that PI3K/Akt/mTOR signalling is relevant to the growth inhibitory response of BC cells to samuraciclib. To test for an association between mTOR function and the growth response to samuraciclib, nuclear GFP expressing MCF7 cells were grown in the presence of 150 nM samuraciclib alone or in combination with 5 nM torin1 for 30 days with cell growth assessed as the number of nuclei, every three days. Interestingly, treatment of MCF7 cells with the mTORC1/2 inhibitor torin1 in combination with samuraciclib did not impact MCF7 cells growth inhibition over 30 days compared to samuraciclib alone (**Figure 3.2A**). However, pre-treating MCF7 cells with torin1, even at a low concentration (5 nM), was sufficient to prevent growth inhibition by samuraciclib. In the pre-treatment experiment, torin1 was washed out after three days and the cells were treated with a combination of torin1 and samuraciclib (150 nM) or with samuraciclib alone. Pre-treatment with torin1 prevented the growth arrest induced by samuraciclib, which became especially pronounced 15 days after beginning treatment with samuraciclib (**Figure 3.2B**). These results show that inhibition of mTORC1/2 prior to treatment with samuraciclib impairs the growth inhibition by samuraciclib, confirming that an active mTOR complex is necessary for the anti-cancer action of samuraciclib in BC cells.

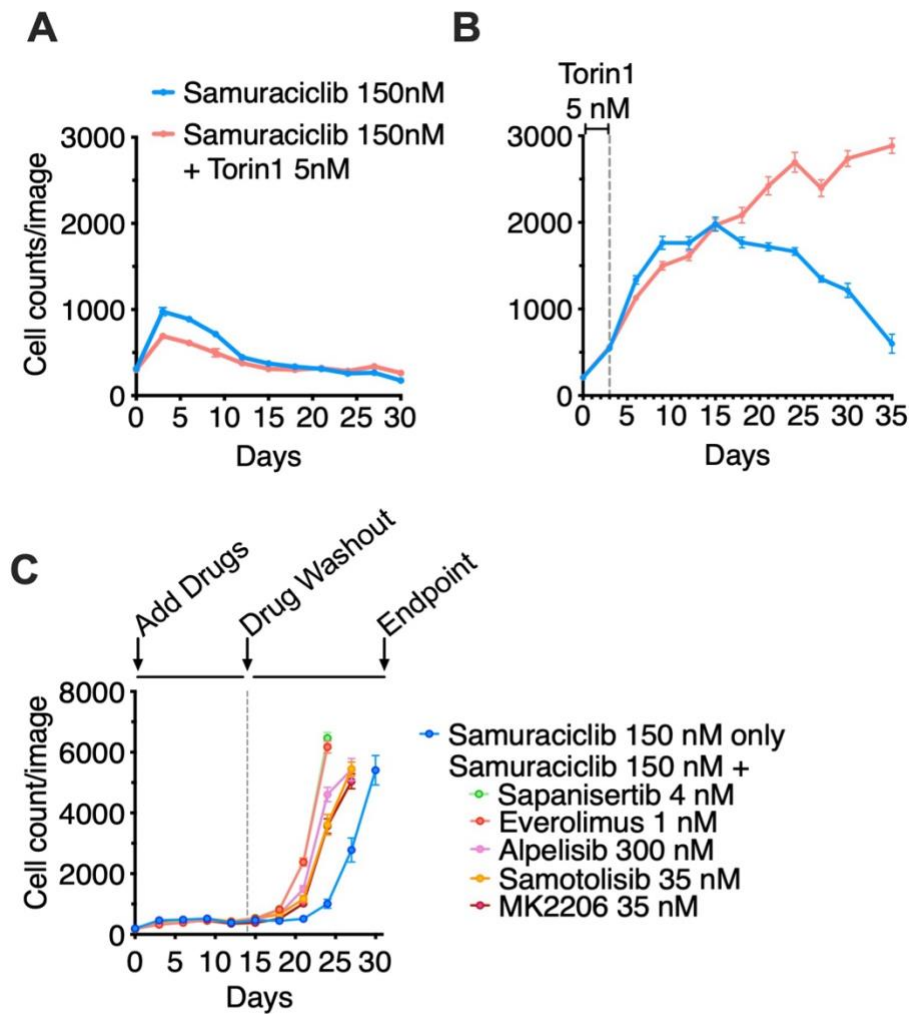


Figure 3.2 Inhibiting the mTOR pathway before treatment with samuraciclib prevents growth inhibition by samuraciclib in MCF7 cells.

(A) Nuclear GFP expressing MCF7 cells were treated with samuraciclib or samuraciclib plus torin1 for 30 days. (B) MCF7 cells were pre-treated with torin1 for 3 days then switched to either samuraciclib alone or samuraciclib and torin1 for 35 days. (C) MCF7-GFP cells were treated with samuraciclib alone or in combination with the indicated PI3K / AKT / mTOR inhibitors for 14 days. Culture medium was changed every three days to replenish the drugs. Drugs were washed out after 14 days and cells were allowed to regrow with media changes every three days. Cell numbers were determined as the number of nuclei based on GFP-positivity from four images per well, measured using IncuCyte Zoom. Error bars show the standard error of the mean (SEM) from one experiment ($n = 1$ biological replicates). These data were generated by Dr Georgina Sava.

Since mTOR is activated by Akt, which is itself activated by PI3K, we predicted that inhibiting PI3K or Akt would also reduce sensitivity to samuraciclib. To investigate this, MCF7 cells were treated with samuraciclib (150 nM) alone or in combination with the mTORC1/2 inhibitor sapanisertib (4 nM), the mTORC1 inhibitor everolimus (1 nM), the PI3K α inhibitor alpelisib (300 nM), the mTORC1/2 and PI3K inhibitor samotolisib (35 nM), or the Akt inhibitor MK-2206 (35 nM) for 14 days. Following the 14-day pre-treatment, the drugs were washed out and cell growth was assessed until cells reached peak confluence in each condition. Cells that had been co-treated all showed more rapid recovery of growth than the cells that had been treated with samuraciclib alone (**Figure 3.2C**). Finally, continued treatment with PI3K, mTOR, or Akt inhibitors showed a clear lessening of the growth inhibitory effects of samuraciclib (**Figure 3.3**). Interestingly, the most potent impact was observed with drugs that most directly inhibited mTOR, specifically the drugs that inhibit mTORC1 and mTORC2. These results show that cell growth inhibition by samuraciclib is suppressed if the PI3K/Akt/mTOR pathway is inhibited.

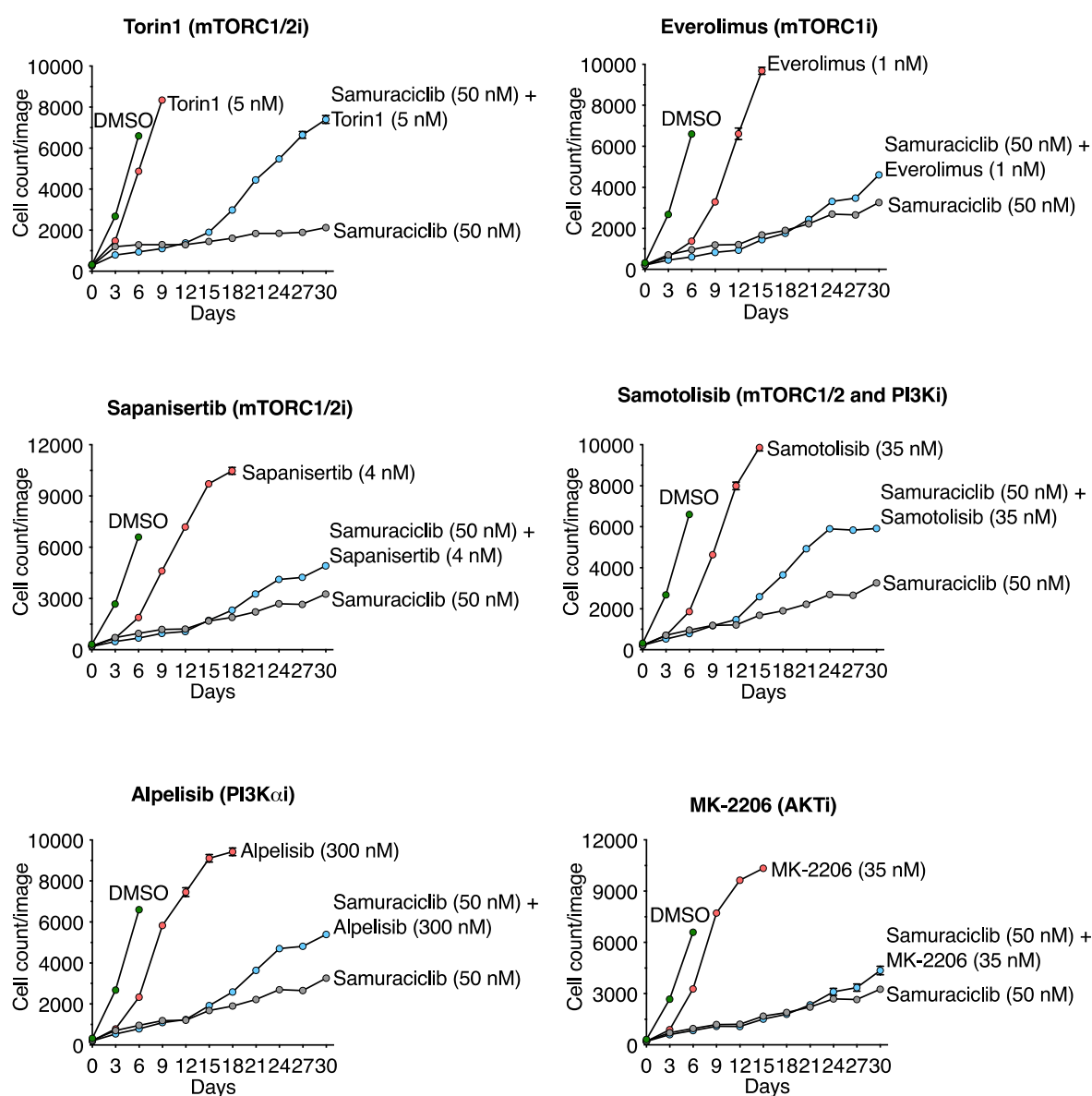


Figure 3.3 PI3K / AKT / mTOR pathway inhibitors reduce samuraciclib-mediated MCF7 cell growth arrest.

MCF7-GFP cells were treated with the indicated drugs over 30 days. The culture medium was changed every three days, to replenish the drug treatments. Cell numbers were measured as the number of GFP-positive nuclei for four images per well using IncuCyte Zoom. Error bars show the SEM for one experiment (n = 1 biological replicates). All treatments were undertaken in the same experiment such that the samuraciclib and DMSO treatment condition is the same data shown in all plots. Data were generated by Dr Georgina Sava.

3.2.2 Activating mutations of PIK3CA enhance the growth-arrest induced by samuraciclib

PIK3CA encodes the catalytic subunit, P110 α , of PI3K, which plays a pivotal role in activating the PI3K/Akt/mTOR pathway (Meric-Bernstam *et al*, 2012), as outlined in **Figure 1.8**. Phosphorylation of PIK3CA occurs in response to growth factors and activates Akt via PIP2 (Hemmings & Restuccia, 2012). Akt, which is a kinase hub receiving and transducing signals via phosphorylation, activates several downstream effectors including mTORC1. As described above, *PIK3CA* knockout was identified as promoting resistance to samuraciclib in the MCF7 CRISPR-Cas9 screening (**Figure 3.1A**). Moreover, PI3K inhibitors also promoted resistance to samuraciclib in BC cell growth assays (**Figure 3.2 – 3.3**). PIK3CA mutations are common in BC, particularly in ER+ BC, where PIK3CA mutations are observed in up to 40% of primary BC tumours and these mutations lead to hyperactivation of P110 α , which is the catalytic subunit of PIK3CA (Zardavas *et al*, 2014). Therefore, I asked whether PIK3CA mutational status can influence response to samuraciclib. Towards this, I investigated growth inhibition in the transformed, but non-tumourigenic, MCF10A cells in which hotspot PIK3CA mutations E545K and H1047R have been inserted into the endogenous PIK3CA gene using CRISPR-Cas9 genome editing. These cell lines have been developed by Dr G. Poulogiannis and have been described (Koundouros *et al*, 2020). These PIK3CA mutations in the helical domain (E545K) or the kinase domain (H1047R) of PIK3CA are the two most common hotspot mutations that result in a constitutively active PIK3CA protein and are associated with oncogenic processes (Meyer *et al*, 2013).

First, I confirmed that the MCF10A cell lines contained the appropriate isogenic mutation through mRNA reverse-transcription and PCR amplification of cDNA followed by Sanger sequencing (**Figure 3.4A**). Next, the cell lines were treated with increasing concentrations of samuraciclib for three days. Day three cell counts revealed a higher sensitivity to samuraciclib of PIK3CA-E545K and -H1047R cell lines compared to PIK3CA WT (**Figure 3.4B**). Growth inhibition was significantly greater in the mutant cells ($P < 0.05$), although this would need confirmation with additional independent experiments. Nevertheless, these results suggest that a hyper-active PI3K promotes sensitivity to samuraciclib.

I also assessed the impact of PIK3CA mutations on recovery of growth inhibition following termination of samuraciclib treatment, as this has potential importance for tumour re-growth following cessation of samuraciclib treatment in patients. For this, I performed growth assays

following long term treatment with increasing concentrations of samuraciclib. Samuraciclib inhibited growth of all three cell lines to similar levels (**Figure 3.4C**), suggesting that the level of PI3K activity does not impact response to samuraciclib during a consistent treatment regime. However, if samuraciclib was washed out after 15 days, cells with WT PIK3CA regrew much more rapidly than the PIK3CA mutant cells (**Figure 3.4D**). These results are consistent with the cell growth results of samuraciclib combination treatments with drugs inhibiting the PI3K/Akt/mTOR pathway (**Figure 3.2 – 3.3**).

PIK3CA mutant cell lines were found to be more sensitive to the PI3K inhibitors samotolisib, dactolisib, and LY-294002 compared to PIK3CA WT. GI_{50} values for these inhibitors measured between 1.2x and 1.9x lower in PIK3CA mutant cell lines compared to WT (**Figure 3.5A**). PIK3CA-WT cells were also less sensitive to torin1, alpelisib, and MK-2206 than the mutant cell lines, with GI_{50} values being consistently lower for the mutant cell lines. Consistent with previous data, the PIK3CA-E545K and -H1047R cell lines were 1.4x and 1.6x more sensitive to samuraciclib than WT cells, respectively.

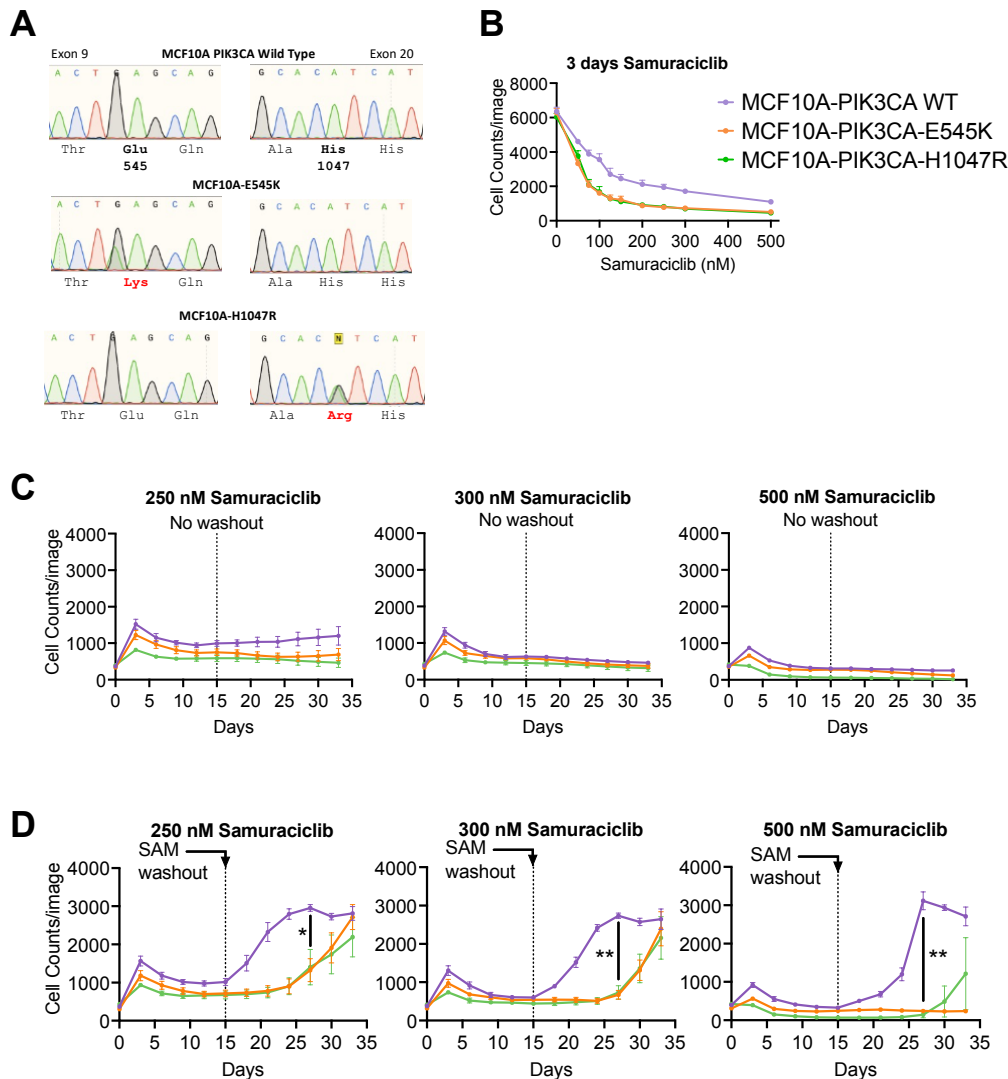


Figure 3.4 Samuraciclib-induced growth arrest is maintained for longer in MCF10A cells expressing activating PIK3CA mutations than in PIK3CA wild-type cells.

The breast cell line MCF10A was engineered using CRISPR/Cas9 genome editing to express the PIK3CA E545K or H1047R mutations in the *PIK3CA* gene. The cell lines were kindly provided by Dr G. Poulogiannis (Institute of Cancer Research). (A) Sanger sequencing was performed to confirm PIK3CA wild-type (WT), E545K, and H1047R mutation status. (B) Day three cell counts from PIK3CA WT, E545K, and H1047R MCF10A cells, lentivirally transduced with nuclear GFP, after treatment with increasing concentrations of samuraciclib. (C) Cell lines outlined in panel B were treated with 250, 300, or 500 nM samuraciclib for 30 days with media changed every three days to replenish drug treatments. (D) Cells were treated as in C, except that after 15 days treatment with samuraciclib the drug was washed out and replaced with drug-free cell media that was changed every three days. Cell numbers were assessed by counting GFP-tagged cells using IncuCyte Zoom. Points represent GFP counts from three independent experiments (n = 3 biological replicates, except n = 1 biological replicates in panel B). Error bars show the SEM; P-values show significance for PIK3CA mutant cell counts to PIK3CA-WT at day 27 (* P<0.01, ** P<0.0001).

I also confirmed the role of an active PI3K/Akt/mTOR pathway in promoting samuraciclib-induced cell growth arrest using a different assay. I studied the incorporation of a thymidine analogue, EdU, in PIK3CA-WT, PIK3CA-E545K, and PIK3CA-H1047R cell lines following treatment with samuraciclib in combination with a PI3K, Akt, or mTOR inhibitor. Actively replicating cells in the S phase of the cell cycle incorporate EdU into their growing DNA chains (Buck *et al.*, 2008). Therefore, EdU-positive cell populations are deemed to be actively proliferating. The concentrations of torin1, alpelisib, and MK2206 were based on GI₅₀ value determination (**Figure 3.5A**), with concentrations approximately 2x lower than the GI₅₀ for PIK3CA-WT cells being used, employing the rationale that this should permit better assessment of combination effects in the WT and mutant MCF10A cells. At these concentrations of these drugs there was little or no inhibition of cell proliferation (**Figure 3.5B – D**). Samuraciclib reduced EdU incorporation in all cell lines, although the reduction only reached significance in PIK3CA-WT cells. Importantly, combining samuraciclib with torin1 resulted in a significantly higher number of proliferating cells compared to samuraciclib alone, again consistent with the data presented in earlier figures. Greater proliferation was also observed when samuraciclib was combined with the Akt inhibitor MK2206, although there was effectively no altered response observed with the PI3Ka inhibitor alpelisib. In both PIK3CA-E545K and PIK3CA-H1047R cell lines, there was also a significant increase in proliferating cells in the combination treatment of MK-2206 with samuraciclib, compared to samuraciclib alone. Thus, the combination of torin1 or MK-2206 with samuraciclib reverts the anti-proliferative effects of samuraciclib. This result further suggests that the inhibitory effects of samuraciclib on cell growth rely on an active PI3K/Akt/mTOR pathway.

To further investigate the differences in PI3K/Akt/mTOR pathway activation at the protein level following combination treatments with samuraciclib and torin1, alpelisib, or MK-2206, I performed immunoblotting in MCF10A PIK3CA-WT, -E545K and -H1047R cells. The cell lines were treated for 48 hours with drugs at the GI₅₀ concentration for mutant cell lines for alpelisib and torin1 and the WT GI₅₀ for samuraciclib (**Figure 3.5A**). A relatively high concentration (0.4 μ M) of samuraciclib was used to ensure effective CDK7 inhibition. At this concentration, there is little or no inhibition of other CDKs or protein kinases (Patel *et al.*, 2018a).

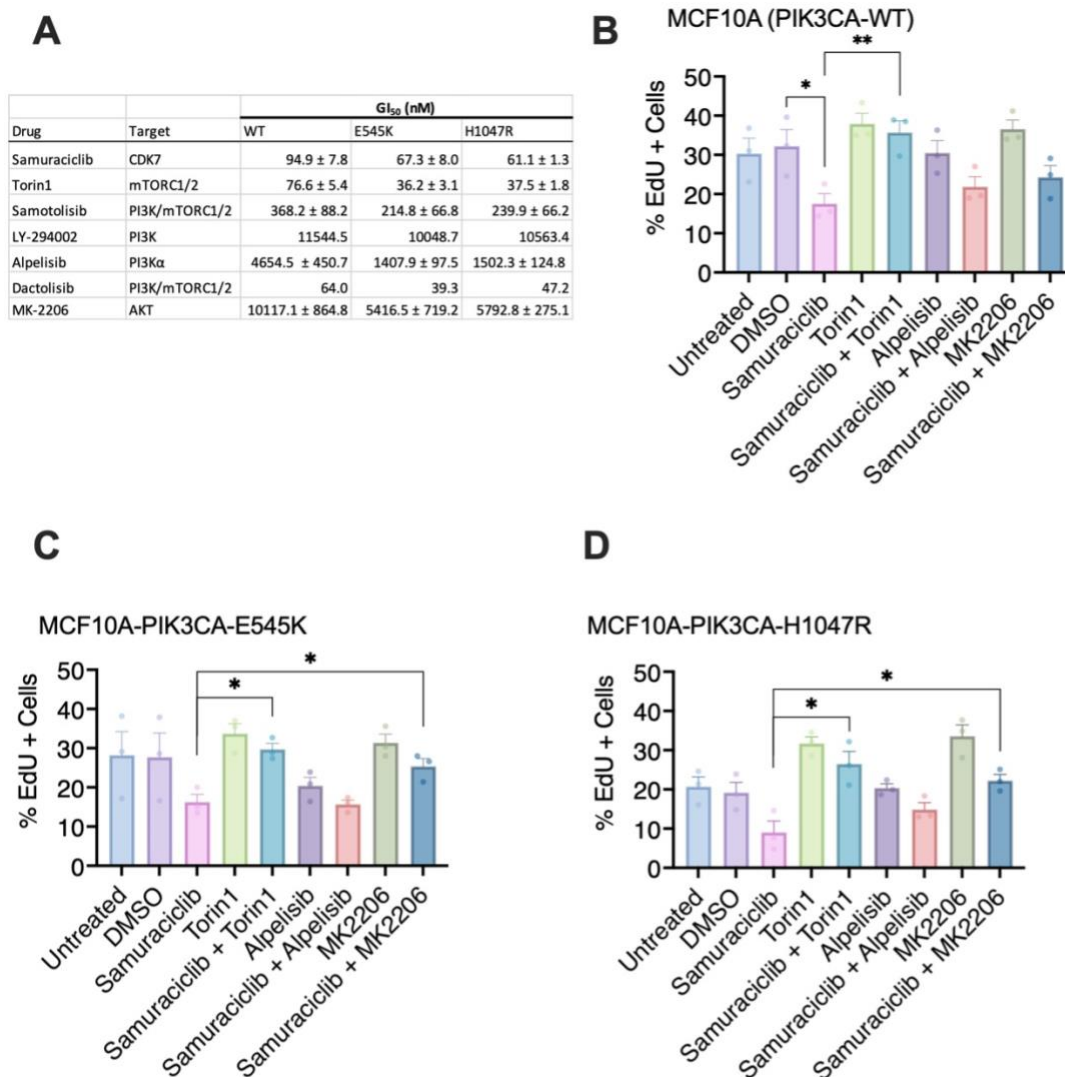


Figure 3.5 mTOR and AKT inhibitors reduce the anti-proliferative effects of samuraciclib in MCF10A cells.

(A) GI₅₀ values (nM) for MCF10A and derivative cell lines (PIK3CA WT, E545K, or H1047R) 50% growth inhibition (GI₅₀) for indicated compounds. Where possible, ± SEM is shown along with the mean day four GI₅₀ values from at least two independent experiments; otherwise, n=1.

(B – D) MCF10A cells were treated with the indicated drugs for 48 hours (samuraciclib 75 nM; torin1 40 nM; alpelisib 2 μM; MK2206 5 μM). After incorporation of EdU, a synthetic thymidine analogue, EdU positive (+) cell populations were measured using flow cytometry to determine proliferating cell numbers. Bars represent the mean percentage of EdU-positive cells from three independent experiments with error bars displaying the SEM. P-values from t-tests with Welch's correction are shown (* P < 0.05, ** P < 0.01).

Akt activation involves phosphorylation at two residues: Ser473 in the C-terminal hydrophobic motif by mTORC2 and Thr308 in the activation loop by the protein kinase PDK1 (Alessi *et al*, 1996; Alessi *et al*, 1997; Sarbassov *et al*, 2005). Through a downstream phosphorylation cascade, activated Akt may block apoptosis and promote cell growth (Manning & Toker, 2017). Immunoblotting showed that Akt phosphorylation at Ser473 was reduced by the PI3K α inhibitor alpelisib, as expected (**Figure 3.6**). Inhibition of Akt phosphorylation was also observed with torin1, which reflects the fact that Akt Ser473 phosphorylation is promoted by mTORC2 (Sarbassov *et al.*, 2005). MK2206 has also been shown to inhibit Ser573 phosphorylation (Hirai *et al.*, 2010).

S6K is activated by the canonical phosphorylation of Thr389 by mTORC1 (Martin *et al*, 2014). Phosphorylation of S6K was inhibited by torin1, alpelisib, and MK2206, again as expected. Samuraciclib did not inhibit Akt phosphorylation but appeared to increase S6K phosphorylation in PIK3CA-WT and mutant cells. The increase in P-S6K was not accompanied by elevated levels of RAPTOR and RICTOR, suggesting that the levels of mTORC1 and mTORC2 are not increased.

As expected, CDK1 phosphorylation was inhibited by samuraciclib. Of particular interest was the finding that the samuraciclib and torin1 combination treatment returned CDK1 phosphorylation to levels similar to control cells, implying that torin1 blocks the ability of samuraciclib to inhibit CDK1 phosphorylation. By contrast, alpelisib or MK2206 treatment did not prevent the inhibition of CDK1 phosphorylation by samuraciclib. CDK2 phosphorylation appeared to be inhibited by samuraciclib, which was not suppressed by torin1, alpelisib, and MK2206. The mechanisms underlying these combination effects require confirmation, probably with more detailed time course and dose-response experiments. Accepting these shortcomings in the current data set, these qualitative data are nevertheless intriguing, suggesting that CDK7 inhibition may increase mTOR complex activity and result in altered control of the G2/M phase of the cell cycle, in which CDK1 is critical.

Collectively, my results show that the cell growth inhibitory effects of samuraciclib are modulated, at least in part, through a mechanism that involves the PI3K/Akt/mTOR pathway. I demonstrated that the inhibition of the PI3K/Akt/mTOR pathway with specific inhibitors impairs the samuraciclib-induced cell growth arrest. I further showed that a hyper-active PI3K/Akt/mTOR pathway via PIK3CA activating mutations enhances the inhibitory effects of

samuraciclib treatment through cell growth assays, GI₅₀ experiments, and EdU incorporation / proliferation assays. Lastly, I validated that PI3K/Akt/mTOR pathway inhibitor and samuraciclib effects by Western blotting. These data imply that BC patients with tumours that have high PI3K/Akt/mTOR pathway activity, which can be gained through activating PI3K mutations, may experience a more sustained benefit from CDK7i, specifically with samuraciclib. Further, activating mutations in Akt, as well as inactivating mutations or loss of heterozygosity (LOH) in repressors of mTOR activity, such as *PTEN*, *LKB1*, and *TSC2* are seen in many cancers (Stemke-Hale *et al*, 2008) and may also promote better patient responses to samuraciclib and other CDK7i. Many drugs targeting PI3K, mTOR, or Akt are in advanced clinical development or are already widely used in advanced BC patients (Paplomata & O'Regan, 2014). These findings would indicate that these should not be combined with CDK7 inhibitors such as samuraciclib.

3.3 Profiling the proteomic response to samuraciclib treatment

3.3.1 Preparation of samples and determining samuraciclib treatment concentrations for quantitative mass spectrometry

CRISPR-Cas9 knockout screening is a powerful tool for identifying genes and signalling pathways that modulate response to the drug of interest. As described above, our genome-wide CRISPR-Cas9 screens have especially identified a key role for the PI3K/Akt/mTOR pathway in promoting the growth inhibitory response to samuraciclib. Defining changes in gene expression is another tool by which it is possible to identify potential biomarkers associated with response as well as genes and signalling pathways that are important for the growth inhibitory responses to the drug of interest. Differential gene expression profiling and pathway enrichment can also identify candidate signalling pathways that could be targeted in combination with the drug of interest. As an example, RNA-seq for global gene expression analysis has revealed that samuraciclib treatment results in the activation of the p53 pathway of BC cell lines (Nguyen *et al.*, in preparation). Interestingly, the phase 1 clinical trial of samuraciclib in advanced ER+ breast cancer patients demonstrated patient response in patients with WT p53 but no response in patients with mutant p53 (Coombes *et al.*, 2023). To profile the molecular consequences of samuraciclib treatment at the protein level, I performed a global proteome analysis of MCF7 cells treated with samuraciclib. This work was performed in collaboration with Dr Tommaso de Marchi (Lund University).

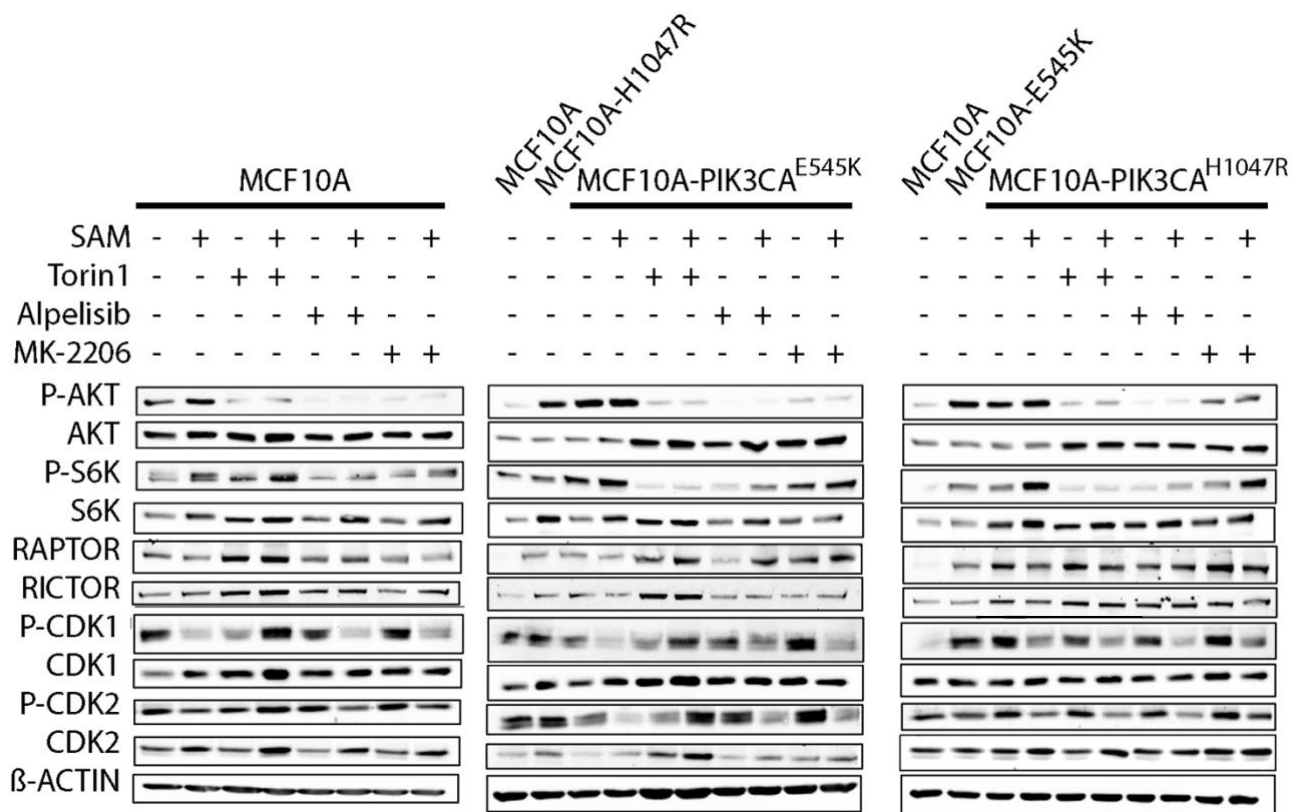


Figure 3.6 Immunoblotting for CDK7 and PI3K/AKT/mTOR pathway demonstrates reduction in phosphorylation of target proteins.

MCF10A or PIK3CA mutant cells were treated with the indicated drugs for 24 hours. The drugs were added to a final concentration of 400 nM (samuraciclib), 250 nM (torin1), 2 μ M (alpelisib), or 1 μ M (MK-2206). Equal volumes of DMSO were added to the no-treatment controls. The specific phosphorylation states of proteins were blotted for: P-AKT (AKT P-Ser473); P-S6K (S6K P-Thr389); P-CDK1 (CDK1 P-Thr161); P-CDK2 (CDK2 P-Thr160). Immunoblotting for β -actin served as the loading control. Note that immunoblotting of MCF10A and mutant cell lysates in the first two lanes of the second and third panels were used to permit cross-comparison between the different cell lines.

Towards determining the appropriate concentrations of samuraciclib, I treated MCF7 cells with samuraciclib at increasing concentrations. Protein lysates were prepared one hour after the addition of samuraciclib, which would identify the most immediate protein changes. I immunoblotted for markers of CDK7 activity, namely phosphorylation at Ser2 and Ser5 in the CTD of Pol-II. The CTD of Pol-II is phosphorylated directly by CDK7 as part of the TFIIH complex at Ser5 of the 52 heptapeptide repeat region (Kelso *et al.*, 2014; Larochelle *et al.*, 2012). Ser2 within the heptapeptide region is an indirect marker of CDK7 activity, since it is phosphorylated by CDK9, which is activated by CDK7 (Glover-Cutter *et al.*, 2009; Larochelle *et al.*, 2012). Immunoblotting of CDK1 at Thr161 was used as a second marker of CDK7 activity (Larochelle *et al.*, 2007). There was evident reduction in Pol-II Ser5 and CDK1 Thr161 phosphorylation with samuraciclib concentrations of 0.5 μ M and above (**Figure 3.7**). Levels of total Pol-II were not altered at these concentrations of samuraciclib, nor was there any reduction in total CDK1. The retinoblastoma (RB) protein is phosphorylated by CDK4/6 and CDK2 during the G1 phase of the cell cycle (Akiyama *et al.*, 1992; Geng *et al.*, 1996; Ohtani *et al.*, 1995). As these CDKs are also activated by CDK7 phosphorylation, immunoblotting for RB phosphorylation is therefore a measure of CDK4/6 and CDK2 activities and therefore an indirect indication of CDK7 inhibition by samuraciclib. There was an apparent dose-dependent reduction in RB phosphorylation with concentrations of samuraciclib at 0.5 μ M or higher.

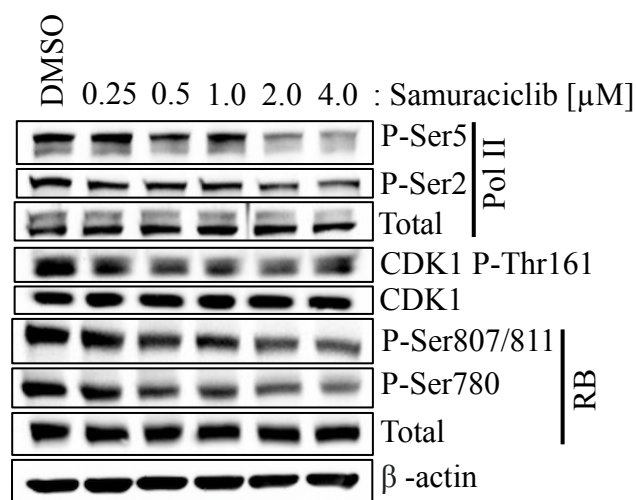


Figure 3.7 Immunoblotting of MCF7 cell protein lysates for identifying concentrations samuraciclib for proteomic studies.

Whole cell lysates prepared from MCF7 cells treated with samuraciclib for one hour were immunoblotted for CDK7 substrates. An equal volume of vehicle (DMSO) was added to control cells. Immunoblotting for β -actin was used as a loading control.

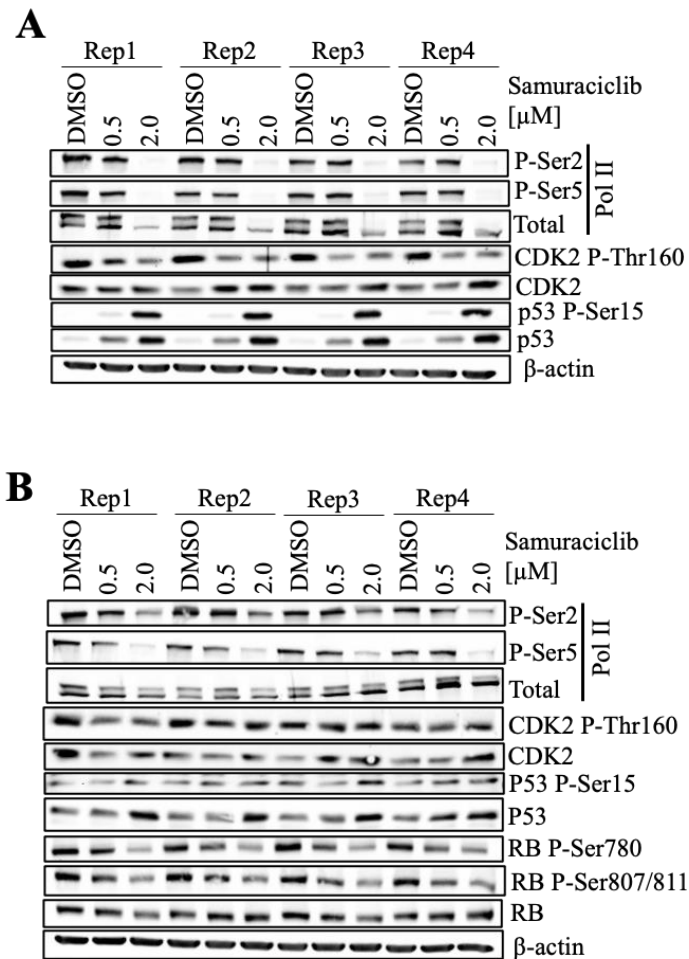


Figure 3.8 Preparation of protein lysates for quantitative proteomic analysis following treatment of MCF7 cells with samuraciclib.

Immunoblotting of four independent biological replicates of MCF7 cells treated with samuraciclib for 24 hours (**A**) or 1 hour (**B**) was performed using antibodies for the protein marks indicated. In all cases an equal volume of vehicle (DMSO) was added to control cells. Immunoblotting for β -actin was used as a loading control.

3.2.2 Proteome changes resulting from treatment of breast cancer cells with samuraciclib

Based on the above data, I selected to treat MCF7 cells with 0.5 μ M samuraciclib as this was the lowest concentration at which Pol-II, CDK1, and RB phosphorylation was reduced. I also treated cells with a higher concentration of 2.0 μ M samuraciclib to maximise possibilities for identifying proteome changes. Independent replicate protein lysates were prepared from MCF7 cells treated for 24 hours following samuraciclib treatment. The two concentrations would provide the possibility of identifying proteome changes in low and high CDK7i conditions. Following treatment, cells were pelleted, frozen, and sent for mass spectrometric proteome analysis. Before sending samples for mass spectrometry analysis, I confirmed appropriate target engagement by immunoblotting an aliquot of each replicate sample. The four independent replicate sample sets show clear dose-dependent reductions in Pol-II P-Ser5 levels following samuraciclib treatment for one and 24 hours (**Figure 3.8A – B**). Interestingly, 2.0 μ M samuraciclib for 24 hours caused a reduction in total Pol-II protein levels. CDK2 phosphorylation followed a similar trend as P-Ser5. The dose-dependent increase in p53 protein and phosphorylation levels, which are most evident at 24 hours, are consistent with unpublished results from our lab (Nguyen *et al.*, in preparation).

Mass spectrometry data was collected and pre-processed to normalized, log2-transformed protein counts by Dr. Tommaso De Marchi (Lund University). For this, cell pellets that I had prepared were used to generate protein lysates, tagged using tandem mass tagging (TMT), and processed for quantitative mass spectrometry. Trypsin-digested and TMT-tagged peptides across the different treatment conditions were pooled for analysis using mass spectrometry. Quantified peptides were compared to known databases in a search that resulted in the identification of >4,000 proteins, after filtering for contaminants, reverse sequences, and the number of unique peptides (minimum of 1) matching to each protein. I received and worked with log2-transformed, median imputed, normalized protein counts throughout my analyses. For subsequent computational analysis, the names of proteins from the 24-hour proteomics data were converted into gene names. To convert gene names to proteins, a table containing gene names and protein was downloaded from the Human Genome Organization (HUGO) database (<https://www.genenames.org/download/custom/>) (Seal *et al.*, 2023). Thus, all subsequent work describing “genes” from proteomics data refers to the gene names of the proteins.

I performed quality control analysis of the samuraciclib-treated (24 hours; 0.5 and 2.0 μ M) quantitative proteomics data to assess the integrity of my data. Spectral counts were collected for 4,617 proteins accounting for approximately 23.9% of all protein-coding genes. Protein levels were normalized into scaled intensities and batch-corrected using Limma (Ritchie *et al.*, 2015). First, I found that the scaled intensities of protein levels followed a normal distribution across all treatment conditions and all replicates (**Figure 3.9A**). The normal distribution of spectral counts was supported by visualizing the scaled intensities using Q-Q plots (**Figure 3.9B**). Second, I visualized samples in reduced dimensions using principal component analysis (PCA). The PCA separated samples according to treatment conditions across the first two principal components (**Figure 3.9C**). These results suggest that my quantitative proteomics data are high quality and support their use to investigate differential protein levels following a 24-hour treatment with samuraciclib compared to vehicle.

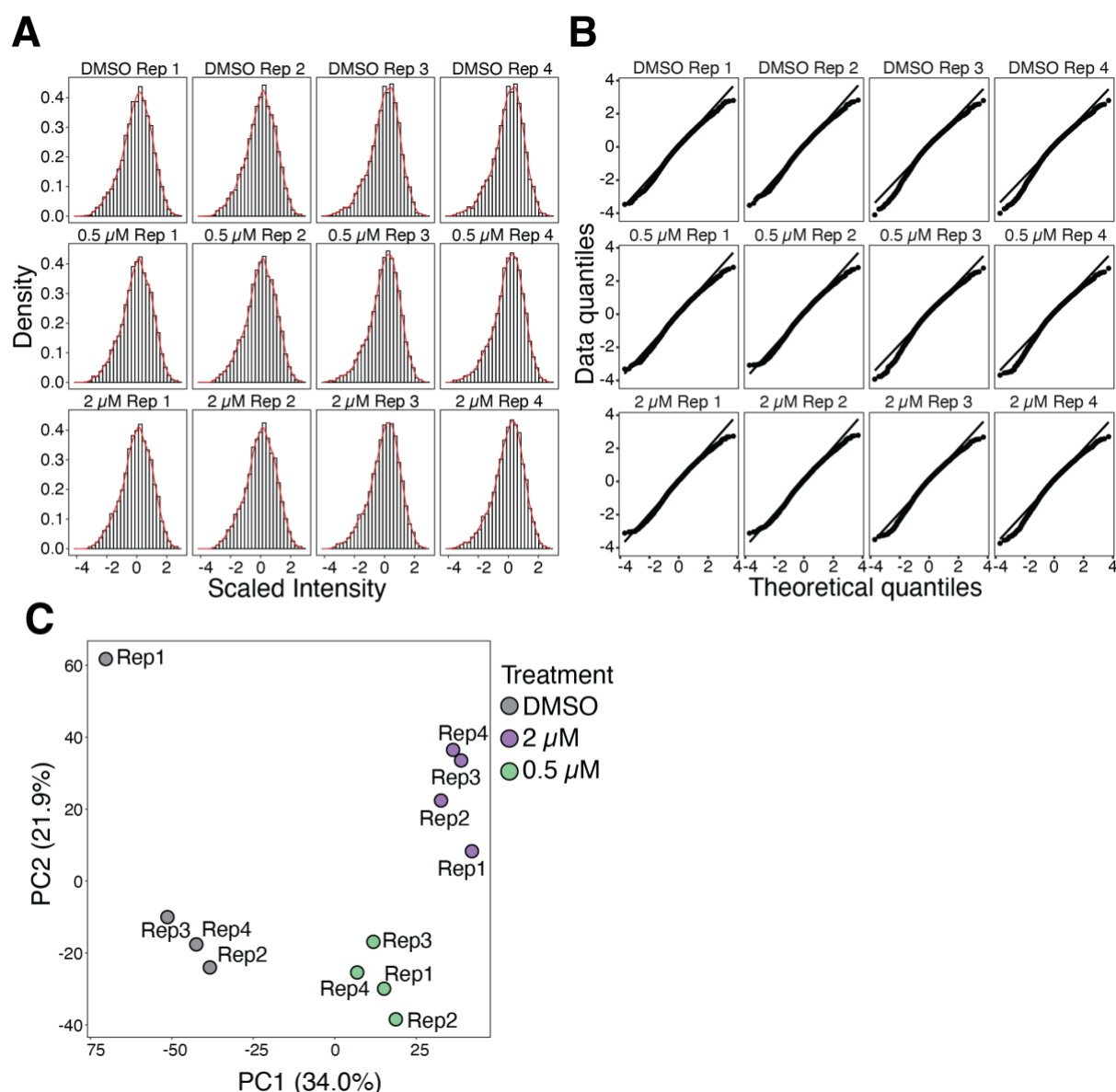


Figure 3.9 Quality control analysis of global proteomic profiling of samuraciclib-treated MCF7 cells.

(A) Protein lysates prepared from MCF7 cells treated with 0.5 μ M or 2 μ M samuraciclib, or an equal volume of DMSO for 24 hours were prepared for whole proteome extraction and quantitative mass spectrometry using tandem mass tagging (TMT). Histograms of protein expression demonstrate that protein quantities follow approximately normal distributions within each treatment and each replicate sample. **(B)** Quantile-quantile (QQ) plots of expected versus actual protein quantiles show approximately normal distributions of protein expression values. **(C)** Principal component analysis (PCA) following batch-effect correction demonstrates that cell treatment conditions cluster by treatment condition.

I performed differential quantitative mass spectrometry analysis (DEQMS) on my normalized and batch-corrected proteomics data to identify significantly differentially expressed proteins (DEPs) following samuraciclib treatment (0.5 and 2.0 μM). Compared to the DMSO (vehicle) treated cells, 1,738 and 2,188 proteins were significantly differentially regulated following 0.5 μM and 2 μM samuraciclib treatment, respectively (P -adjusted < 0.05) (**Figure 3.10A**). Of the 0.5 μM samuraciclib DEPs, 53% (921/1,738) were upregulated and 47% (817/1,738) were downregulated. Similarly, 59% (1,285/2,188) of the 2.0 μM samuraciclib DEPs represented downregulated proteins. The majority (71%; 1,238/1,738) of the DEPs for the 0.5 μM treatment were also differential in the 2.0 μM condition and represented 57% (1,238/2,188) of the 2.0 μM samuraciclib DEPs. When comparing the up- and down-regulated proteins separately, 75% (694/921) of the 0.5 μM samuraciclib up-regulated proteins were also upregulated with 2.0 μM samuraciclib. The downregulated DEPs in common between the two samuraciclib treatments represented 60% (490/903) of the 0.5 μM DEPs and 54% (490/903) of the 2.0 μM DEPs. Generally, significantly DEPs that were downregulated showed a greater fold-change (FC) in the 2 μM condition compared to the 0.5 μM condition (**Figure 3.10B**) with a log₂ mean FC of -0.45 compared to -0.31 for the 0.5 μM condition ($P = 2.78\text{E-}17$, Mann-Whitney U-test). In total, 6 proteins were significantly downregulated ≥ 2 -fold ($\log_2\text{FC} \leq -1$) with 0.5 μM samuraciclib, while 68 proteins were downregulated with 2.0 μM samuraciclib (**Table 3.1 – 3.2**). Although not necessarily passing the FC cut-off, the four out of six significantly down-regulated proteins with 0.5 μM samuraciclib were also significantly downregulated with 2.0 μM , namely UBE2T, PAF15, SPC25, TEDC1. UBE2T is an E2 ubiquitin ligase, central to the activity of the Fanconi's anaemia complex in maintenance of genomic stability and DNA inter-strand crosslink repair (Lemonidis *et al*, 2022). PAF15, encoded by the PCLAF (PCNA clamp associated factor) gene has been implicated in G1/S progression and in inhibiting cellular quiescence (Kim *et al*, 2021). SPC25 is a mitosis-associated spindle-assembly checkpoint regulatory protein whose expression is predictive of hepatocellular carcinoma tumour progression and metastasis (Shi *et al*, 2022). TEDC1 is a tubulin complex protein whose expression is correlated with improved BC patient survival (Dai *et al*, 2019).

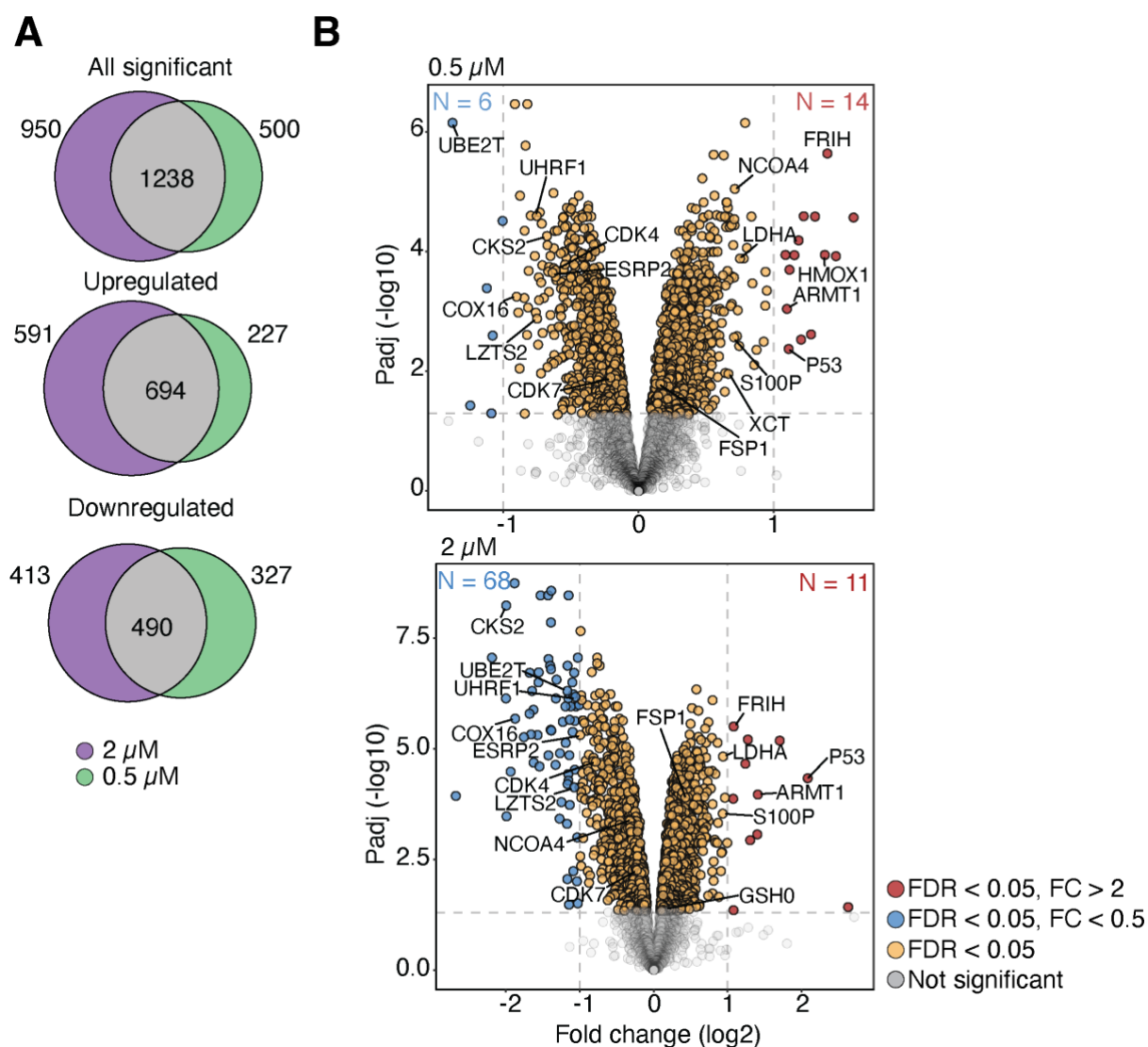


Figure 3.10 Quantitative mass spectrometry of samuraciclib-treated MCF7 cells identifies alterations in protein levels.

(A) Volcano plots showing differential protein levels, comparing samuraciclib-treated cells with DMSO-treated cells. **(B)** Venn diagrams show significantly (P -adjusted < 0.05) differential proteins common between 0.5 μ M and 2 μ M samuraciclib treatment.

Table 3.1 Differentially expressed proteins (DEPs; P-adjusted < 0.05; $1 \leq \log_2FC \leq -1$) identified in MCF7 cells treated with 0.5 μ M samuraciclib for 24 hours.

Protein	Gene name	log2FC	P-adjusted
UBE2T	UBE2T	-1.3759997	7.05E-07
H2A1C	H2AC6	-1.2448311	0.03730214
PAF15	PCLAF	-1.122217	0.00040813
TEDC1	TEDC1	-1.0788358	0.00252666
SPC25	SPC25	-1.0055249	3.07E-05
FBX41	FBXO41	-1.087976	4.64E-02
DDX28	DDX28	1.08861633	0.00011349
ARMT1	ARMT1	1.09956451	0.00090663
NDKA	NME1	1.59274002	2.69E-05
SPB5	SERPINB5	1.46213861	0.00011904
FRIH	FTH1	1.39872738	2.29E-06
HMOX1	HMOX1	1.38012739	0.00011349
EPHA2	EPHA2	1.30763145	2.59E-05
MCFD2	MCFD2	1.27793117	0.00241588
TMED5	TMED5	1.22355457	2.56E-05
ADK	ADK	1.2044005	0.00295392
SPCS3	SPCS3	1.18573709	6.49E-05
SPHM	SGSH	1.15444822	0.00011449
CLH2	CLTCL1	1.11833664	0.00020094
P53	TP53	1.11226197	0.00423478

Table 3.2 Differentially expressed proteins (DEPs; P-adjusted < 0.05; $1 \leq \log_2\text{FC} \leq -1$) identified in MCF7 cells treated with 2 μM samuraciclib for 24 hours.

Protein	Gene name	log2FC	P-adjusted		Protein	Gene name	log2FC	P-adjusted
BAMBI	BAMBI	-2.6778548	0.00011582		RPF1	RPF1	-1.1684714	0.00876953
DBLOH	DIABLO	-2.1886635	8.73E-08		CRIP1	CRIP1	-1.1661229	6.27E-05
HTRA2	HTRA2	-2.0001443	7.26E-07		UTP6	UTP6	-1.1658266	1.32E-07
CKS2	CKS2	-1.996114	5.79E-09		NSA2	NSA2	-1.1568778	3.71E-05
AROS	RPS19BP1	-1.9917751	0.00033501		RN223	RNF223	-1.1562762	7.26E-07
COXM1	CMC1	-1.9366708	3.27E-05		TIM16	PAM16	-1.1545704	5.20E-05
CEBPB	CEBPB	-1.8827014	1.81E-09		RAVR1	RAVER1	-1.1527651	3.42E-09
COX16	COX16	-1.8752988	2.08E-06		FBX41	FBXO41	-1.1465616	0.03311912
UIF	FYTTD1	-1.7569552	5.44E-06		OPA1	OPA1	-1.1418646	1.40E-05
MICU1	MICU1	-1.6795962	1.61E-06		UBL5	UBL5	-1.1374109	2.21E-06
CHCH5	CHCHD5	-1.6786494	1.91E-07		PNO1	PNO1	-1.1363862	0.00018566
TIM8A	TIMM8A	-1.6594932	4.75E-06		KDM5B	KDM5B	-1.1220381	1.10E-06
TOB1	TOB1	-1.6465371	4.93E-07		MO4L1	MORF4L1	-1.1010869	3.16E-07
UT14A	UTP14A	-1.6277707	1.31E-06		RBM3	RBM3	-1.0970471	4.15E-06
F136A	FAM136A	-1.625181	2.04E-05		CA131	C1orf131	-1.0890927	5.87E-07
TIM8B	TIMM8B	-1.5713754	4.88E-06		ZC3H3	ZC3H3	-1.08794	0.00578504
CHTOP	CHTOP	-1.5600362	3.16E-07		CYC	CYCS	-1.0764373	1.91E-07
RMD1	RMDN1	-1.5558493	1.87E-07		LZTS2	LZTS2	-1.0762317	7.43E-05
PAF15	PCLAF	-1.5455885	2.51E-05		COXM2	CMC2	-1.0693348	2.97E-05
DCA13	DCAF13	-1.5311065	3.42E-09		SLX9	SLX9	-1.0669553	2.35E-06
HEM6	CPOX	-1.4291718	3.42E-09		UHRF1	UHRF1	-1.0582507	6.70E-07
BMP7	BMP7	-1.4250329	1.41E-05		JAK1	JAK1	-1.0532618	1.10E-06
LACTB	LACTB	-1.423209	9.38E-08		CDC20	CDC20	-1.0387724	0.00984653
CLPB	CLPB	-1.4016712	1.32E-07		TTC19	TTC19	-1.0356684	0.00100427
PGAM5	PGAM5	-1.39449	3.71E-06		SPART	SPART	-1.0275831	8.73E-08
GATA3	GATA3	-1.3910504	1.40E-08		PYRD	DHODH	-1.0273194	0.03102598
NDUA4	NDUFA4	-1.3902199	1.59E-07		CX6B1	COX6B1	-1.0099161	1.03E-06
FA32A	FAM32A	-1.3873837	2.68E-09		SURF6	SURF6	-1.0034399	7.39E-05
QKI	QKI	-1.3864713	3.87E-06		OPLA	OPLAH	2.62965939	0.03754881
PDIP3	POLDIP3	-1.3282347	2.30E-05		P53	TP53	2.08235095	4.62E-05
OXA1L	OXA1L	-1.3243211	7.26E-07		NDKA	NME1	1.70461009	6.54E-06
PGES2	PTGES2	-1.3150542	2.74E-07		ARMT1	ARMT1	1.40765975	0.00010794
DDX56	DDX56	-1.2741978	0.0003783		MCFD2	MCFD2	1.40056251	0.00085767
GRPE2	GRPEL2	-1.2689626	1.26E-05		ADK	ADK	1.30454133	0.00116136
FND3B	FND3B	-1.2488022	0.00016001		TMED5	TMED5	1.27379821	6.17E-06
NDUA8	NDUFA8	-1.2301367	2.46E-06		SPCS3	SPCS3	1.23942778	2.17E-05
RARB	RARB	-1.1939423	1.10E-06		FRIH	FTH1	1.08045505	3.13E-06
HAX1	HAX1	-1.1904948	7.36E-06		BAF	BANF1	1.07935832	0.04412553
CCHL	HCCS	-1.1730321	0.00049456		CLH2	CLTCL1	1.07477306	0.00013434
UBE2T	UBE2T	-1.1690297	4.93E-07					

With a stringent cut-off of $\log_2\text{FC} \geq 1$, just 11 proteins were upregulated with 2.0 μM samuraciclib, which was similar to the number of proteins (14) that were upregulated with 0.5 μM samuraciclib. Nine of these 11 proteins were common between the two treatment conditions. Among the nine proteins are P53, already shown to be up-regulated by immunoblotting (**Figure 3.8A – B**). Other proteins include ARMT1 (acidic residue methyltransferase 1), which has little described about its function, but it has been reported to methylate PCNA, implicating it in DNA replication and DNA damage response (Perry *et al*, 2015).

Interestingly, several of the significantly downregulated proteins have been implicated in regulation of mTOR activity, including UBE2T, UHRF1, CKS2, and LZTS2 (**Table 3.3 – 3.4**). UBE2T is the only protein that passes the FC cut-off with both 0.5 and 2.0 μM samuraciclib; however, UHRF1, CKS2 and LZTS2 are all significant DEPs in both treatment conditions. UBE2T, in addition to being implicated in the Fanconi's Anaemia complex, has been reported to regulate cell proliferation and autophagy via the PI3K/Akt/mTOR pathway (Zhu *et al*, 2021). UHRF1, which was downregulated 2-fold by 2.0 μM samuraciclib, influences mTOR-mediated autophagy by regulating histone methylation (Li *et al*, 2021b). CKS2 (2.0 μM samuraciclib $\log_2\text{FC} = -1.996$; 0.5 μM samuraciclib $\log_2\text{FC} = -0.667$) cooperates with mTORC1 to coordinate the assembly of the transcription complex eIF4F (Gandin *et al*, 2016). LZTS2 is a putative tumour suppressor gene which reportedly inhibits cell proliferation through a mTOR/Akt-dependent mechanism and has also been implicated in the regulation of β -catenin activity (Lu *et al*, 2021; Thyssen *et al*, 2006).

Table 3.3 Differentially expressed proteins (P-adjusted < 0.05, log2FC > 1) identified in MCF7 cells treated with 0.5 μ M samuraciclib for 24 hours and associated with mTOR or ferroptosis.

Protein	Gene name	log2FC	P-adjusted
Proteins Downregulated			
UBE2T	UBE2T	-1.3759997	7.05E-07
PAF15	PCLAF	-1.122217	0.00040813
FBX41	FBXO41	-1.087976	0.0504648
COX16	COX16	-0.9015947	0.00056612
UHRF1	UHRF1	-0.752409	2.54E-05
LZTS2	LZTS2	-0.7498279	0.00131744
CKS2	CKS2	-0.6771937	5.49E-05
RN223	RNF223	-0.6735127	7.79E-05
CEBPB	CEBPB	-0.5532876	2.69E-05
BAMBI	BAMBI	-0.5322934	0.26319908
HAX1	HAX1	-0.5259104	0.00285197
LACTB	LACTB	-0.4877634	0.00034211
RBM3	RBM3	-0.4600723	0.00249196
PNO1	PNO1	-0.4121018	0.06495225
HEM6	CPOX	-0.3997565	0.00011906
UT14A	UTP14A	-0.3881517	0.02168838
GATA3	GATA3	-0.3784037	0.00036931
UTP6	UTP6	-0.3643866	0.00081781
PGAM5	PGAM5	-0.3537651	0.02966636
DBLOH	DIABLO	-0.349721	0.02303989
UBL5	UBL5	-0.3461452	0.00895451
FND3B	FND3B	-0.3288703	0.15815889
RMD1	RMDN1	-0.2576571	0.0323897
CX6B1	COX6B1	-0.1616731	0.08629755
NDUA4	NDUFA4	-0.1589947	0.11271329
NDUA8	NDUFA8	-0.1395865	0.27270141
CYC	CYCS	-0.1171794	0.14228726
PGES2	PTGES2	-0.0689135	0.49751604
JAK1	JAK1	-0.0547996	0.57882274
Proteins Upregulated			
PYRD	DHODH	0.08129068	0.87213779
OPLA	OPLAH	0.28679748	0.83361267
ARMT1	ARMT1	1.09956451	0.00090663
P53	TP53	1.11226197	0.00423478
CLH2	CLTCL1	1.11833664	0.00020094
SPCS3	SPCS3	1.18573709	6.49E-05
ADK	ADK	1.2044005	0.00295392
TMED5	TMED5	1.22355457	2.56E-05
MCFD2	MCFD2	1.27793117	0.00241588
HMOX1	HMOX1	1.38012739	0.00011349
FRIH	FTH1	1.39872738	2.29E-06
NDKA	NME1	1.59274002	2.69E-05

Bold rows were significant in both 0.5 μ M and 2 μ M samuraciclib proteomics.

Table 3.4 Differentially expressed proteins (DEPs; P-adjusted < 0.05, log2FC > 1) identified in MCF7 cells treated with 2 μ M samuraciclib for 24 hours and associated with mTOR or ferroptosis.

Protein	Gene name	log2FC	P-adjusted
Proteins Downregulated			
BAMBI	BAMBI	-2.6778548	0.00011582
DBLOH	DIABLO	-2.1886635	8.73E-08
CKS2	CKS2	-1.996114	5.79E-09
CEBPB	CEBPB	-1.8827014	1.81E-09
COX16	COX16	-1.8752988	2.08E-06
UT14A	UTP14A	-1.6277707	1.31E-06
RMD1	RMDN1	-1.5558493	1.87E-07
PAF15	PCLAF	-1.5455885	2.51E-05
HEM6	CPOX	-1.4291718	3.42E-09
LACTB	LACTB	-1.423209	9.38E-08
PGAM5	PGAM5	-1.39449	3.71E-06
GATA3	GATA3	-1.3910504	1.40E-08
NDUA4	NDUFA4	-1.3902199	1.59E-07
PGES2	PTGES2	-1.3150542	2.74E-07
FND3B	FND3B	-1.2488022	0.00016001
NDUA8	NDUFA8	-1.2301367	2.46E-06
HAX1	HAX1	-1.1904948	7.36E-06
UBE2T	UBE2T	-1.1690297	4.93E-07
UTP6	UTP6	-1.1658266	1.32E-07
RN223	RNF223	-1.1562762	7.26E-07
FBX41	FBXO41	-1.1465616	0.03311912
UBL5	UBL5	-1.1374109	2.21E-06
PNO1	PNO1	-1.1363862	0.00018566
RBM3	RBM3	-1.0970471	4.15E-06
CYC	CYCS	-1.0764373	1.91E-07
LZTS2	LZTS2	-1.0762317	7.43E-05
UHRF1	UHRF1	-1.0582507	6.70E-07
JAK1	JAK1	-1.0532618	1.10E-06
PYRD	DHODH	-1.0273194	0.03102598
CX6B1	COX6B1	-1.0099161	1.03E-06
HMOX1	HMOX1	-0.0836504	0.70004988
Proteins Upregulated			
CLH2	CLTCL1	1.07477306	0.00013434
FRIH	FTH1	1.08045505	3.13E-06
SPCS3	SPCS3	1.23942778	2.17E-05
TMED5	TMED5	1.27379821	6.17E-06
ADK	ADK	1.30454133	0.00116136
MCFD2	MCFD2	1.40056251	0.00085767
ARMT1	ARMT1	1.40765975	0.00010794
NDKA	NME1	1.70461009	6.54E-06
P53	TP53	2.08235095	4.62E-05
OPLA	OPLAH	2.62965939	0.03754881

Bold rows were significant in both 0.5 μ M and 2 μ M samuraciclib proteomics.

Considering the association of the key anti-ferroptotic protein, GPX4, with resistance to samuraciclib in both the MCF7 and NALM-6 CRISPR screens, it was interesting to see several ferroptosis-related proteins identified in the proteomics screen. Samuraciclib treatment upregulated key mediators and regulators of ferroptosis (Hassannia *et al*, 2019) (**Table 3.3 – 3.4**). In particular, the ferritin heavy chain (FRIH / FTH1) was one of the most significant up-regulated protein in the 0.5 μ M (log2 FC = 1.399, P-adjusted = 2.29E-06) and the 2.0 μ M (log2 FC = 1.08, P-adjusted = 3.13E-06) treatments (Tian *et al*, 2020). HMOX1, also a key player in regulation of ferroptosis, was upregulated in 0.5 μ M samuraciclib (log2 FC = 1.38, P-adjusted = 0.0001) conditions, although it did not reach significance in 2.0 μ M samuraciclib (Hassannia *et al.*, 2019; Liao *et al*, 2023). Other key proteins in ferroptosis that were significantly up-regulated, but not specified in tables 3.3-3.4, were lactate dehydrogenase (LDHA; log2 FC = 0.75 and 0.94; P-adjusted = 1.31E-04 and 1.49E-05 for 0.5 μ M and 2 μ M samuraciclib, respectively) (Yang *et al*, 2023) and NCOA4 (log2 FC = 0.71 and -0.30; P-adjusted = 8.95E-06 and 7.49E-04 and for 0.5 μ M and 2 μ M samuraciclib, respectively) (Mancias *et al*, 2014). NCOA4 is a key driver of intracellular ferritin sequestration and degradation and as such is an important regulator of intracellular iron homeostasis.

3.2.3 Western blot confirmation of protein level changes following treatment of breast cancer cells with samuraciclib

To confirm the results of the proteomics analysis, I selected several proteins that were strongly up- or down-regulated by samuraciclib. To further extend the proteomics carried out in MCF7 cells, I also assessed the influence of PI3K and p53 on expression of the selected proteins, as PI3K/Akt/mTOR and p53 signalling are both important for response to samuraciclib (Coombes *et al.*, 2023; Wilson *et al.*, 2023). As MCF7 cells carry the E545K mutation in the PIK3CA gene, I used MCF7 cells in which the E545K mutation has been corrected to WT PIK3CA (a kind gift of Dr J Lauring) (Beaver *et al*, 2013). MCF7 cells in which the p53 gene has been disrupted using CRISPR-Cas9 genome editing has been developed in our lab and has been described previously (Periyasamy *et al*, 2017). PIK3CA-WT and p53-null MCF7 cell lysates were assessed by Western blotting following 0.5 μ M and 2 μ M samuraciclib addition for 24 hours. Immunoblotting for Pol-II and CDK2 phosphorylation, as well as RB phosphorylation, confirmed CDK7 inhibition (**Figure 3.11**). A dose dependent reduction in Pol-II and CDK2 phosphorylation was confirmed by quantification of the results of three independent biological replicate series (**Figure 3.12**), the first of which is shown in **Figure 3.11**. Interestingly, total RB levels also reduced with samuraciclib treatment in the PIK3CA-WT and p53-null MCF7

cells. The reason for this difference from MCF7 cells is unclear and was not further explored. Levels of CDK7, MAT1, and Cyclin H remained unchanged across all treatment conditions and in all cell lines. Also as expected, p53 levels increased in a dose-dependent manner in MCF7 and PIK3CA-WT MCF7 cells and no p53 was observed in the p53-null MCF7 cells.

Immunoblotting for hits other than p53 mostly confirmed expected reductions or increases in protein levels. Levels of UBE2T were decreased by samuraciclib, with the reductions being significant for 0.5 and 2.0 μM treatments in agreement with the proteomics findings (**Figure 3.12B**). In the proteomic analysis, levels of UHRF1, LZTS2, COX16, and CKS2, were observed to be reduced for 2.0 μM and 0.5 μM samuraciclib but did not reach the $\log_2 \text{FC} \leq -1$ cut-off for 0.5 μM samuraciclib. Immunoblotting confirms these findings, except in the case of UHRF1 where levels were significantly reduced for both 0.5 and 2.0 μM samuraciclib (**Figure 3.12B**). In the case of UHRF1, the immunoblotting results showed a stronger FC, which is in fact consistent with the proteome analysis, since $\log_2 \text{FC} = -0.75$ (P-adjusted = $2.54\text{E-}05$) for 0.5 μM samuraciclib. The levels of these proteins were similar in the PIK3CA-WT and p53-null cells and levels were reduced to similar extents with samuraciclib. A notable exception was LZTS2, levels of which were considerably higher in the PIK3CA-WT cells than in the other cell lines, although its protein levels remained sensitive to samuraciclib in these cells.

In the case of up-regulated proteins, levels of ARMT1 and LDHA were apparently increased in all cell lines (**Figure 3.11A**), although quantification of $n = 3$ replicates showed that this increase was not significant for MCF7 cells (**Figure 3.12A**). NDKA (NME1, NM23-H1) was among the most highly upregulated proteins by FC ($\log_2 \text{FC} = 1.59$, P-adjusted = $2.69\text{E-}05$) with 0.5 μM samuraciclib and was also upregulated ($\log_2 \text{FC} = 1.70$, P-adjusted = $6.54\text{E-}06$) for 2.0 μM samuraciclib (**Table 3.3 – 3.4**). In agreement with these results, NDKA levels were increased in the three cell lines in a dose-dependent manner by samuraciclib, the results being statistically significant for 0.5 samuraciclib (**Figure 3.12A**). NME1 (NDKA) has been reported as a p53-regulated gene (Chen *et al*, 2003). NME1 upregulation could therefore be due to p53 activation by samuraciclib treatment. However, NDKA levels were higher in p53-null MCF7 cells than in the isogenic p53-WT MCF7 cells and levels of NDKA increased with samuraciclib, indicating that its upregulation is independent of p53 (**Figure 3.11A**). NME1 has been associated with a caspase-independent cell death pathway activated by granzyme A (Fan *et al*, 2003) and to be a suppressor of metastasis (Jarrett *et al*, 2012; Steeg *et al*, 1988).

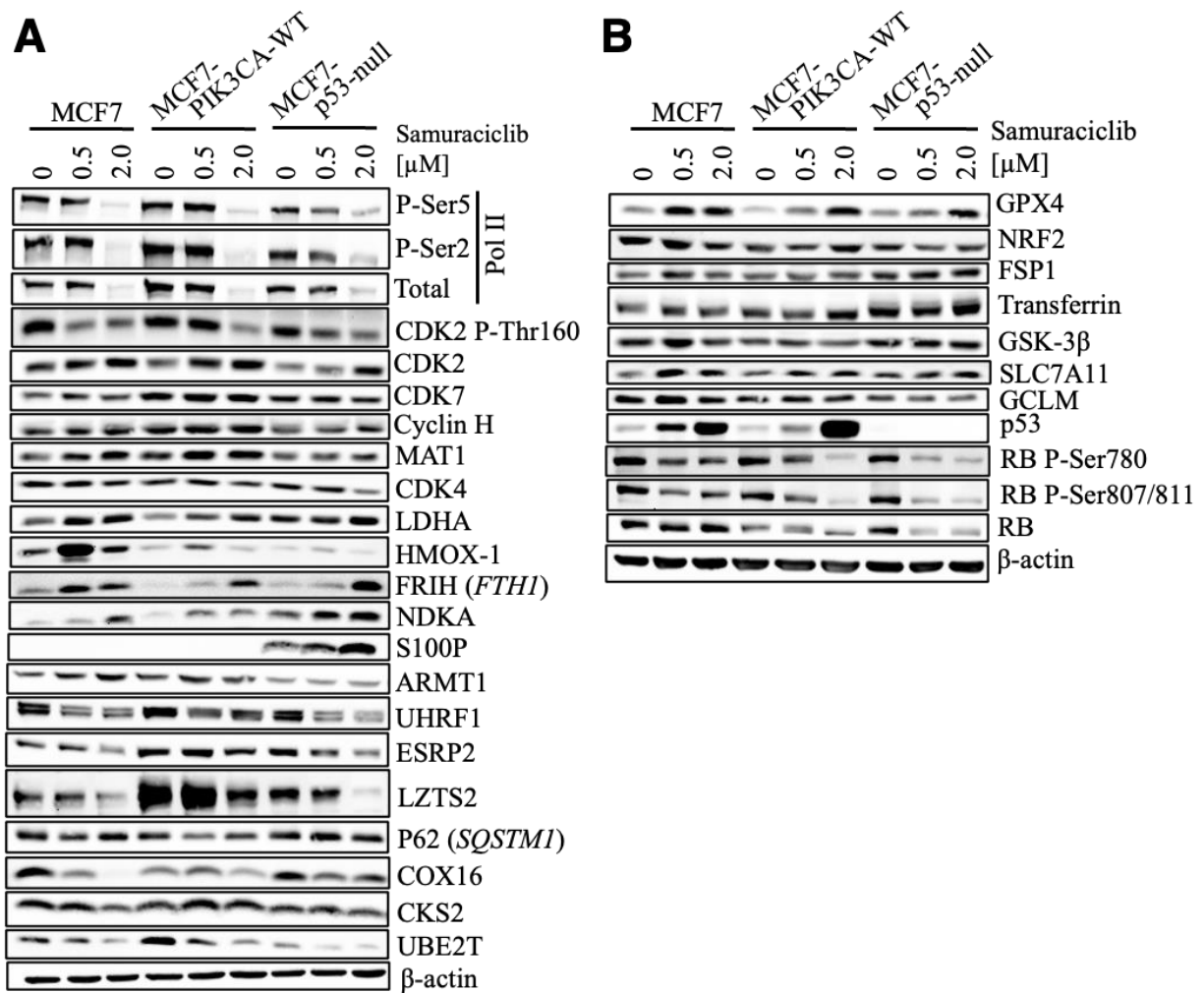


Figure 3.11 Western blotting confirms differential levels of proteins identified from proteomic analysis.

(A) Protein lysates prepared from the indicated cell lines, treated for 24 hours with 0.5 μM or 2 μM of samuraciclib were immunoblotted using antibodies for select proteins identified in the quantitative proteomics analysis. (B) Proteins that have been associated with ferroptosis but were not identified as differentially expressed in the proteomics analysis were assessed using protein lysates generated alongside those in A.

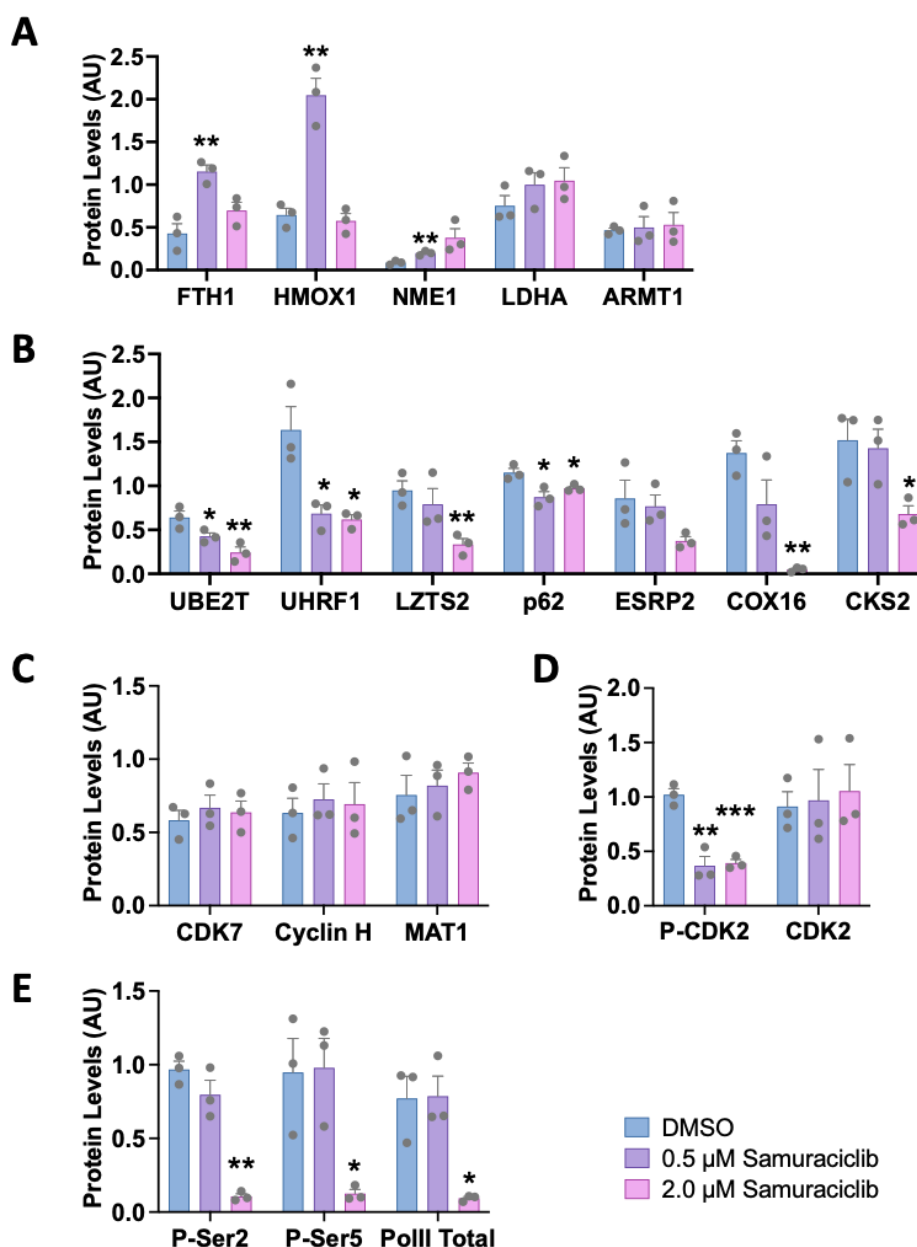


Figure 3.12 Quantitation of Western blotting following treatment with samuraciclib confirms differential expression of proteins identified in quantitative proteome analysis. Protein lysates prepared from MCF7 cells treated with 0.5 μ M or 2 μ M of samuraciclib for 24 hours were immunoblotted. Bars show the mean protein levels from three independent experiments. Filled circles show the quantified expression for each replicate. Images from one of the three experiments are shown in the previous figure. (A – B) Examples of proteins identified as up-regulated (A) and downregulated (B) are shown. (C) Expression levels of CAK complex components CDK7, cyclin H, and MAT1. (D – E) Levels of total or phosphorylated CDK2 and Pol-II are shown. Western blots were quantified using ImageJ. Protein levels are normalized to β -actin. Error bars represent the SEM. P-values represent significance of differential expression compared to the DMSO control (Welch's t-test; * $P < 0.05$, ** $P < 0.01$).

Immunoblotting also confirmed the increased levels of proteins linked with ferroptosis. Levels of FRIH (FTH1) and HMOX1 were increased with both concentrations of samuraciclib, although the results were only significant for 0.5 μ M samuraciclib (**Figure 3.11A, 3.12A**). LDHA levels were also increased with samuraciclib treatment, although as stated above, the increase was not significant (**Figure 3.12A**). I also assessed expression of other ferroptosis proteins that were not detected in the mass spectrometry analysis. The key proteins involved in the anti-ferroptosis response, GPX4 (Forcina & Dixon, 2019), and to a lesser extent NRF2 and FSP1 (Anandhan *et al.*, 2023; Doll *et al.*, 2019), showed increased levels after samuraciclib treatment across all cell lines. Additionally, immunoblotting for other ferroptosis mediators (transferrin, GSK-3 β , SLC7A11, or GCLM) did not evidence altered levels.

Taken together, the immunoblotting data confirm many of the strong up- and down-regulated proteins identified in the quantitative proteomic analysis of MCF7 cells treated with samuraciclib. The most interesting hits related to proteins implicated in ferroptosis, specifically the ferritin heavy chain FRIH (FTH1) and HMOX1.

3.2.4 Cancer and ferroptosis pathways are significantly enriched in the 24-hour proteomic data

I performed an integrative pathway enrichment analysis using DEQMS data from the 0.5 μ M and 2 μ M samuraciclib conditions to identify dysregulated pathways and pathway themes from both treatment conditions (**Figure 3.13 and Figure 3.14, Supplementary tables 1 – 3**). I used the pathway enrichment software ActivePathways in R to enrich for pathways from the gene sets of Reactome, gene ontology biological processes (GOBP), and Hallmarks (Paczkowska *et al.*, 2020; Subramanian *et al.*, 2005). Many pathways were enriched in both 0.5 μ M and 2 μ M samuraciclib while some pathways were identified only when combining P-values from both conditions (*i.e.*, pathways with the “combined” label). The top ten most significantly enriched pathways from the Reactome and GOBP gene sets were enriched in both 0.5 μ M and 2 μ M conditions, and 9/10 pathways from the Hallmarks gene set were enriched in both 0.5 μ M and 2 μ M conditions (**Figure 3.13, Supplementary tables 1 – 3**). The top enriched GOBP pathways all related to RNA processing, as did several of the Reactome pathways, which may reflect the transcriptional role of CDK7. The top Hallmarks pathways are enriched in pathways associated with cell proliferation, including MYC targets, E2F targets and G2/M. Among highly enriched Hallmark pathways is MTORC1 signalling, which is consistent with the CRISPR/Cas9 screen data and the in vitro studies described earlier.

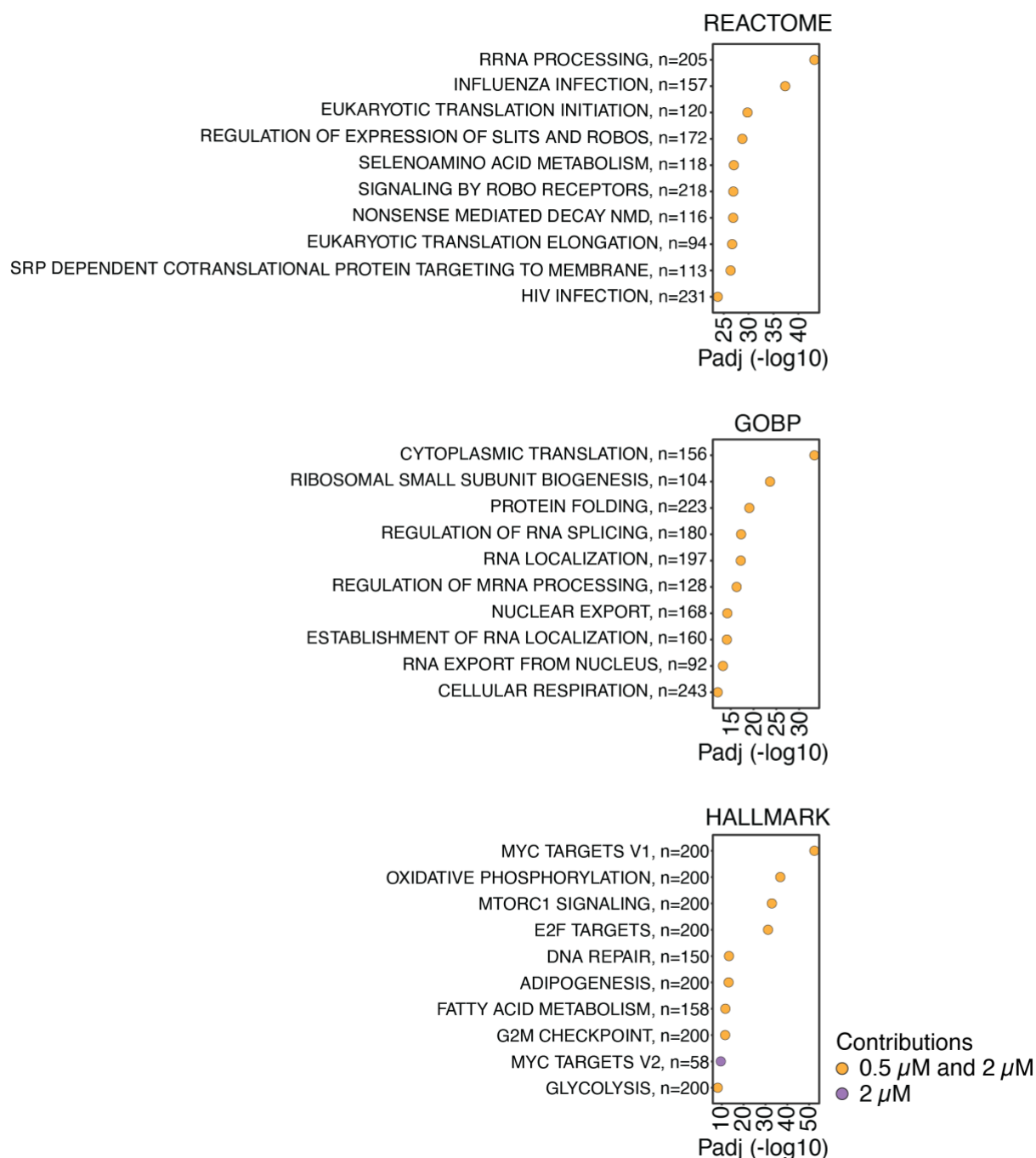


Figure 3.13 Top enriched pathways from proteomics data for 24-hour samuraciclib treatment of MCF7 cells.

Shown are the Holm family-wise error rate-adjusted P-values and data sources of pathway enrichment for all significantly enriched pathways. Differentially expressed gene P-values from quantitative proteomics analysis of MCF7 cells following 0.5 μ M or 2 μ M samuraciclib treatment for 24 hours were merged using the ActivePathways method of gene set enrichment analysis. Gene Ontology Biological Processes (GOBP), Reactome, and Hallmark gene sets were used in the enrichment.

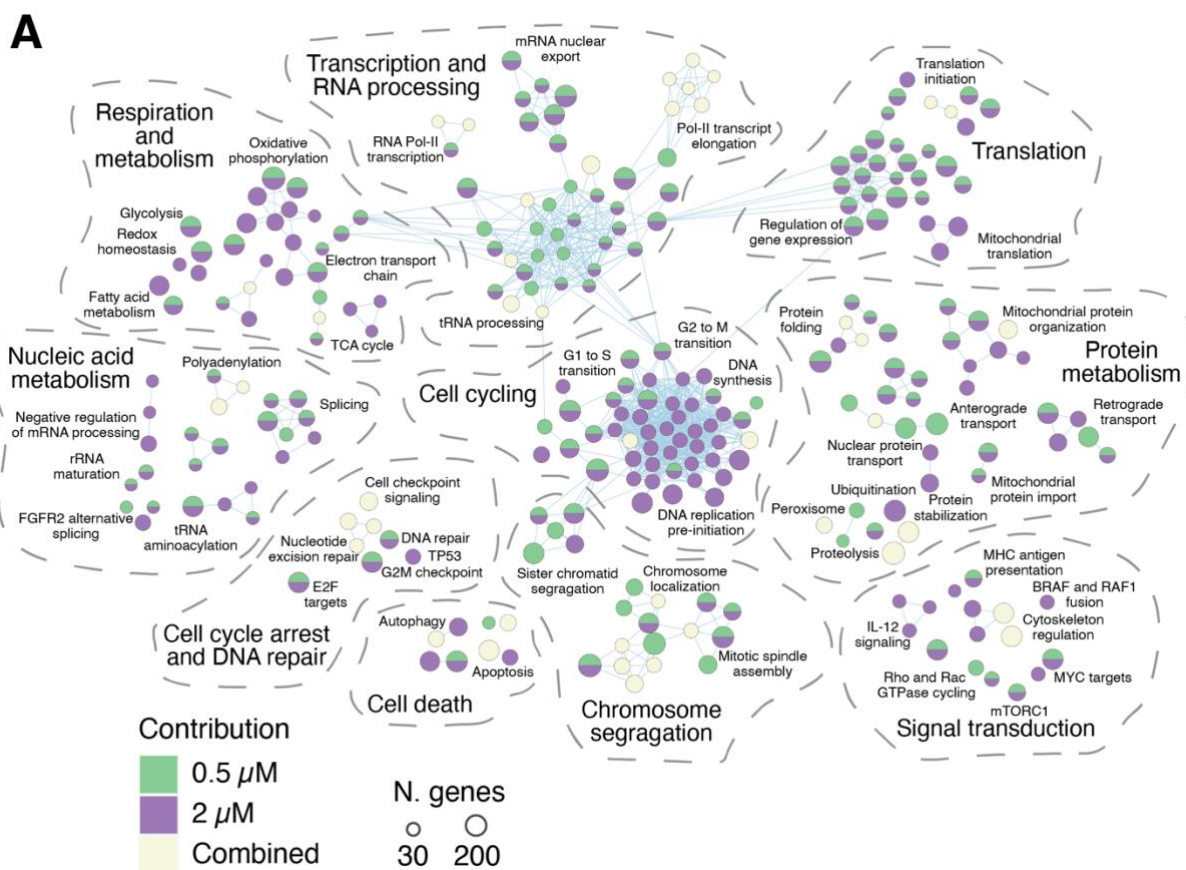


Figure 3.14 Pathway enrichment analysis of differentially expressed proteins following samuraciclib treatment of MCF7 cells.

Integrative pathway enrichment map of significantly altered pathways. P-values from differential proteins identified in a quantitative proteomics analysis of the samuraciclib-treated MCF7 cells for 24 hours were merged using the ActivePathways method of gene set enrichment analysis. The gene sets of Gene Ontology Biological Processes (GOBP), Reactome, and Hallmarks were used in the enrichment analysis. Node size reflects the number of genes comprising that gene set. Gene sets with at least 60% of genes overlapping are connected by edges. Node colours represent the data source that enriched for that pathway. The “combined” contribution indicates that the pathway is enriched only when P-values from both data sources are combined.

Next, I visualized the enriched pathways by creating an enrichment map and labelling groups of pathways based on their biological themes (**Figure 3.14**). First, I found several dysregulated pathway themes relating to cell growth and division such as cell cycling, chromosome segregation, and nucleic acid metabolism. The themes of cell death and cell cycle arrest revealed possible mechanisms of cell death for samuraciclib. Interestingly, several pathway themes were enriched that relate to mechanisms of ferroptosis. For example, Hallmarks oxidative phosphorylation ($P = 1.65\text{E-}37$), Hallmarks fatty acid metabolism ($P = 2.20\text{E-}12$), and multiple mitochondrial pathways such as GOBP apoptotic mitochondrial changes ($P = 6.00\text{E-}03$) were enriched in both 0.5 μM and 2 μM conditions and may be related to the mitochondrial functions important for ferroptosis (Gao *et al*, 2019). Similarly, many programmed cell death pathways were enriched in both conditions such as Reactome programmed cell death ($P = 2.34\text{E-}08$) and GOBP regulation of intrinsic apoptotic signalling ($P = 1.66\text{E-}03$). Collectively, these results suggest that many pathways associated with cancer processes and regulated cell death, specifically suggesting a theme of ferroptosis, are enriched from proteomics data following samuraciclib treatment for 24 hours.

3.2.5 Multi-omic analysis of samuraciclib treatment enriches the same pathways and biological themes as the 24-hour proteomic data

I sought to identify consistent signals of mTOR pathway and ferroptosis gene activity across different BC cell datasets following samuraciclib treatment. To do this, I merged RNA-sequencing and 24-hour proteomics data following samuraciclib treatment (**Figure 3.15**). First, I independently analyzed the RNA-sequencing data from MCF7 cells treated with 0.5 μM samuraciclib or equal volumes of DMSO for six hours in four replicates. I performed differential gene expression analysis using EdgeR to identify significantly differentially expressed genes after treatment, compared to the DMSO control. 3,819/12,358 genes (31% of all protein-coding genes detected) were significantly differentially expressed in the RNA-sequencing dataset (**Figure 3.15A**). In the RNA-sequencing dataset, I found several significantly differentially expressed genes known to be highly sensitive to CDK7 inhibition (Van Nguyen *et al*, unpublished), including *KDM8*, *ARID2*, *ZYMND8*, *MEPCE*, and *HEXIM1*, which play an important role in transcription (Cao *et al*, 2021; Hsia *et al*, 2010; Jiang *et al*, 2020; Lee *et al*, 2020; Michels & Bensaude, 2018). Further, I found several genes associated with ferroptosis, such as *HMOX1*, *FTH1*, and *NCOA4* (Anandhan *et al*, 2023; Hou *et al*, 2016; Tang *et al*, 2021a; Tian *et al*, 2020).

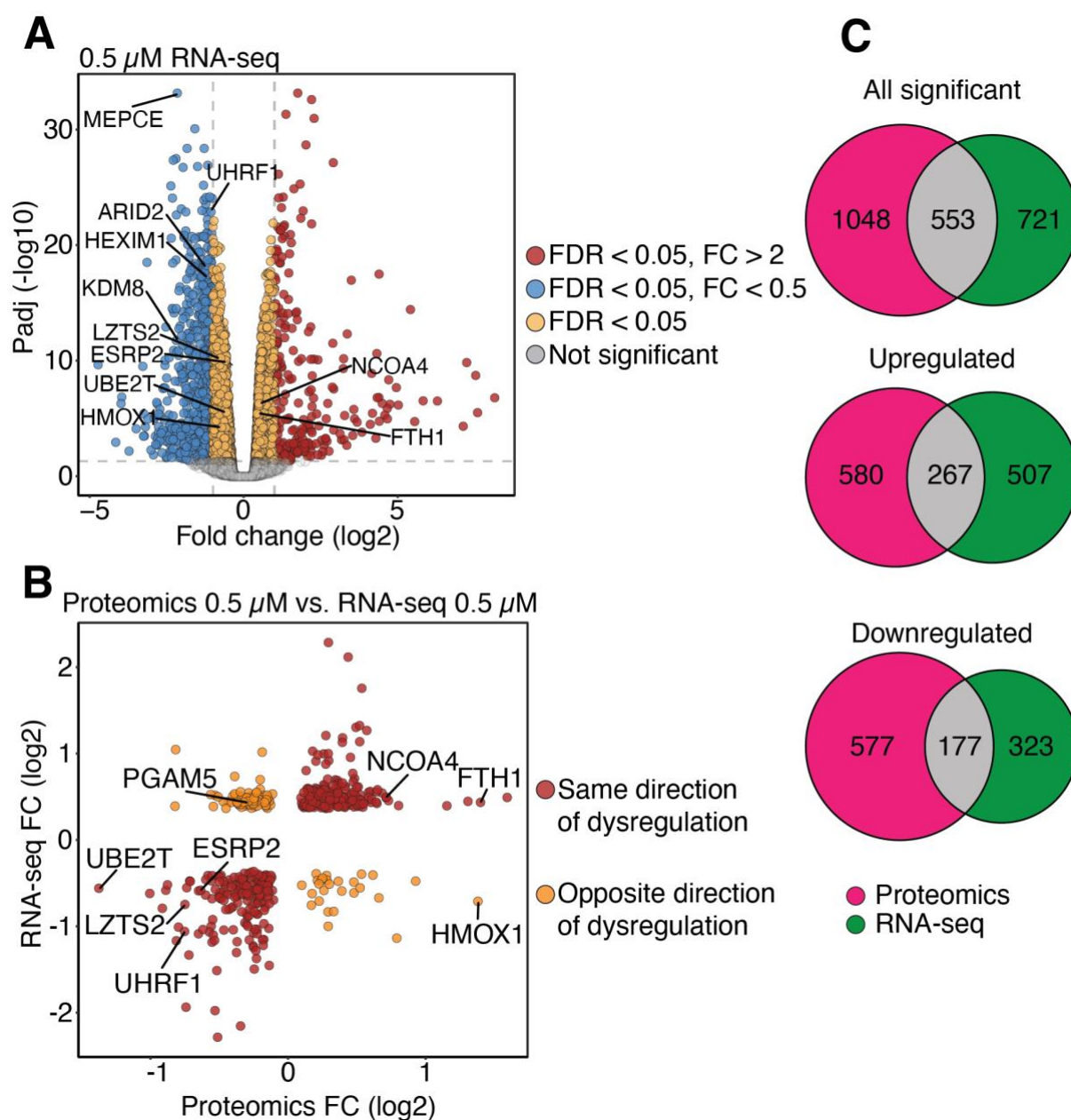


Figure 3.15 Differential gene expression analysis of quantitative proteomic data and whole transcriptome RNA-sequencing data following samuraciclib treatment.

(A) Volcano plot of differential gene expression analysis from RNA-sequencing data following 6-hour treatment of MCF7 cells with 0.5 μ M samuraciclib. Gene counts for four replicates of DMSO and four replicates of samuraciclib-treated cells were compared using differential gene expression analysis in EdgeR by requiring a minimum average count of one across all conditions and a minimum fold-change of 1.5x. (B) Genes significantly associated with samuraciclib treatment in MCF7 cells identified from quantitative proteomics (0.5 μ M samuraciclib for 24 hours) compared to RNA-sequencing. FC (log2) of significantly differentially expressed genes from both datasets are shown. (C) Venn diagrams of commonly differentially enriched genes between proteomics and RNA-sequencing. Shown are the numbers of differentially enriched genes from each data source (P-adjusted < 0.05, no FC cut-off).

Presumably, changes in protein levels following samuraciclib treatment are largely correlated with changes at the transcriptome (RNA-seq) level. Thus, I investigated commonly significantly differentially enriched genes between the proteomics data and the RNA-sequencing data for MCF7 cells treated with samuraciclib. I visualized significantly differentially expressed proteins and genes (P-adjusted < 0.05) commonly identified in both the proteomic and RNA-seq datasets (**Figure 3.15B**). I found 553 genes significantly differentially expressed in common between the proteomics and RNA-sequencing data with 444 genes (80% of all significant genes in common) showing the same FC direction in both datasets (**Figure 3.15C**). Many genes that were significantly associated with mTOR or ferroptosis activity were significant and showed the same FC direction in both the proteomics and RNA-sequencing dataset. The mTOR proteins UBE2T, UHRF1, and LZTS2 were all significantly downregulated in both data sets while the ferroptosis proteins FTH1 and NCOA4 were significantly upregulated. POLR2A was significantly downregulated, albeit only slightly, in the proteomics data and was upregulated in the RNA-seq data. Similarly, HMOX1 was significantly downregulated in the RNA-seq data and upregulated in the proteomics data. Overall, these results indicate that there is a largely consistent signal of gene and protein expression dysregulation across the proteomics and RNA-seq data in MCF7 cells following samuraciclib treatment.

Next, I looked at significantly enriched pathways from a combined proteomics and RNA-seq dataset. The RNA-seq data measured a more immediate cellular responses to samuraciclib treatment as it measured the transcriptomic response after six hours of treatment. Proteomics data measured the response after a longer period (24 hours) and at the proteomic level, which, according to the central dogma, is presumably slower to respond than the transcriptomic level. Consequently, I hypothesized that the pathways enriched in both the proteomics and RNA-seq datasets would be high confidence processes associated with the response to samuraciclib, and not just cellular responses to stress or artefacts of the specific dataset. Thus, to profile the molecular pathways commonly disrupted in response to samuraciclib treatment in the short- and longer-term, I used ActivePathways to perform an integrative pathway enrichment on GOBP, Reactome, and Hallmarks gene sets using the P-values from both the proteomics and RNA-seq datasets combined.

This multi-omic pathway enrichment resulted in 230 significantly enriched pathways, which were visualized in an enrichment map (**Figure 3.16, Supplementary tables 4 – 6**). Most

pathways were enriched from the RNA-seq data (202 / 230 pathways, or 88%), while fewer pathways were enriched in the proteomics data (152 / 230, or 66%). Thus, most pathways (124 / 230, or 54%) were enriched in both datasets independently or by combining P-values from both datasets. This indicates that the RNA-seq data detected more significantly disrupted genes than the proteomics data, which is likely due to a great number and depth of genes profiled in transcriptomics dataset than proteomics data. Importantly, the same general pathway themes were enriched across both datasets as in the proteomics dataset alone, such as cell cycling, transcription and RNA processing, respiration and metabolism, and cell death and cell cycle arrest. This indicates that the biological themes dysregulated by samuraciclib treatment are detected at both the transcriptomic and proteomic levels.

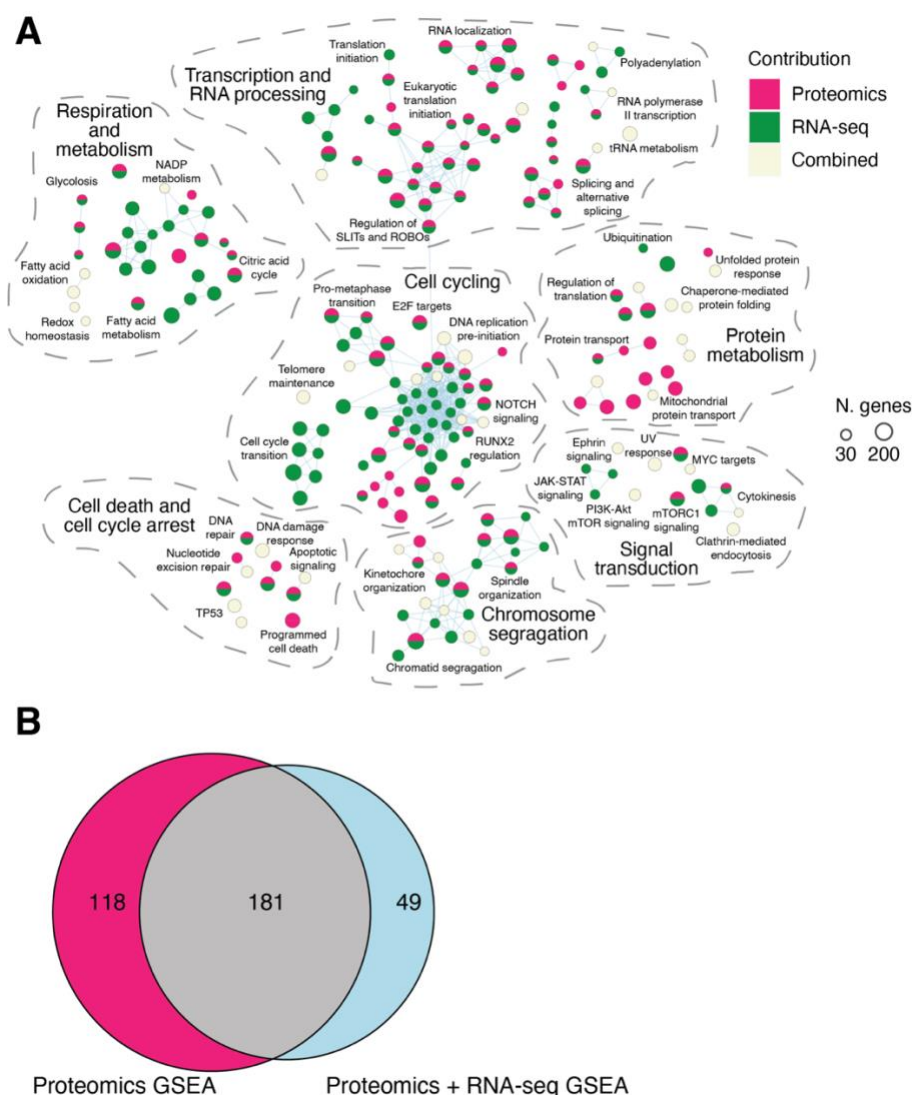


Figure 3.16 Pathway enrichment analysis from differential expression analysis of quantitative proteomics and RNA-sequencing following samuraciclib treatment of MCF7 cells.

(A) Integrative pathway enrichment map of significantly dysregulated pathways. P-values from differentially expressed proteins identified in quantitative proteomics and RNA-sequencing analyses of MCF7 cells following 0.5 μ M samuraciclib treatment for 24 hours or 6 hours, respectively, were merged using the ActivePathways method of gene set enrichment analysis. The gene sets of Gene Ontology Biological Processes (GOBP), Reactome, and Hallmarks were used in the enrichment analysis. Node size reflects the number of genes comprising that gene set. Gene sets 60% genes shared are connected by edges. **(B)** Venn diagram of the enriched pathways found with proteomics data alone versus proteomics and RNA-sequencing data combined. Circle size reflects total number of significantly enriched pathways.

Notably, in both the proteomics and RNA-seq datasets, Hallmarks mTORC1 was enriched ($P = 4.13\text{E-}15$; **Figure 3.17**). Similarly, ferroptosis-related pathway themes were enriched in the merged pathway enrichment, including Hallmarks oxidative phosphorylation ($P = 9.45\text{E-}35$), Hallmarks fatty acid metabolism ($P = 4.45\text{E-}13$), Reactome programmed cell death ($P = 1.37\text{E-}03$) and GOBP regulation of intrinsic apoptotic signalling ($P = 1.93\text{E-}05$) (**Figure 3.17**, **Supplementary tables 4 – 6**). 181 pathways that were enriched from the proteomics dataset were also identified in the combined dataset (**Figure 3.16B**). When looking at the top ten most significantly enriched pathways across the gene sets of GOBP, Reactome, and Hallmarks, 25 / 30 of these pathways were enriched in both treatment conditions (**Figure 3.17**). Collectively, these results indicate that the cellular response to samuraciclib treatment is detectable across proteomics and RNA-seq datasets, and that mTOR and ferroptosis is implicated in this response.

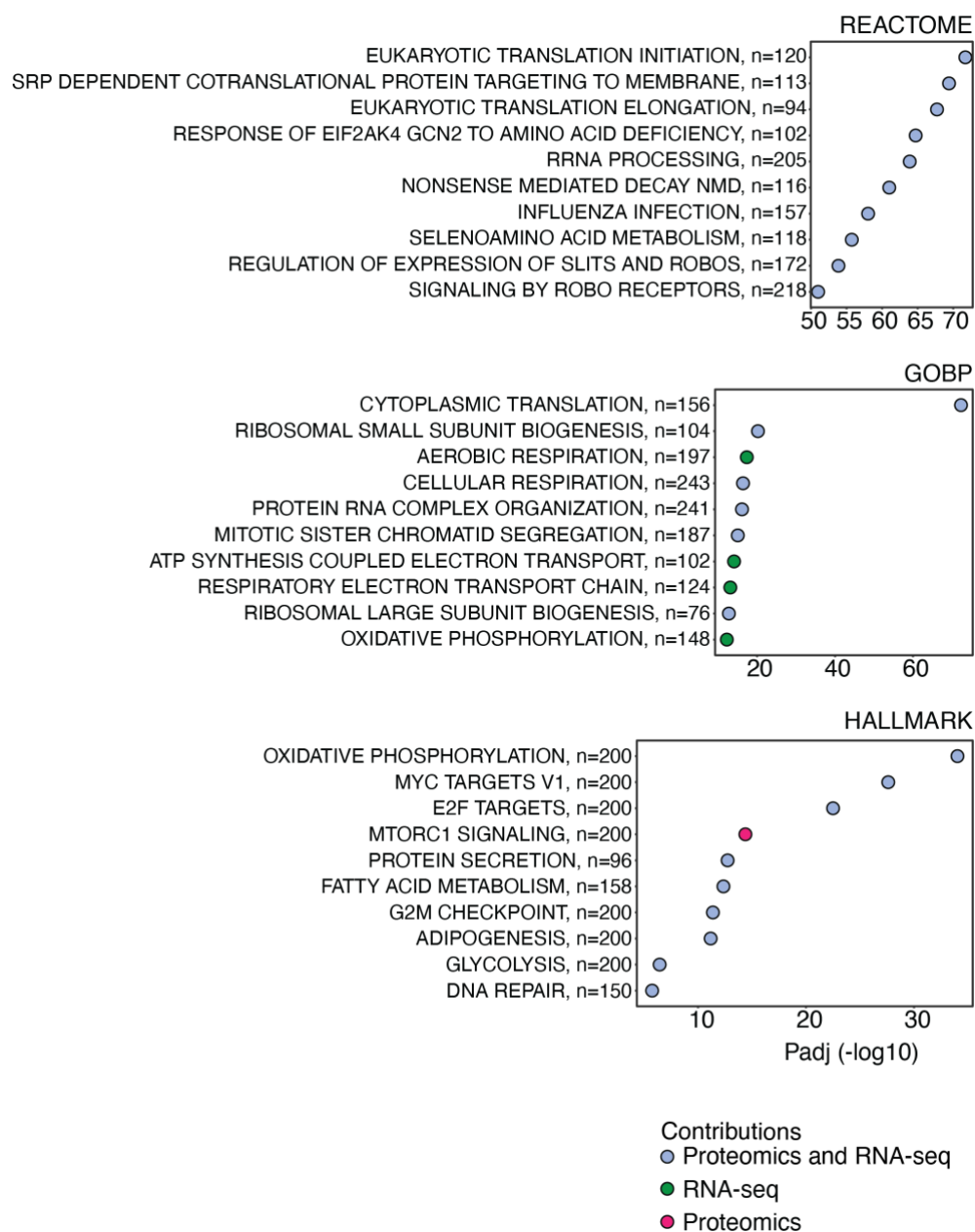


Figure 3.17 Top enriched pathways from proteomics and RNA-seq screens following samuraciclib treatment.

Shown are the Holm family-wise error rate-adjusted P-values and data sources of pathway enrichment for all significantly enriched pathways. Differentially expressed gene P-values from quantitative proteomics and RNA-sequencing of MCF7 cells following 24-hour or 6-hour 0.5 μ M samuraciclib treatment were merged using the ActivePathways method of pathway enrichment. Gene sets from Gene Ontology Biological Processes (GOBP), Reactome, and Hallmarks were used for the enrichment. Shown are adjusted P-values and enrichment data sources for all significant pathways.

3.4 Ferroptosis activation in the response to samuraciclib

3.4.1 Ferroptosis is dysregulated following samuraciclib treatment

There was evidence of ferroptosis processes being altered following samuraciclib treatment across multi-omics datasets. The CRISPR-Cas9 screen had identified gRNAs targeting a ferroptosis gene as significantly differentially enriched following samuraciclib treatment (**Figure 3.1**). Namely, *GPX4*, which encodes a central ferroptosis suppressor and the dysregulation of which is a hallmark of ferroptosis, was significantly downregulated following 150 nM and 500 nM samuraciclib treatment in MCF7 and NALM-6 cell lines, respectively (Forcina & Dixon, 2019; Ingold *et al.*, 2018). Quantitative proteomics analysis identified several ferroptosis proteins as differentially expressed in samuraciclib-treated MCF7 cells including the proteins FRIH (*FTH1*), HMOX1, and LDHA (**Figure 3.10**) (Anandhan *et al.*, 2023; Hou *et al.*, 2016; Tang *et al.*, 2021a; Tian *et al.*, 2020). There were many differentially expressed ferroptosis genes in RNA-sequencing data previously generated in our lab, including *FTH1* and *HMOX1* (**Figure 3.15**). The dysregulation of ferroptosis processes was also apparent at the molecular pathway level. Pathway enrichment analysis revealed pathways associated with ferroptosis processes, including oxidative phosphorylation, fatty acid metabolism, and programmed cell death, which were enriched across the different datasets (**Figure 3.14**). These results collectively indicate that ferroptosis processes may be dysregulated following samuraciclib treatment.

For a more detailed understanding of what ferroptosis processes are dysregulated, another pathway enrichment was performed using the 24-hour samuraciclib proteomic data (0.5 μ M and 2 μ M; **Figure 3.18**). The GOBP list of gene sets describes a single ferroptosis pathway labelled “Ferroptosis” that includes five genes: *AIFM2*, *GPX4*, *SLC39A7*, *SLC7A11*, and *TMEM164*. The 24-hour proteomics analysis detected three of these genes: *AIFM2*, a ferroptosis suppressor (Doll *et al.*, 2019), *SLC39A7*, a zinc transporter that is essential for ferroptosis in BC and renal cancer cells (Chen *et al.*, 2021a), and *SLC7A11*, the System Xc⁻ cystine / glutamate antiporter (Shih *et al.*, 2006). Each of these three genes were significantly differentially expressed in at least one 24-hour proteome samuraciclib treatment condition. However, pathway enrichment analysis would require gene sets with P-values for more than five different genes to test if a gene set is statistically significantly enriched (Paczkowska *et al.*, 2020). As a result, I analyzed the ferroptosis processes and gene lists defined in the FerrDB database (Zhou *et al.*, 2023b), which included the gene sets of ferroptosis drivers, suppressors,

markers, and unclassified gene lists. Of note, some genes in FerrDb appear in more than one ferroptosis gene list because some genes function as both ferroptosis drivers and suppressors, depending on the context. For example, *HMOX1* exhibits both ferroptosis driver and ferroptosis suppressor effects (Chiang *et al*, 2018; Kwon *et al*, 2015; Liao *et al.*, 2023). To understand which ferroptosis processes are consistently dysregulated following samuraciclib treatment, I performed a pathway enrichment using ActivePathways with the P-values from the 0.5 μ M and 2 μ M samuraciclib conditions (Paczkowska *et al.*, 2020).

The ActivePathways pathway enrichment method first merges P-values from both the 0.5 μ M and 2 μ M treatment conditions such that a single P-value exists for each gene. Pathways are enriched by determining if more genes associated with a specific pathway are significant ($P < 0.05$) than expected by chance. Once a pathway is significantly enriched, the ActivePathways package then determines if the P-values from either treatment condition (0.5 μ M or 2 μ M) were sufficient to enrich the pathway, or if the pathway could only be enriched using the merged P-values. The gene sets used in this analysis included all GOBP, Reactome, and Hallmarks gene sets in addition to the four gene sets defined in the FerrDB. Pathway enrichment analysis found that ferroptosis drivers ($P\text{-adjusted} = 3.2\text{E-}03$) and ferroptosis suppressors ($P\text{-adjusted} = 1.39\text{E-}08$) were significantly enriched from differentially expressed genes in both samuraciclib conditions (**Figure 3.18A**). Ferroptosis drivers were enriched using merged P-values from both 0.5 μ M and 2 μ M samuraciclib conditions combined, while ferroptosis suppressors were enriched independently using P-values from each treatment condition.

The ferroptosis drivers and suppressors gene lists were visualized to check for consistency in the direction of differential expression across proteomics and RNA-sequencing (RNA-seq) datasets (**Figure 3.18A**). Comparing other datasets allows us to expand our understanding of how differentially expressed proteins correlate with their respective genes (RNA-seq). Thus, I looked at all genes that were significantly differentially expressed in at least one data source (*i.e.*, 0.5 μ M or 2 μ M proteomics), which consisted of 60 ferroptosis driver genes and 66 ferroptosis suppressor genes. There were 117 unique, significantly differentially expressed genes, and there were 9 genes defined as both a ferroptosis driver and suppressor.

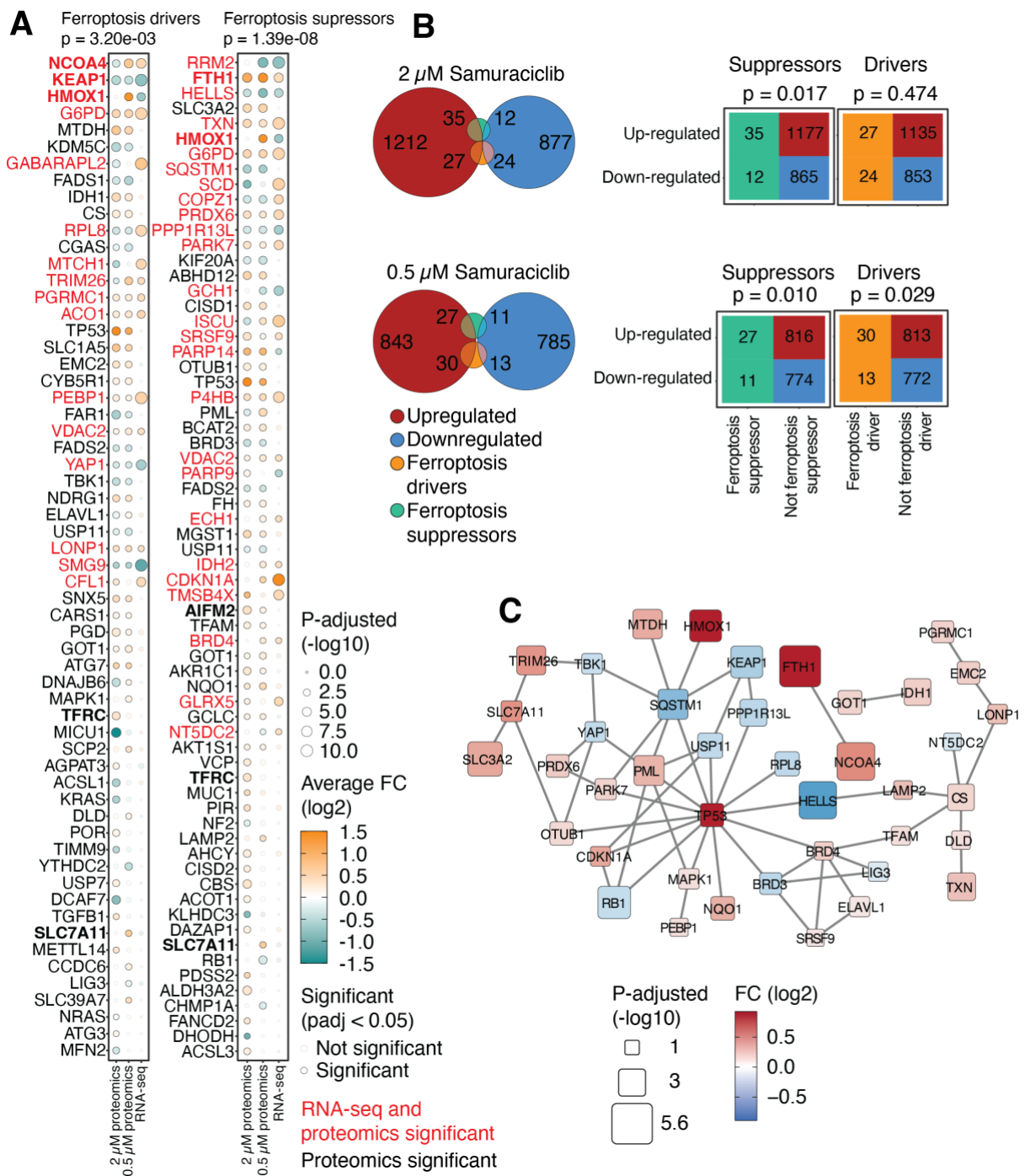


Figure 3.18 Ferroptosis pathways and proteins are dysregulated during samuraciclib treatment.

(A) The gene sets of ferroptosis suppressors and ferroptosis drivers were the two ferroptosis gene sets enriched from quantitative proteomics data using the ActivePathways method of pathway enrichment. P-values from pathway enrichment were calculated from differential expression of proteins using quantitative mass spectrometry of MCF7 cells following 24 hours of treatment with 0.5 μ M or 2 μ M samuraciclib compared to DMSO controls. Shown are P-values and FC from 0.5 μ M and 2 μ M proteomics and 0.5 μ M RNA-sequencing (6-hour samuraciclib treatment). Proteins in bold are well-established ferroptosis mediators. **(B)** Venn diagrams show the direction of differential protein expression for ferroptosis drivers and suppressors following samuraciclib treatment of MCF7 cells. Shown are the number of significantly differentially expressed genes ($P < 0.05$, no FC cut-off, DEQMS) and their direction in each condition of samuraciclib treatment. **(C)** Kinase-substrate interaction network of ferroptosis drivers and suppressors. Shown are proteins that are significantly differentially expressed in at least one of the two samuraciclib treatment conditions. The combined P-value and average FC between both conditions is shown (Fisher's method). Kinase-substrate interactions were acquired from the BioGrid database for high-confidence human kinase-substrate interactions (BIOGRID-ORGANISM-Homo_sapiens-4.4.228.mitab.txt) and visualized in Cytoscape.

Generally, ferroptosis genes in the proteomics and RNA-sequencing datasets were consistently dysregulated in the same direction. For example, the ferroptosis driver *FTH1* was significantly upregulated in both 24-hour proteomics data (P-adjusted = 2.98E-08 and 4.90E-08, log2FC = 1.40 and 1.08 for 0.5 μ M and 2 μ M, respectively) and the RNA-sequencing data (P-adjusted = 3.89E-6, log2FC = 0.435). Similarly, the ferroptosis suppressor *KEAPI* was significantly downregulated in both 24-hour proteomics data (P-adjusted = 1.07E-04 and 1.00E-05, log2FC = -0.416 and -0.528 for 0.5 μ M and 2 μ M, respectively) and the RNA-sequencing data (P-adjusted = 4.80E-16, log2FC = -0.790). Overall, 30 significantly differentially expressed genes had directional agreement in at least one of the proteomics treatment conditions and the RNA-sequencing datasets, and only 9 genes had directional disagreement between the proteomics and RNA-sequencing datasets.

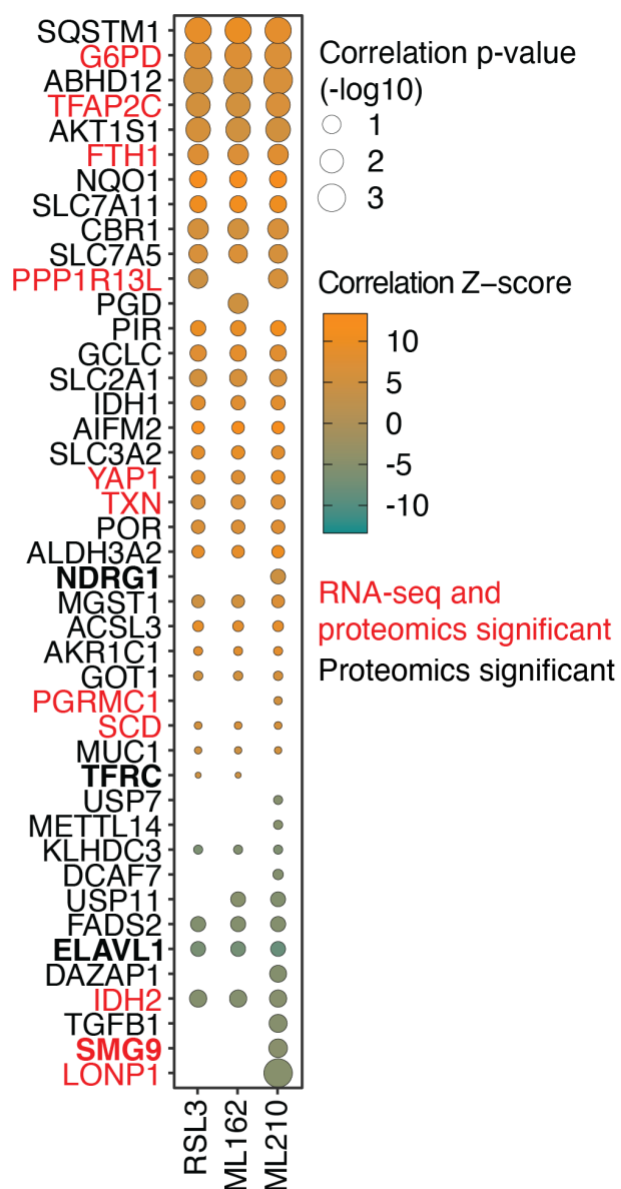
Ferroptosis drivers and ferroptosis suppressors are expected to have opposing mechanisms on cells with driver genes promoting ferroptosis and suppressor genes preventing ferroptosis. Thus, the direction of ferroptosis driver and suppressor gene differential expression in the 24-hour proteomics dataset may reveal whether ferroptosis is upregulated or downregulated following samuraciclib treatment (**Figure 3.18B**). Significantly differentially expressed genes (P-adjusted < 0.05) from the 24-hour 0.5 μ M and 2 μ M samuraciclib treatment were classified as upregulated or downregulated depending on their log2FC. There were 129 unique genes from the FerrDb database that were significantly enriched in the 24-hour proteomics dataset, hereafter referred to as the “prioritized ferroptosis genes”. This consisted of 60 ferroptosis driver genes, 66 suppressor genes, 2 marker genes, and 22 unclassified genes. I then compared the ratio of significantly upregulated ferroptosis drivers to significantly downregulated drivers in the 0.5 μ M or 2 μ M 24-hour proteomics treatment condition, using Fisher’s exact test (Fisher, 1934). Specifically, I asked if there are more ferroptosis driver genes upregulated than expected by chance, while accounting for the fact that there are more genes upregulated than downregulated across the measured proteome. I then did the same with ferroptosis suppressor genes from FerrDb. Ferroptosis suppressor genes were significantly upregulated in both 2 μ M (P = 0.017) and 0.5 μ M (P = 0.010) samuraciclib conditions. Ferroptosis driver genes were significantly upregulated only in the 0.5 μ M condition (P = 0.029). This result shows that ferroptosis suppressors proteins are upregulated in response to both samuraciclib treatment conditions and ferroptosis driver proteins are upregulated in 0.5 μ M samuraciclib treatment after 24 hours.

The kinase-substrate interactions between ferroptosis drivers and suppressors that were significantly differentially expressed in the 24-hour proteomics dataset were visualized (**Figure 3.18C**). High-confidence kinase-substrate interaction data from the BioGRID database was downloaded, which contains manually-curated kinase-substrate interactions in the human proteome (Oughtred *et al.*, 2021). The 60 ferroptosis driver protein and 66 suppressor proteins that were differentially expressed in the 24-hour proteomics dataset were narrowed down to proteins that had known kinase-substrate interactions with other drivers or suppressors. In total, there were 56 interactions between the 41 ferroptosis proteins. 12 ferroptosis proteins were downregulated in the 24-hour proteomics dataset, while the rest were upregulated. Collectively, these results demonstrate on the intricate interactions across ferroptosis proteins that were dysregulated in the proteomics data.

Knowing that samuraciclib treatment dysregulates ferroptosis, I asked whether samuraciclib treatment is associated with genes whose basal mRNA expression levels correlate with GPX4 inhibitor (GPX4i) sensitivity (**Figure 3.19A**). The significantly differentially expressed ferroptosis proteins from both treatment conditions of my proteomics analysis (24 hours, 0.5 μ M and 2 μ M samuraciclib), as defined in the FerrDb database, were considered the prioritized gene set. The prioritized gene set consisted of a unique set of genes associated with any ferroptosis process: drivers, suppressors, markers, and unclassified. 126 / 129 (98%) genes of this prioritized gene set from the 24-hour proteomics analysis had a corresponding gene that was detected in sufficient quantities in the 6-hour, 0.5 μ M samuraciclib treatment RNA-sequencing data. 96 / 126 (76%) of genes commonly detected between the proteomics and RNA-sequencing data had the same direction of differential expression. The prioritized gene set was subset to genes whose basal mRNA expression levels were significantly correlated to GPX4i sensitivity across >600 cancer cell lines, as defined in the dataset downloaded from the Cancer Therapeutics Response Portal (CTRP) (Aksoy *et al.*, 2017). The CTRP dataset consisted of correlation Z-scores and P-values for genes whose basal mRNA expression levels were significantly correlated with GPX4i sensitivity. This analysis was based on a similar analysis that was previously described in Rodencal *et al.* (Rodencal *et al.*, 2023). Genes whose mRNA expression correlated with sensitivity to at least one of the three GPX4i's (*i.e.*, ML210, RSL3, and ML162) were considered (Eaton *et al.*, 2020; Karaj *et al.*, 2022; Wang *et al.*, 2023). A positive Z-score means that low expression of the gene is associated with GPX4i sensitivity while a negative Z-score means that high expression of the gene is associated with GPX4i sensitivity. 43 / 129 (33.3%) prioritized genes from 24-hour 0.5 μ M or 2 μ M samuraciclib

treatment conditions were correlated at the transcriptomic level with sensitivity to at least one GPX4i across the >600 cell lines. 31 / 43 (72%) of the genes significantly correlated with sensitivity were positively correlated with GPX4i treatment, while 12 genes were negatively correlated with treatment across the cell lines. Lastly, I confirmed the consistent differential expression of these genes between the proteomics and RNA-seq datasets and found, as expected, 80% of the genes had consistent directional agreement between proteomics and RNA-seq (**Figure 3.19B**). Overall, this suggests that a relatively small fraction of proteins dysregulated following samuraciclib treatment have basal mRNA levels significantly correlated with GPX4i sensitivity.

A



B

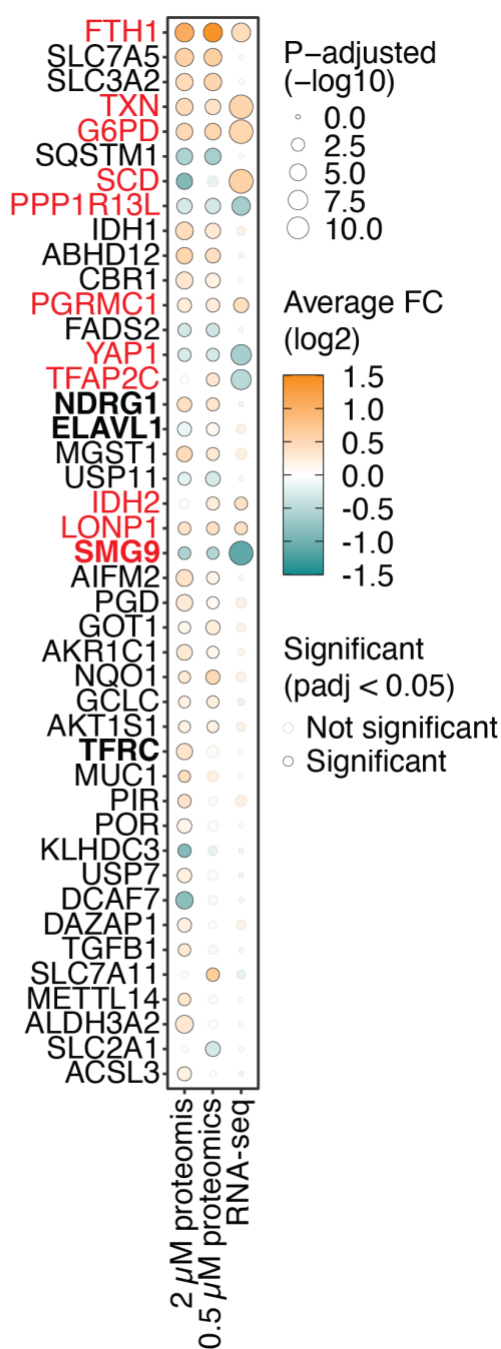


Figure 3.19 Ferroptosis pathway proteins differentially expressed during samuraciclib treatment are correlated with GPX-inhibitor (GPX4i) sensitivity.

(A) Proteins associated with ferroptosis that are significantly differentially expressed in MCF7 cells during samuraciclib treatment are significantly correlated with multiple GPX4i sensitivity screens. Ferroptosis-associated proteins that were differentially expressed in MCF7 cells following samuraciclib treatment for 24 hours (0.5 μ M and 2 μ M) were examined in the cancer therapeutics response portal (v2.1, accessed 15 December 2023) for correlations with GPX4 inhibitor sensitivity across >600 cancer cell lines. Shown are the Pearson correlation P-values and correlation Z-scores across significantly correlated ferroptosis-associated proteins. A positive correlation Z-score indicates low expression of the gene is associated with sensitivity to the GPX4i, while a negative Z-score means that high expression of the gene is associated with GPX4i sensitivity. Proteins in bold are high-confidence CDK7 targets, as defined by Rimel *et al* (Rimel *et al*, 2020). **(B)** Significantly differentially expressed ferroptosis-associated proteins from (A) that correlate with GPX4 inhibitor sensitivity. Shown are P-values and FC from 0.5 μ M and 2 μ M proteomics (24-hour samuraciclib treatment) and 0.5 μ M RNA-sequencing (6-hour samuraciclib treatment).

3.4.2 Ferroptosis protein levels are up-regulated following CDK7 inhibition

The CRISPR Cas9 genome-wide screens of MCF7, as well as NALM-6 cells reveal that knockout of GPX4 increases sensitivity to samuraciclib. Moreover, the samuraciclib proteomics data identify FRIH (*FTH1*) as one the most significant and highly upregulated proteins. Levels of HMOX1 are also increased by samuraciclib. *FTH1* and *HMOX1* are important for $\text{Fe}^{2+}/\text{Fe}^{3+}$ balance and storage, which is itself critical for redox balance and implicated in the cell death process known as ferroptosis. Together, these data are indicative of altered iron metabolism in cells treated with samuraciclib. To determine if altered levels of FRIH and HMOX1 were due to CDK7 inhibition or are off-target effects of samuraciclib, I treated MCF7 cells for 48 hours with 5 nM SY-1365, a covalent CDK7 inhibitor (Hu *et al.*, 2019). The concentration of 5 nM SY-1365 was chosen from GI_{50} experiments generated in our lab (data not shown), so that it results in similar growth inhibition to 150 nM samuraciclib. At this concentration of SY-1365, Pol-II Ser5 phosphorylation was modestly, albeit not significantly, reduced (**Figure 3.20A – B**). Immunoblotting was performed for proteins associated with ferroptosis regulation, including GPX4, FSP1, FTH1, NRF2, GCLM, and HMOX1 (Anandhan *et al.*, 2023; Doll *et al.*, 2019; Forcina & Dixon, 2019; Tang *et al.*, 2021a; Tian *et al.*, 2020) (**Figure 3.20A**). Levels of each protein, with the exception of NRF2, were increased for SY-1365 treatment (**Figure 3.20C**). Immunoblotting for samuraciclib-treated cells demonstrated the expected increases in levels of each protein. Collectively, these results show that CDK7 inhibition with samuraciclib or SY-1365 generally increases expression of ferroptosis suppressors.

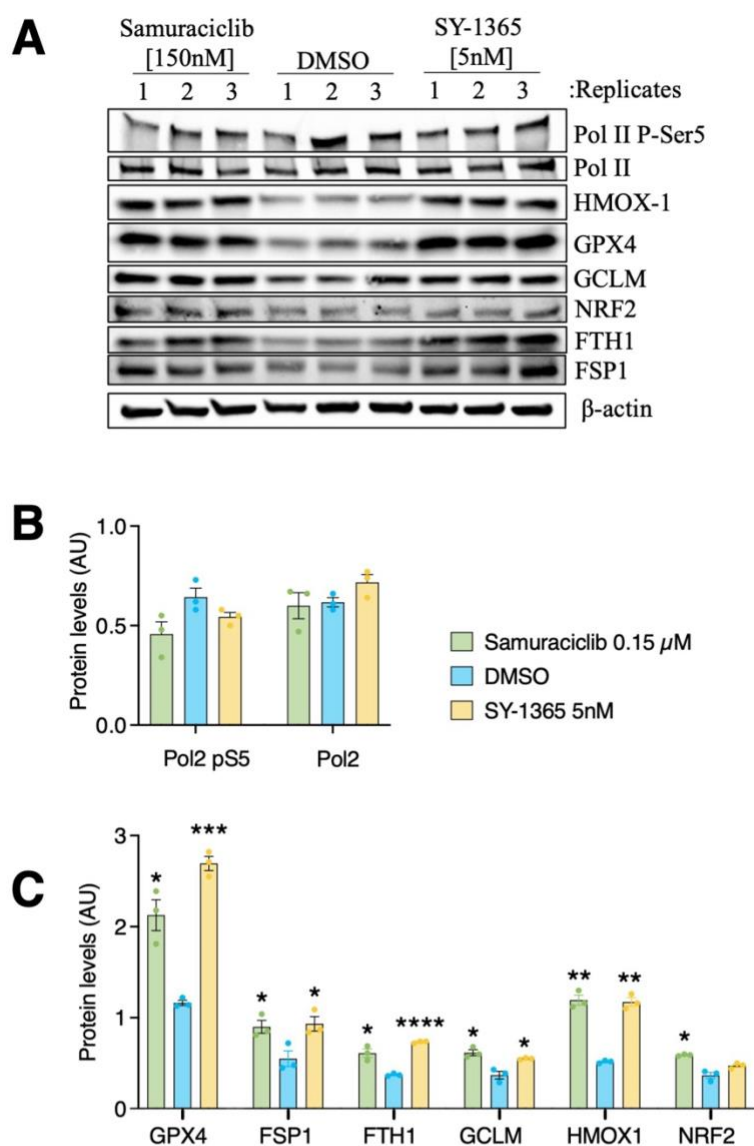


Figure 3.20 The covalent CDK7i SY-1365, increases levels of ferroptosis associated proteins up-regulated by samuraciclib.

(A) Protein lysates were prepared from MCF7 cells treated with the CDK7 inhibitors samuraciclib (0.15 μ M) or SY-1365 (5 nM) for 48 hours. β -actin was used as a loading control (n=3 biological replicates). (B-C) Quantification of levels of each protein are shown relative to the β -actin control. Bars represent mean protein levels across three biological replicates, while points show the specific levels from each replicate. Western blots were quantified using ImageJ. Error bars represent the SEM. P-values from t-tests with Welch's correction compare treatment conditions with the respective DMSO control (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

3.4.3 Samuraciclib sensitizes breast cancer cells to ferroptosis

To further explore the role of ferroptosis in samuraciclib response, I used cell growth assays and treatment combinations of samuraciclib and drugs that either inhibit (Ferrostatin-1 (F1)) or induce (RSL3) ferroptosis. RSL3 is a GPX4 inhibitor that induces ferroptosis in many cell types (Sui *et al.*, 2018). However, in certain cell types, including MCF7, RSL3 treatment alone is insufficient to induce ferroptosis (Park *et al.*, 2023). F1 is a lipid ROS scavenger that selectively prevents ferroptosis by inhibiting lipid peroxidation (Zilka *et al.*, 2017). I treated MCF7 cells with combinations of samuraciclib (0.15 μ M), RSL3 (1 μ M), and F1 (1.5 μ M) and monitored cell growth for 33 days or until cells reached confluence. These concentrations of RSL3 and F1 were chosen based on experimental data previously generated in our lab (data not shown). MCF7 cells treated with F1 alone, a combination of RSL3 + F1, or, importantly, RSL3 alone, had a growth rate comparable to DMSO control treated cells, indicating that neither F1, nor RSL3 impact MCF7 cell growth (**Figure 3.21A**). All cells treated with samuraciclib were growth inhibited, regardless of co-treatment with RSL3 and F1.

I then repeated this experiment but first pre-treated cells for nine days with samuraciclib prior to the addition of F1 and/or RSL3. After pre-treatment, cells treated with RSL3 alone showed markedly reduced cell numbers compared to the DMSO control (**Figure 3.21B**). This impaired growth was reversed in the RSL3 + F1 condition, demonstrating RSL3-directed induction of ferroptotic cell death in cells pre-treated with samuraciclib. Continued treatment with samuraciclib over the 33 days growth experiment showed maintenance of growth inhibition and progressive reductions in cell numbers over the treatment period. Interestingly, a more pronounced reduction in cell numbers was apparent when samuraciclib was combined with RSL3, an effect that was abrogated by F1.

Cell counts at day 15 taken from panel A, where cells were treated continuously for 15 days with indicated drugs (**Figure 3.21C**) or from panel B, where cells were pre-treated with samuraciclib followed by six days of treatment with the indicated drugs (**Figure 3.21D**), were used for statistical comparisons. Statistical tests compare all treatment conditions to the respective DMSO, where cells were either continuously treated with DMSO for 15 days (**Figure 3.21C**), or for nine days with samuraciclib, followed by six days of DMSO (**Figure 3.21D**). If significance is not specified, the treatment did not cause a significant change compared to the vehicle DMSO. At day 15, all treatment conditions containing samuraciclib were confirmed to significantly reduce cell numbers compared to the DMSO control,

irrespective of whether the cells were pretreated with samuraciclib or not. Treatment with RSL3 over the 15-day period, with no samuraciclib pre-treatment (**Figure 3.21C**), did not impact cell numbers compared to the DMSO control. By contrast, pre-treatment with samuraciclib for the first 9 days, significantly increased their sensitivity to GPX4 inhibition by RSL3, as indicated by significantly reduced cell numbers compared to the DMSO control (**Figure 3.21D**). A more pronounced growth inhibition following co-treatment with samuraciclib and RSL3 was observed. Again, this effect of RSL3 was prevented by F1. Replotting these data for a direct comparison of cells that were pre-treated with samuraciclib for nine days, or where cells were treated continuously for 15 days with the indicated drug combinations (**Figure 3.21E**), shows a clear and statistically significant reduction in cell numbers when RSL3 was added on day 9, after samuraciclib pre-treatment. This reduction in cell numbers was abrogated by F1 in all co-treatments with RSL3. Collectively, these results suggest that samuraciclib does not induce ferroptosis but acts to sensitise cancer cells to ferroptosis.

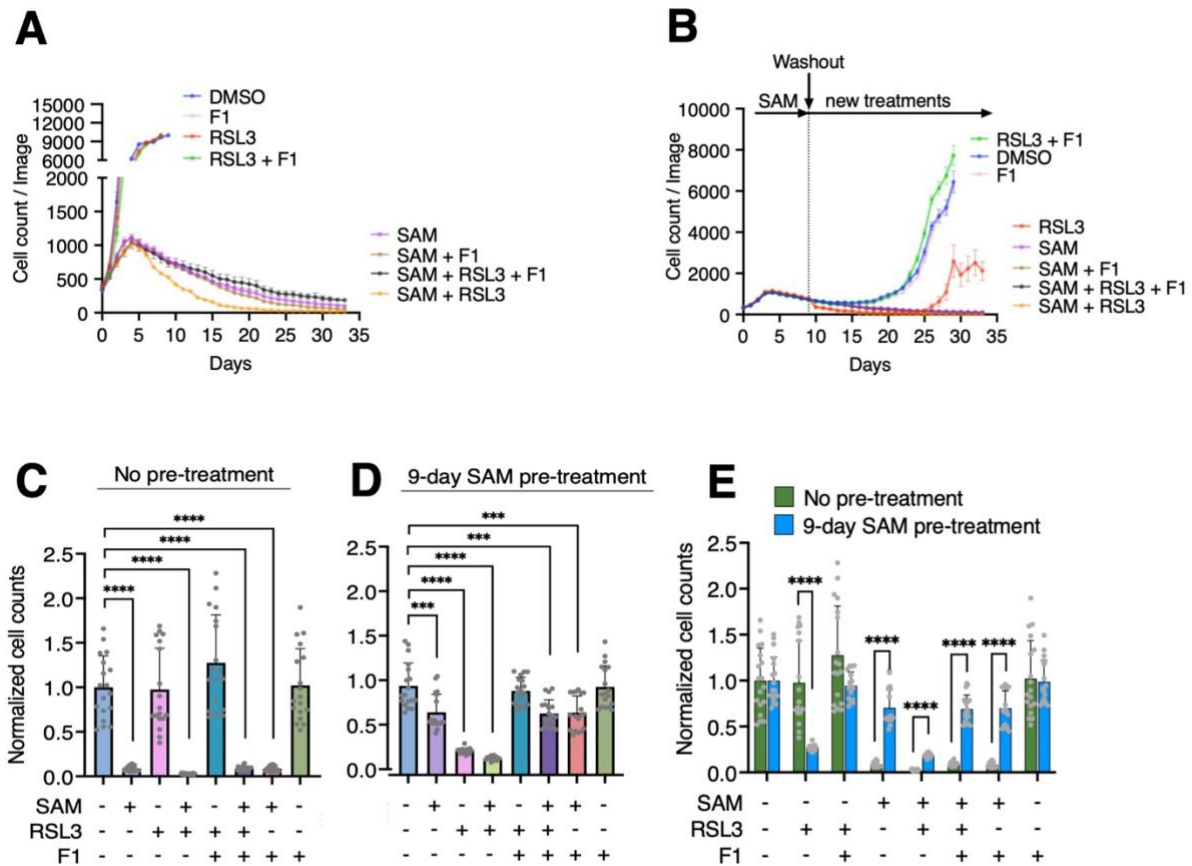


Figure 3.21 MCF7 cells are sensitized to ferroptosis by samuraciclib.

(A) GFP-tagged MCF7 cells were treated with combinations of the CDK7 inhibitor samuraciclib (SAM; 0.15 μ M), the ferroptosis inducer (RSL3; 1 μ M), and the ferroptosis inhibitor Ferrostatin-1 (F1; 1.5 μ M) over a period of 33 days. Cell numbers were defined as the number of GFP-positive nuclei in four images per well, measured using IncuCyte S3 every 24 hours. (B) MCF7 cells were grown for nine days in the presence of 0.15 μ M samuraciclib, then washed and treated with different combinations of drugs over 33 days, as shown. (A-B) The culture medium was changed every three days, to replenish the drug treatments. (C-D) Cell counts at day 15 from panels A (no pre-treatment) and B (9-day SAM pre-treatment), indicative of 15 days of continuous treatment with indicated drugs or nine days of pre-treatment with samuraciclib followed by six days of treatment with the indicated drugs, were used for statistical comparisons. (E) Replotted data (C-D) for a direct comparison. Cell numbers are shown relative to day zero, normalized to DMSO. Bars represent the mean normalized cell counts with error bars displaying the standard error of the mean (SEM). P-values from t-tests with Welch's correction are shown (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001). Shown are the results of all three independent biological experiments. Each experiment was done with n = 6 internal replicates.

3.4.4 Samuraciclib sensitizes HR+ and TNBC breast cancer cells to ferroptosis

To understand if the samuraciclib-induced sensitization to ferroptosis occurs in other breast cancer cell lines, I repeated the same experiment in another ER+, HER2- cell line, T47D, as well as a triple-negative breast cancer cell line, MDA-MB-453. The GI₅₀ values I determined for T47D (172 nM) and MDA-MB-453 (179 nM) cells were several-fold higher than those for MCF7 (22 nM) cells (data not shown). Nevertheless, I used 150 nM samuraciclib for these experiments, given the fact that this concentration is similar to the GI₅₀ values for T47D and MDA-MB-453 cell. Samuraciclib inhibited T47D cell growth over the time course of treatment (**Figure 3.22**). T47D cells showed some sensitivity to RSL3 in the absence of samuraciclib. Notwithstanding, as observed for MCF7 cells, samuraciclib sensitised T47D cells to RSL3. Thus, combined treatment with samuraciclib and RSL3 reduced T47D cell numbers much more potently than samuraciclib or RSL3 alone. The RSL3 sensitivity of T47D cells was reversed with F1, whereas F1 did not impact growth inhibition by samuraciclib. Statistical analyses of day 15 results are summarised in **Figure 3.22C – E**.

MDA-MB-453 cells were not growth inhibited by RSL3 (**Figure 3.23A**). Samuraciclib at a concentration of 150 nM only partially, but continuously until day 33, blunted cell growth. The reasons for the poor growth inhibition of MDA-MB-453 cells is unclear and may suggest a level of ER-dependency for CDK7 inhibitors. Nevertheless, co-treatment with samuraciclib and RSL3 strongly inhibited growth of MDA-MB-453 cells (**Figure 3.23A**). Treatment with samuraciclib, washout on day 9, followed by treatment with RSL3 inhibited cell growth until about day 20, at which point RSL3 treated cells regrew (**Figure 3.23B**). This contrasts with the results in T47D cells, where the RSL3 treatment irreversibly led to progressive loss in cell numbers. Statistical analyses of day 21 results are summarised in **Figure 3.23C – E**.

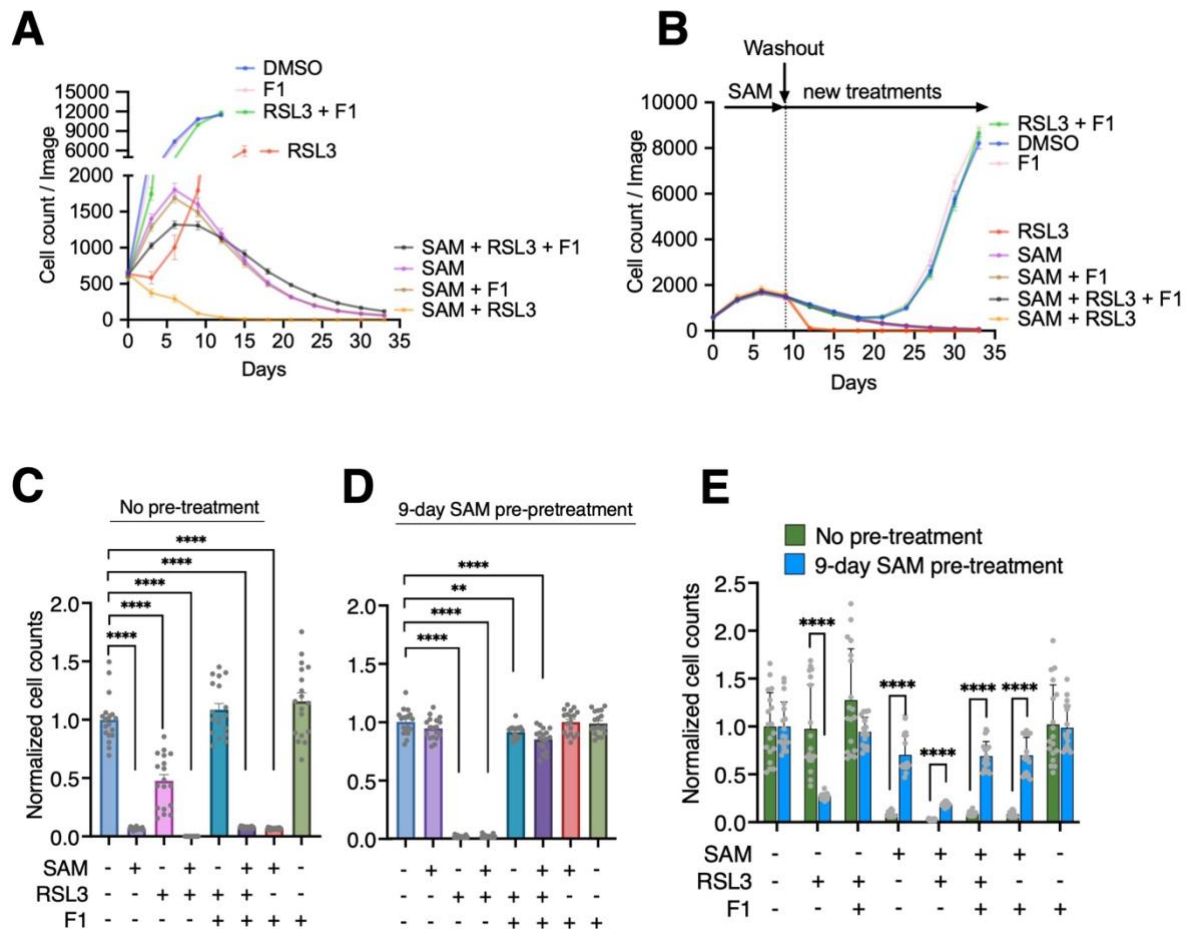


Figure 3.22 T47D cells are sensitized to ferroptosis by samuraciclib.

Growth curves show T47D cell growth is impaired following treatment with samuraciclib (SAM) in combination with RSL3. (A-B) The experiments were undertaken for 33 days, exactly as described for MCF7 cells in Figure 3.21. Cell numbers were assessed by IncuCyte every three days. (C-D) Day 15 cell counts from the growth curves shown in A and B are plotted, as described in Figure 3.21. (E) Replotted data (C-D) for a direct comparison. Bars represent the mean normalized cell count with error bars displaying the standard error of the mean (SEM). P-values from t-tests with Welch's correction are shown (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). Shown are the results of all three independent biological experiments. Each experiment was done with $n = 6$ internal replicates. Samuraciclib (SAM); 0.15 μM . RSL3; 1 μM . Ferrostatin-1 (F1); 1.5 μM .

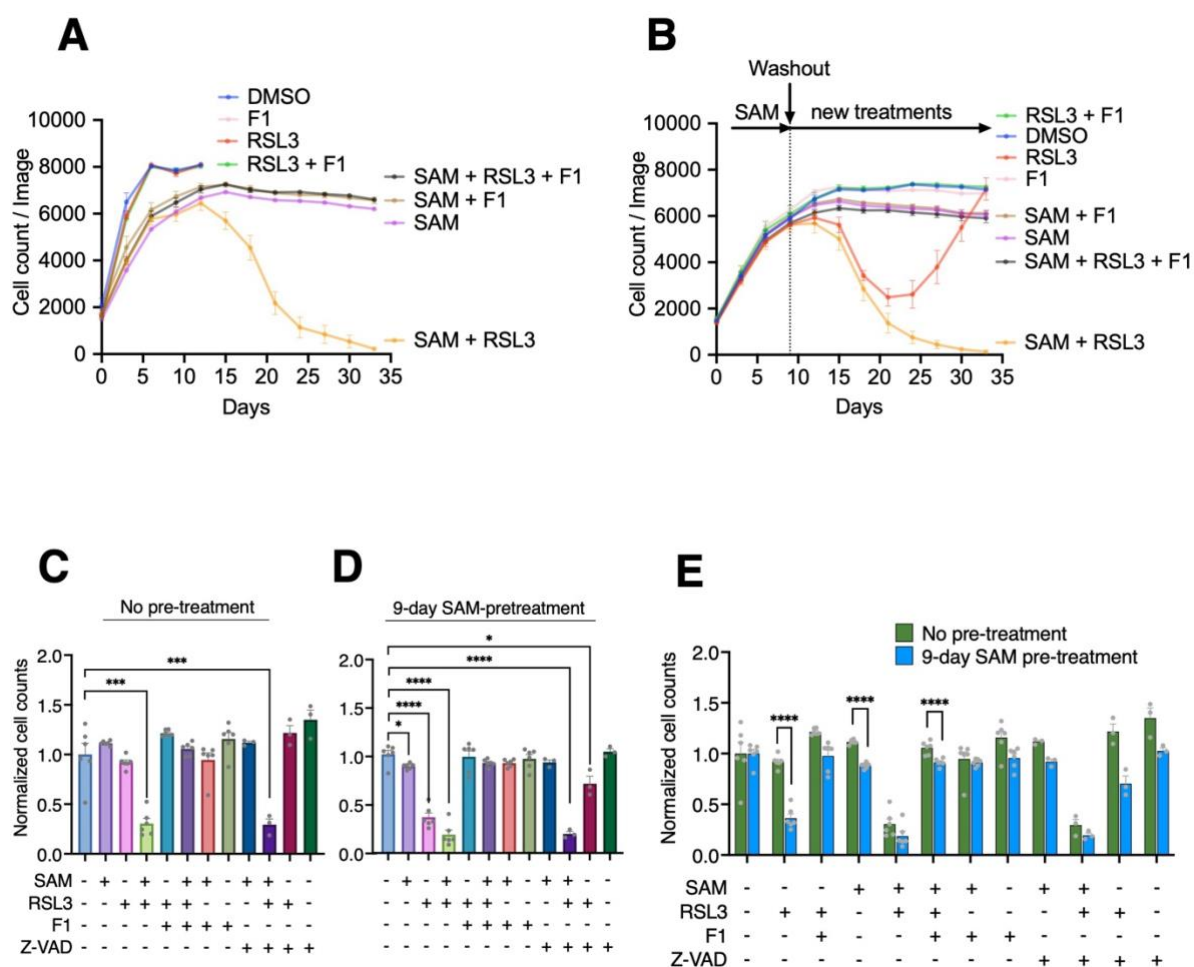


Figure 3.23 Samuraciclib sensitizes the ER-negative MDA-MB-453 cells to ferroptosis by RSL3.

Growth curves show MDA-MB-453 cell growth is impaired following treatment with samuraciclib (SAM) in combination with RSL3. (A-B) The experiments were undertaken for 33 days, exactly as described for MCF7 cells in Figure 3.21, with additional treatments with the apoptosis inhibitor (Z-VAD(OMe)-FMK / Z-VAD; 10 μ M). Cell numbers were assessed by IncuCyte every three days. (C-D) Day 21 cell counts from the growth curves shown in A and B are plotted, as described in Figure 3.21. (E) Replotted data (C-D) for a direct comparison. Bars represent the mean normalized cell count with error bars displaying the standard error of the mean (SEM). P-values from t-tests with Welch's correction are shown (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). Shown are the results of one independent biological experiment done with $n = 6$ internal replicates ($n=3$ for Z-VAD conditions). Samuraciclib (SAM); 0.15 μ M. RSL3; 1 μ M. Ferrostatin-1 (F1); 1.5 μ M.

Considering the above findings altogether, these growth studies demonstrate that CDK7 inhibition sensitises ER+ and triple-negative breast cancer cell lines to ferroptosis. This is seen even in MCF7 cells, which have been reported to be resistant to ferroptosis (Park *et al.*, 2023). The temporary growth inhibition by RSL3 seen in ER+ T47D, but not the ER+ MCF7 cells, is intriguing. The most likely explanation for the discrepancy between the two ER+ cell lines is p53 mutation status. While MCF7 cells contain a functional p53 protein localized in the nucleus, T47D cells harbor a missense mutation in p53, which is localized in the cytoplasm (Schafer *et al.*, 2000). The tumor suppressor p53 has been heavily implicated in regulating ferroptosis, with its role in promoting and suppressing ferroptosis being highly context-dependent (Kastenhuber & Lowe, 2017). For example, in HT-1080 fibrosarcoma cells, stabilization of p53 by nutlin-3 treatment, reduced SLC7A11 mRNA expression and xCT protein levels (Tarangelo *et al.*, 2018). Perturbation of SLC7A11, a component of the cystine-glutamate antiporter (system xCT), decreases intracellular cystine uptake, which abrogates downstream synthesis of GSH, diminishing the ability of GPX4 to prevent ferroptosis (Dixon *et al.*, 2012). However, in vascular smooth muscle cells, p53 has been shown to upregulate SLC7A11, protecting cells from ferroptosis (Ma *et al.*, 2021). Thus, different cell types have distinct gene expression profiles and signalling pathways, which together dictate cell behavior after p53 induction, making the effects of p53 on ferroptosis cell-type specific. This can be readily tested with additional ER+ and TNBC cell lines with varying p53 status to determine if this is a cell line specific effect or is generalizable to p53 status. A clearer model system would be to compare isogenic p53 mutant versus wild-type cell lines. Notwithstanding, these results demonstrate that addition of the RSL3 potently enhances the anti-tumour effects of samuraciclib, most likely by promoting ferroptosis-directed cell death.

3.4.5 CDK7 inhibition sensitizes breast cancer cells to GPX4i-induced ferroptosis not apoptosis

Ferroptosis is a mechanism of cell death that is distinct from apoptosis (Dixon *et al.*, 2012). Our previous work has demonstrated that samuraciclib does not drive apoptotic mediated cancer cell death (Patel *et al.*, 2018a). In agreement with these findings, I used the apoptosis inhibitor Z-VAD, an irreversible caspase inhibitor (Slee *et al.*, 1996). Z-VAD did not impact growth inhibition of samuraciclib-treated MDA-MB-453 cells (**Figure 3.23C – D**). There was a small increase in cell number when Z-VAD was combined with RSL3, which was greater for the no-pretreatment cells (**Figure 3.23C**). In both samuraciclib-pretreated and no pre-treatment conditions, the addition of Z-VAD to RSL3 increased cell numbers significantly compared to

RSL3 alone (P-value = 0.023 and 0.046, respectively) (data not shown). This could suggest that some effects of RSL3 are mediated by apoptosis rather than ferroptosis. However, the increase in cell numbers for Z-VAD alone in the no-pre-treatment conditions, suggests that the effect seen in RSL3-treated cells may in fact reflect a reduction in background apoptosis in MDA-MB-453 cells. Nevertheless, no reduction in cell number was observed when Z-VAD was combined with samuraciclib and RSL3. Taken together, these results indicate that the profound and progressive loss in cell number observed for the combination of samuraciclib and RSL3 is not due to caspase-dependent apoptosis but is mediated by ferroptotic cell death. Note that Z-VAD was used at a concentration of 10 μ M, selected based on prior work in our lab (G Sava *et al* unpublished), but its effectiveness at inhibiting apoptosis should be confirmed, for example by treating with drugs such as doxorubicin, which are known to induce apoptosis in cancer cells.

I then confirmed the results for the GPX4 inhibitor and ferroptosis inducer RSL3 with ML210, a covalent GPX4 inhibitor (Eaton *et al.*, 2020). As was the case for RSL3, ML210 enhanced growth inhibition by samuraciclib in the continuous treatment setting (**Figure 3.24A**). ML210 inhibited MCF7 cell growth when added following washout of samuraciclib after 9 days (**Figure 3.24B**), again as seen for RSL3. The statistically significant differences seen for RSL3 were maintained with ML210 treatment (**Figure 3.24C – E**). Of note is the increased cell number in the no pre-treatment conditions seen for the apoptosis inhibitor Z-VAD, as observed in MDA-MB-453 cells. Other than this effect of Z-VAD, I did not observe any impact of Z-VAD on growth inhibition by samuraciclib with RSL3, again in agreement with results in MDA-MB-453 cells.

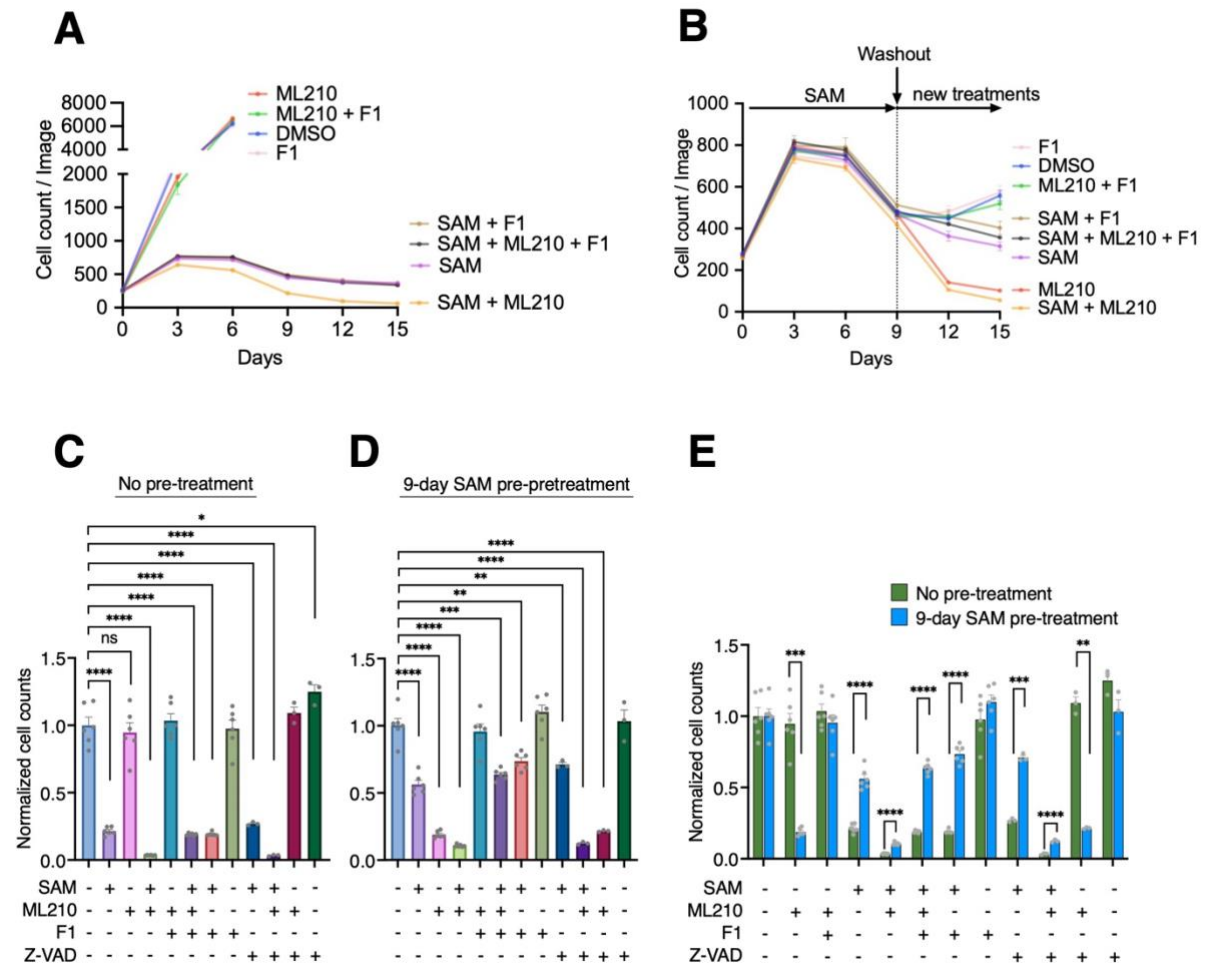


Figure 3.24 Samuraciclib-treated MCF7 cells are sensitized to ferroptosis by ML210.

Growth curves show MCF7 cell growth is impaired following treatment with samuraciclib (SAM) in combination with ML210. (A-B) The experiments were undertaken for 15 days, exactly as described for MCF7 cells in Figure 3.21, with additional treatments of a ferroptosis inhibitor (Ferrostatin-1 / F1; 1.5 μ M), and an apoptosis inhibitor (Z-VAD(OMe)-FMK / Z-VAD; 10 μ M). Cell numbers were assessed by IncuCyte every three days. (C-D) Day 15 cell counts from the growth curves shown in A and B are plotted, as described in Figure 3.21. (E) Replotted data (C-D) for a direct comparison. Bars represent the mean normalized cell count with error bars displaying the standard error of the mean (SEM). P-values from t-tests with Welch's correction are shown (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). Shown are the results of one independent biological experiment done with $n = 6$ internal replicates ($n=3$ for Z-VAD conditions). Samuraciclib (SAM); 0.15 μ M. ML210; 2 μ M. Ferrostatin-1 (F1); 1.5 μ M.

The covalent CDK7 inhibitor SY-1365 was used to confirm on-target action of samuraciclib in MCF7 cells. SY-1365 inhibited MCF7 growth over the 21-day treatment period (**Figure 3.25A**). Growth inhibition by SY-1365 was enhanced by RSL3 and this effect was partly reversed by F1. In the pre-treatment setting, RSL3 shortly inhibited cell growth (**Figure 3.25B**). The combination of SY-1365 with RSL3 again gave the greatest level of growth inhibition. These results indicate that CDK7 inhibition sensitizes cells to ferroptosis, which can be induced by inhibition of GPX4.

In contrast, inhibition of SLC7A11 using the ferroptosis-inducers sorafenib (**Figure 3.26**) or sulfasalazine (**Figure 3.27**) (Dixon *et al.*, 2014; Lachaier *et al.*, 2014) did not sensitize cells to ferroptosis after samuraciclib pre-treatment. SLC7A11 is a subunit of the system xCT antiporter that mediates the cystine/glutamate antiporter activity, importing cystine for glutathione (GSH) biosynthesis and antioxidant defence by GPX4. Similarly, buthionine sulfoximine (BSO), an irreversible inhibitor known to cause ferroptosis in cancer cells by inhibiting glutamate–cysteine ligase (GCL), the rate-limiting enzyme responsible for GSH synthesis (Yang *et al.*, 2014), did not sensitize cells to ferroptosis (**Figure 3.28**). Taken together, these findings show that samuraciclib pre-treatment sensitizes MCF7 cells to GPX4 inhibition, and not SLC7A11 or GCL inhibition.

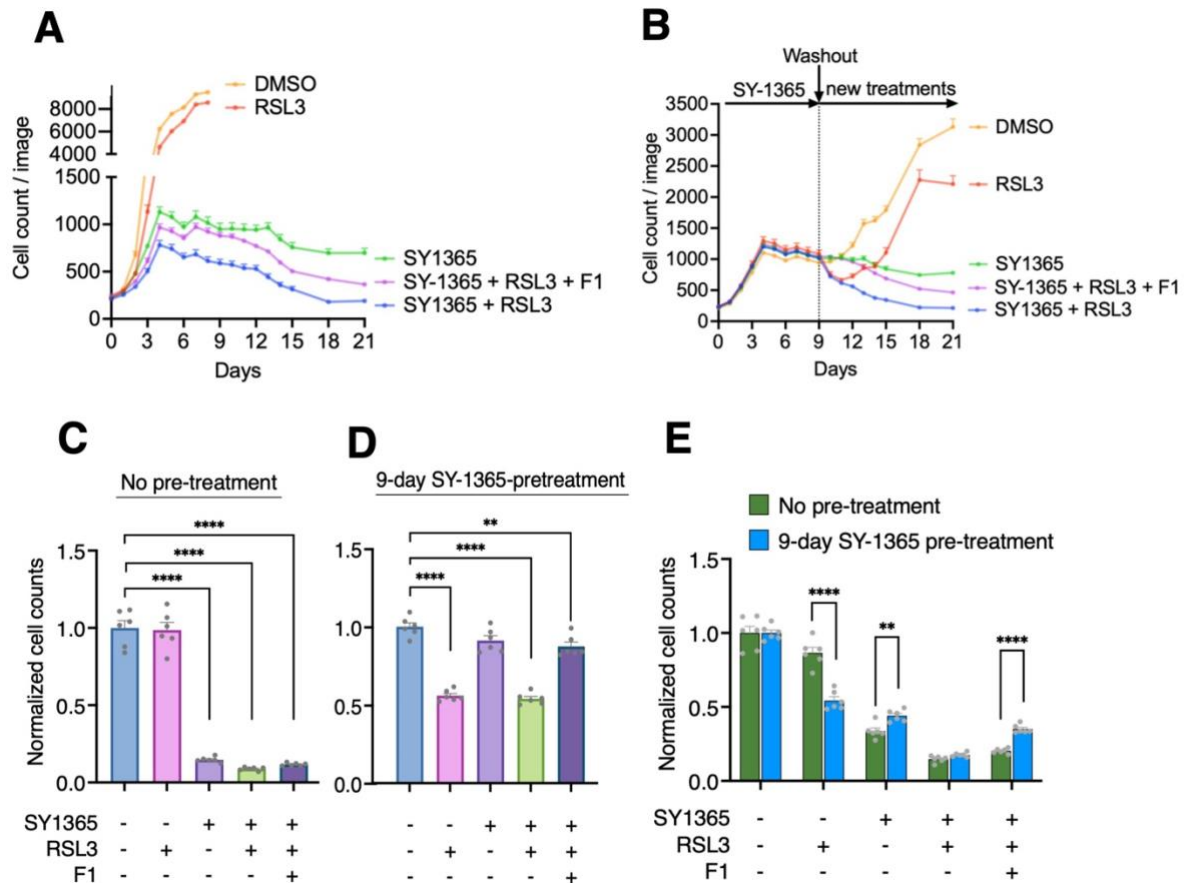


Figure 3.25 MCF7 cells are sensitized to ferroptosis by RSL3 following treatment with the covalent CDK7 inhibitor, SY-1365.

Growth curves show MCF7 cell growth is impaired with treatment conditions containing SY-1365 in combination with RSL3. (A-B) The experiments were undertaken for 21 days, as described for MCF7 cells in Figure 3.21. Cell numbers were assessed by IncuCyte every 24 hours. (C-D) Day 15 cell counts from the growth curves shown in A and B are plotted, as described in Figure 3.21. (E) Replotted data (C-D) for a direct comparison. Bars represent the mean normalized cell count with error bars displaying the standard error of the mean (SEM). P-values from t-tests with Welch's correction are shown (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). Shown are the results of one independent biological experiment done with $n = 6$ internal replicates. SY-1365; $0.005 \mu\text{M}$. RSL3; $1 \mu\text{M}$. Ferrostatin-1 (F1); $1.5 \mu\text{M}$.

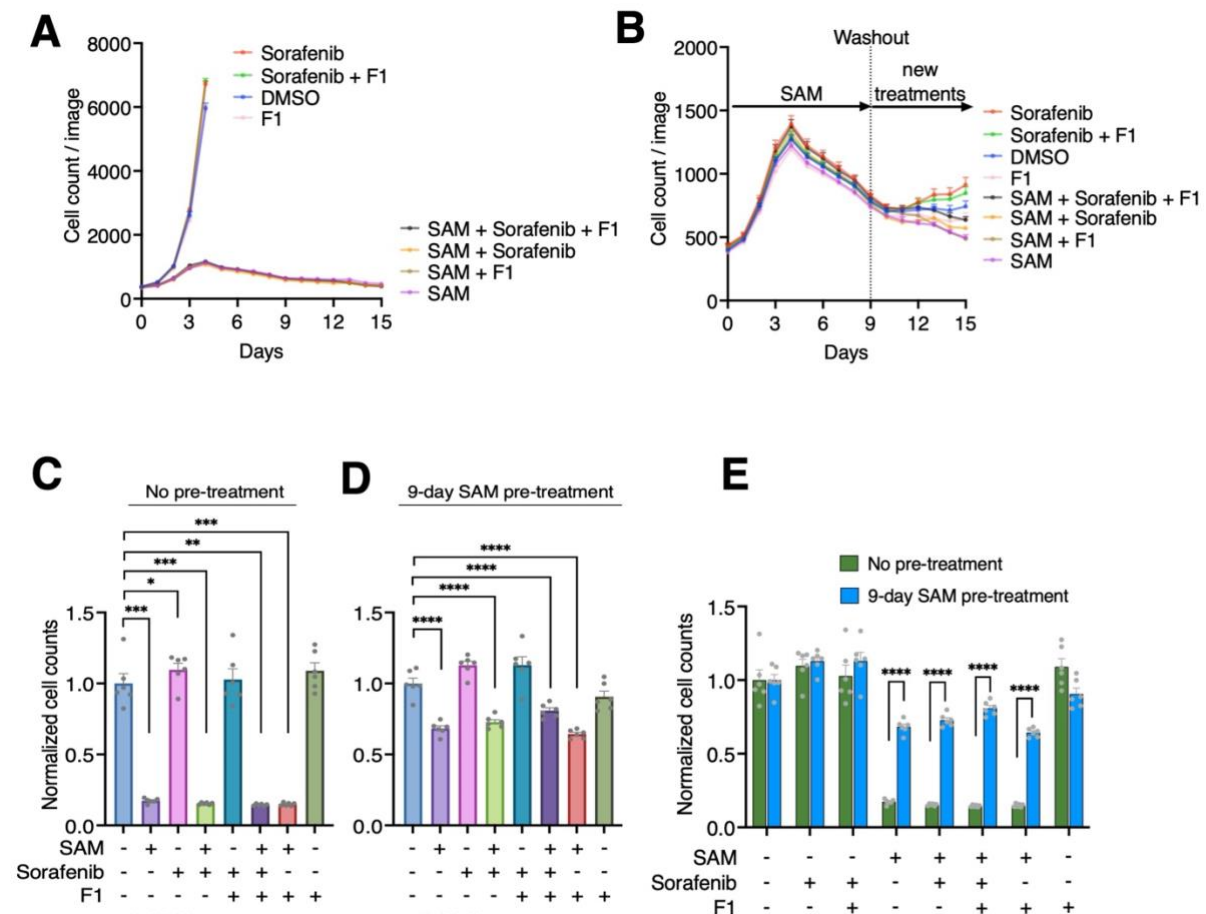


Figure 3.26 MCF7 cells are not sensitized to ferroptosis by sorafenib following treatment with samuraciclib.

Growth curves show MCF7 cell growth is impaired with treatment conditions containing samuraciclib only. (A-B) The experiments were undertaken for 15 days, as described for MCF7 cells in Figure 3.21. Cell numbers were assessed by IncuCyte every 24 hours. (C-D) Day 15 cell counts from the growth curves shown in A and B are plotted, as described in Figure 3.21. (E) Replotted data (C-D) for a direct comparison. Bars represent the mean normalized cell count with error bars displaying the standard error of the mean (SEM). P-values from t-tests with Welch's correction are shown (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). Shown are the results of one independent biological experiment done with $n = 6$ internal replicates.

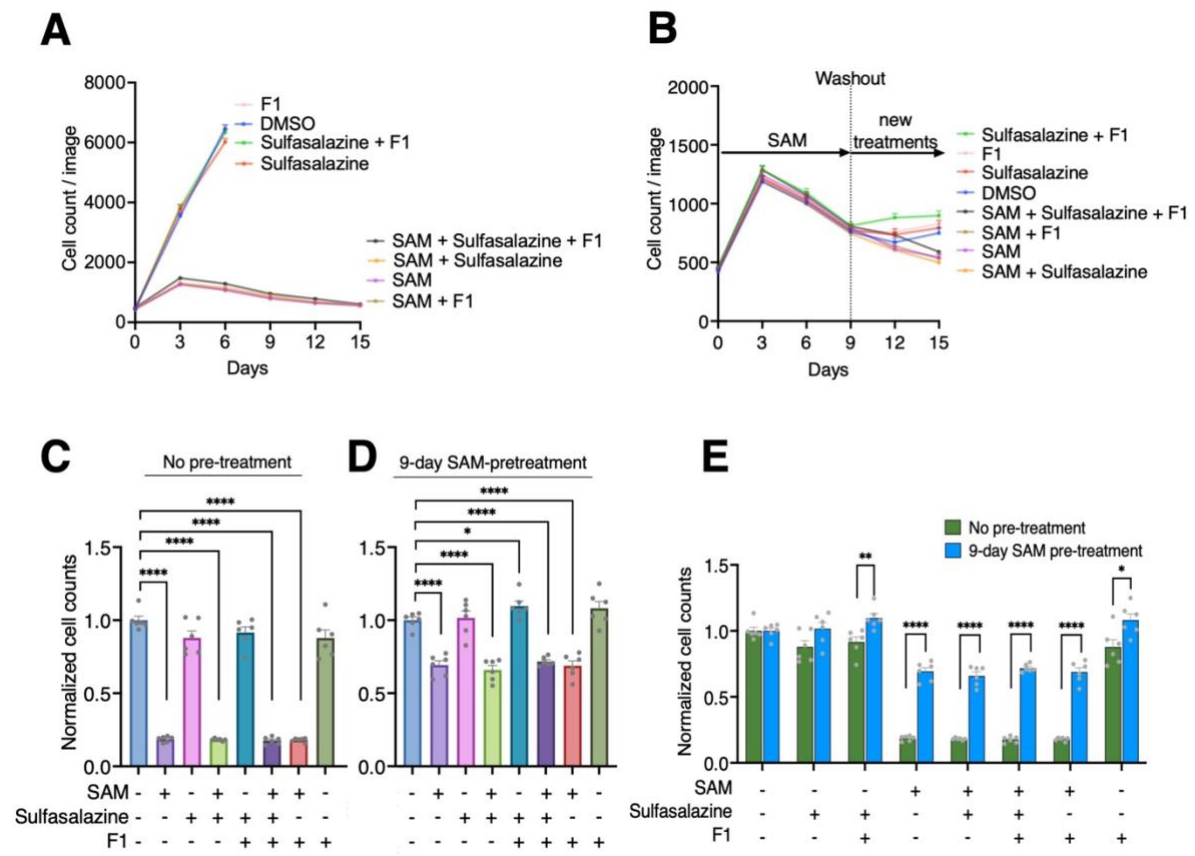


Figure 3.27 MCF7 cells are not sensitized to ferroptosis by sulfasalazine following treatment with samuraciclib.

Growth curves show MCF7 cell growth is impaired with treatment conditions containing samuraciclib only. (A-B) The experiments were undertaken for 15 days, as described for MCF7 cells in Figure 3.21. Cell numbers were assessed by IncuCyte every 24 hours. (C-D) Day 15 cell counts from the growth curves shown in A and B are plotted, as described in Figure 3.21. (E) Replotted data (C-D) for a direct comparison. Bars represent the mean normalized cell count with error bars displaying the standard error of the mean (SEM). P-values from t-tests with Welch's correction are shown (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). Shown are the results of one independent biological experiment done with $n = 6$ internal replicates.

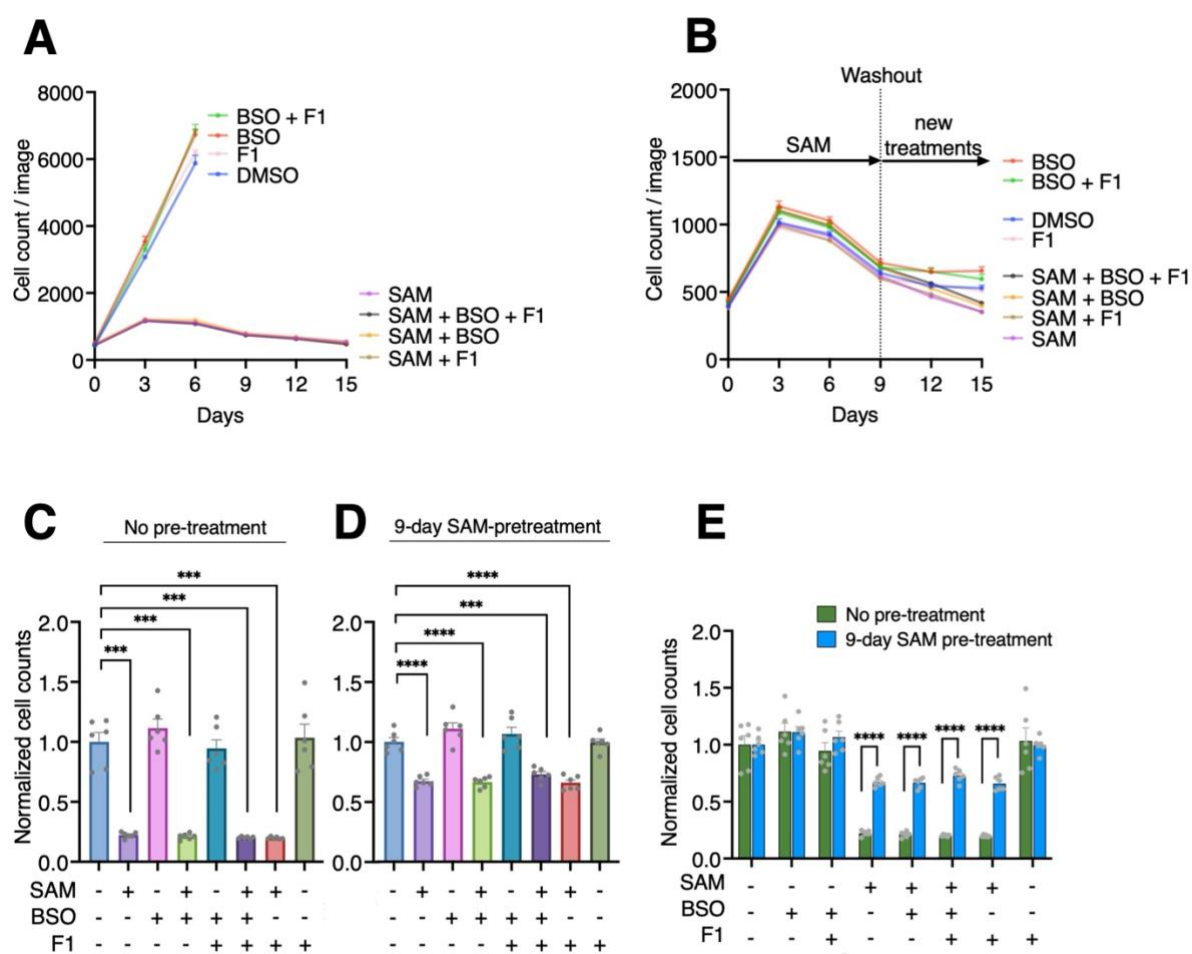


Figure 3.28 MCF7 cells are not sensitized to ferroptosis by BSO following treatment with samuraciclib.

Growth curves show MCF7 cell growth is impaired with treatment conditions containing samuraciclib only. (A-B) The experiments were undertaken for 15 days, as described for MCF7 cells in Figure 3.21. Cell numbers were assessed by IncuCyte every 24 hours. (C-D) Day 15 cell counts from the growth curves shown in A and B are plotted, as described in Figure 3.21. (E) Replotted data (C-D) for a direct comparison. Bars represent the mean normalized cell count with error bars displaying the standard error of the mean (SEM). P-values from t-tests with Welch's correction are shown (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). Shown are the results of one independent biological experiment done with $n = 6$ internal replicates.

3.4.6 Mechanisms of CDK7 inhibition sensitizing cells to ferroptosis

NRF2 is a transcription factor that binds to antioxidant response elements under cell stress conditions to express proteins involved in glutathione synthesis, ROS elimination, and genes involved in the prevention of lipid peroxidation and free iron accumulation (Chen *et al.*, 2009; Taguchi *et al.*, 2011; Yamamoto *et al.*, 2018). As such, NRF2 promotes expression of genes that regulate ferroptosis. I determined whether samuraciclib alters NRF2 transcriptional activity, using MCF7 cells stably expressing an AREc32 NRF2 reporter gene (MCF7-AREc32) (Wang *et al.*, 2006). MCF7-AREc32 cells have stable integration of a firefly luciferase gene containing eight copies of the antioxidant response element (ARE). Cells were treated with samuraciclib for 24 hours (**Figure 3.29A**) or 48 hours (**Figure 3.29B**). There was a roughly dose-dependent increase in reporter gene activity at 24 and 48 hours, although reporter activity was strongly inhibited 1.0 and 2.0 μ M samuraciclib, possibly due to the transcription-repressive effects of samuraciclib at the higher concentrations, or potent growth inhibition at the higher concentrations. Tert-butyl hydroperoxide (TBHQ), a known NRF2 activator served as a positive control. These results show that samuraciclib treatment can increase NRF2 transcriptional activity (at least at low drug concentrations), possibly as an anti-ferroptosis response mechanism. This result also suggests that at higher concentrations samuraciclib may reduce NRF2 activity and thus render cells more vulnerable to GPX4 inhibition and ferroptosis.

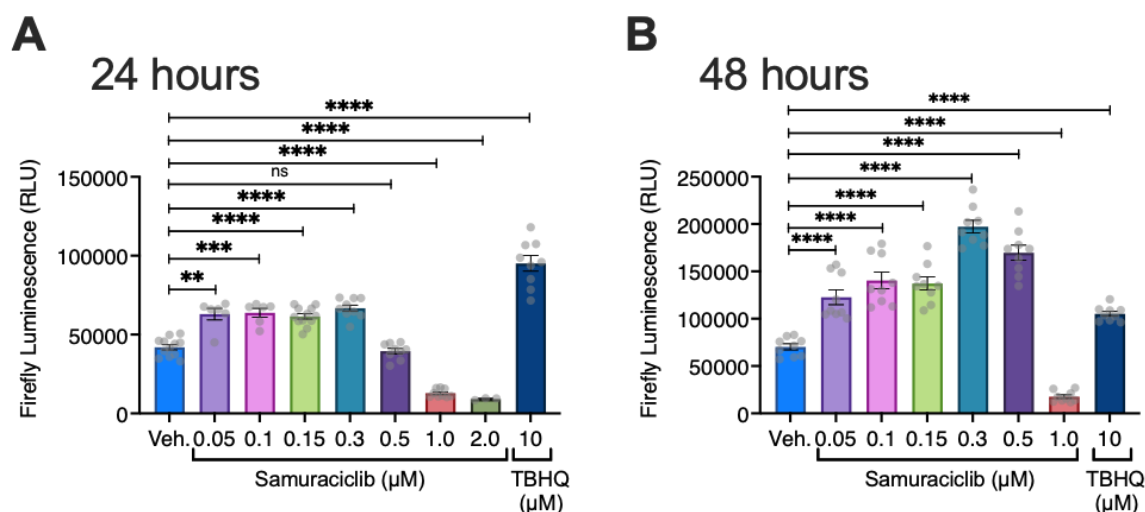


Figure 3.29 Samuraciclib treatment stimulates NRF2 activity in MCF7 cells.

(A-B) MCF7 cells stably expressing NRF2 firefly luciferase reporter genes (MCF7-AREc32) were treated with the indicated concentrations of samuraciclib (SAM). Firefly luciferase activity was determined 24 (A) or 48 (B) hours following samuraciclib addition. Tert-Butyl hydroperoxide (TBHQ; 10 μ M), a known inducer of NRF2, was used as a positive control. Each experiment was undertaken in triplicate. Bars represent mean NRF2 activity. Error bars represent the SEM. P-values from t-tests with Welch's correction compare treatment conditions with the DMSO control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

Lipid peroxidation is one of the hallmarks of ferroptosis (Dixon & Stockwell, 2019). To better understand if CDK7 inhibition sensitizes cells to ferroptosis or induces ferroptosis itself, I measured ROS production following treatments with ferroptosis inducers and CDK7 inhibitors. Specifically, I first asked if intracellular ROS production is increased after a nine-day pre-treatment with samuraciclib or SY-1365 followed by GPX4 inhibition (**Figure 3.30**). To test this, I pre-treated MCF7 cells with samuraciclib (0.15 μ M), SY-1365 (5 nM), or an equal volume of DMSO (control) for nine days. Following the pre-treatment, cells were treated for four hours with combinations of samuraciclib or SY-1365, as indicated, and the ferroptosis inducers ML210 or RSL3 along with the intracellular ROS reporter, CellROX green. Tert-Butyl hydroperoxide was used as a positive control for ROS production. Across all pre-treatment conditions, four-hour treatments with RSL3 increased ROS significantly more than was seen in the DMSO control. However, regardless of whether cells were pre-treated with a CDK7 inhibitor, or DMSO, RSL3 and the combination of RSL3 and samuraciclib or SY-1365 resulted in significantly higher ROS levels compared to DMSO or the CDK7i alone.

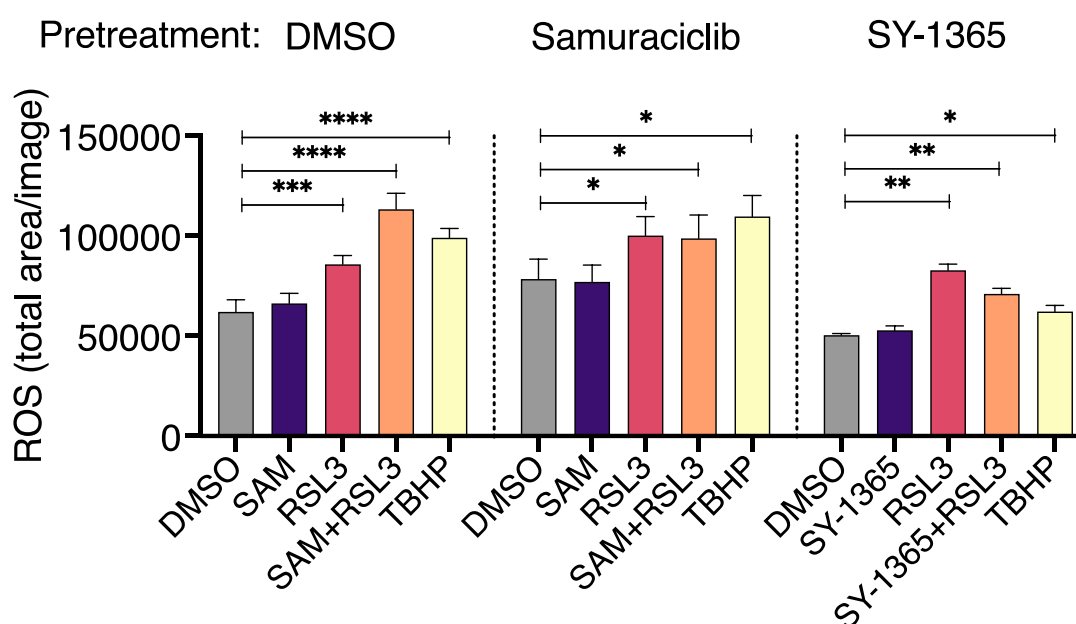
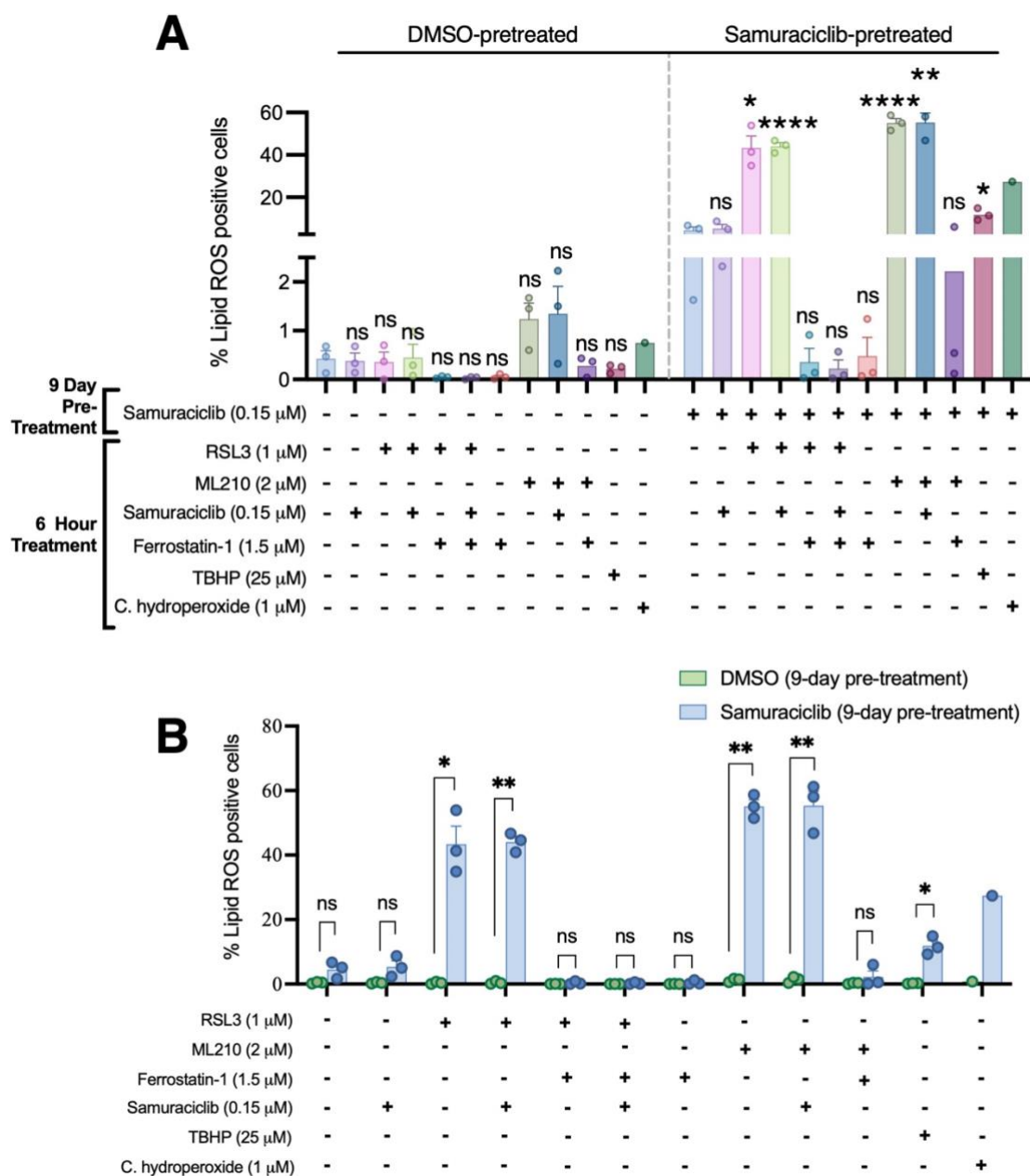


Figure 3.30 Samuraciclib increases cellular reactive oxygen species (ROS) levels in MCF7 cells following treatment with RSL3.

MCF7 cells were pre-treated with DMSO or with the CDK7 inhibitors samuraciclib (SAM) (0.15 μ M) or SY-1365 (5 nM) for nine days. Following pre-treatment, different combinations of samuraciclib (0.15 μ M), the ferroptosis inducer RSL3 (1 μ M) or ML210 (2 μ M), and the positive control tert-Butyl hydroperoxide (TBHP; 25 μ M) were added simultaneously with the ROS reporter CellROX green (2.5 μ M) for four hours. After incubation, images using the GFP channel were taken on IncuCyte S3. Bars show the mean ROS area per image from two biological (n=1 for SY-1365 data) replicates with n=6 internal replicates. Error bars display the SEM. P-values from t-tests with Welch's correction compare treatment conditions with the respective DMSO control (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

Intracellular ROS levels are a general indicator of cellular stress and is not specifically a hallmark of ferroptosis (Schieber & Chandel, 2014). To more directly measure if samuraciclib increases lipid peroxidation, the BODIPY C11 lipid peroxidation sensor was used in fluorescent activated cell sorting (FACS) analysis. As before, cells were pre-treated with DMSO or samuraciclib for nine days then treated with combinations of RSL3, ML210, F1, and samuraciclib for six hours before lipid peroxidation was measured using BODIPY Green C11 581/591, which fluoresces green following interrogation with a blue laser (Drummen *et al*, 2002; Martinez *et al.*, 2020). A nine-day pre-treatment with DMSO resulted in no observable increase in lipid peroxidation following any of the six-hour drug combination treatments (**Figure 3.31A**). On the other hand, pre-treatment for nine days with samuraciclib significantly increased lipid peroxidation after a six-hour treatment with RSL3 or ML210. F1 inhibited lipid peroxidation by RSL3 or ML210. Of note, the positive control TBHP only increased lipid peroxidation in cells pre-treated with samuraciclib. Cumene hydroperoxide (C. hydroperoxide), a second inducer of lipid peroxidation, similarly only showed activity following samuraciclib pre-treatment; although it was only tested in one experiment and thus was not statistically analysed and requires confirmation. Interestingly, addition of samuraciclib for 6 hours was insufficient to increase lipid peroxidation. As expected, any drug combination that included F1 effectively showed no lipid peroxidation, except for a combination with ML210, which did not decrease lipid peroxidation in one outlier experiment. Comparing total lipid peroxidation between samuraciclib pre-treatment and DMSO pre-treatment conditions showed that the 9-day samuraciclib pre-treatment significantly increased lipid peroxidation across all conditions containing RSL3 or ML210, with or without samuraciclib (**Figure 3.31B**). These results indicate that prolonged samuraciclib treatment sensitizes cells to lipid peroxidation induced by a GPX4 inhibitor and consequently sensitizes cells to ferroptosis.



Recently, reduced expression of *EMP2* and *MBOAT1* have been implicated in sensitizing HT-1080^N fibrosarcoma cells to ferroptosis by GPX4 inhibition after treatment with a CDK4/6 inhibitor, palbociclib (Rodencal *et al.*, 2023). In this analysis, Rodencal *et al.* demonstrated that CDK4/6 inhibition with palbociclib and MEK1/2 inhibition with Pimasertib, both of which cause cell cycle arrest at G1, sensitized cells to ferroptosis by GPX4 inhibition. Differential gene expression analysis of RNA-sequencing results following palbociclib and pimasertib treatments identified *MBOAT1*, *GPD2*, *EMP2*, and *MYO19* as candidate genes whose reduced expression sensitizes cells to ferroptosis. Subsequent analyses demonstrated that down-regulation of *MBOAT1* and *EMP2* sensitizes cells to ferroptosis. Thus, Rodencal *et al.* suggested that CDK4/6 inhibition sensitizes cells to ferroptosis via the downregulation of *MBOAT1* and *EMP2*. Considering that CDK4/6 is a substrate of CDK7, CDK7 inhibition reduces CDK4/6 activity, and induces cell cycle arrest in G1 (Wilson *et al.*, 2023), I determined whether CDK7 inhibition also sensitizes cells to ferroptosis by downregulating *MBOAT1* and *EMP2* gene expression (**Figure 3.32**). I treated MCF7 cells with samuraciclib or SY-1365 for 48 hours and then performed reverse-transcriptase quantitative PCR (RT-qPCR) to measure the mRNA levels of *MBOAT1* and *EMP2*. *MBOAT1* expression was only a little increased by samuraciclib and not at all by SY-1365. CDK7i increased *EMP2* expression, albeit not significantly. While still preliminary, these results suggest that CDK7 inhibition sensitizes cells to ferroptosis through a different mechanism than down-regulation of *MBOAT1* and *EMP2*.

After finding evidence of ferroptosis processes being dysregulated following samuraciclib treatment, I experimentally confirmed that samuraciclib, and by extension to SY-1365, CDK7 inhibition sensitizes ER+ and triple negative breast cancer cells to ferroptosis. My analyses have elucidated a better understanding of how CDK7 inhibition sensitizes cells to ferroptosis. I demonstrated that samuraciclib is itself insufficient for ferroptosis in breast cancer cells, but that prolonged treatment with samuraciclib sensitizes cells to ferroptosis, which can be achieved by inhibition of GPX4. I showed that key ferroptosis-promoting mechanisms, including dysregulated activity of the anti-ferroptotic NRF2 pathway and increased lipid peroxidation, are induced by CDK7i. Collectively, my results indicate that CDK7 inhibition sensitizes cells to ferroptosis. These findings reveal a possible novel approach to BC therapy that could utilize this samuraciclib-induced vulnerability to drive cancer cell death.

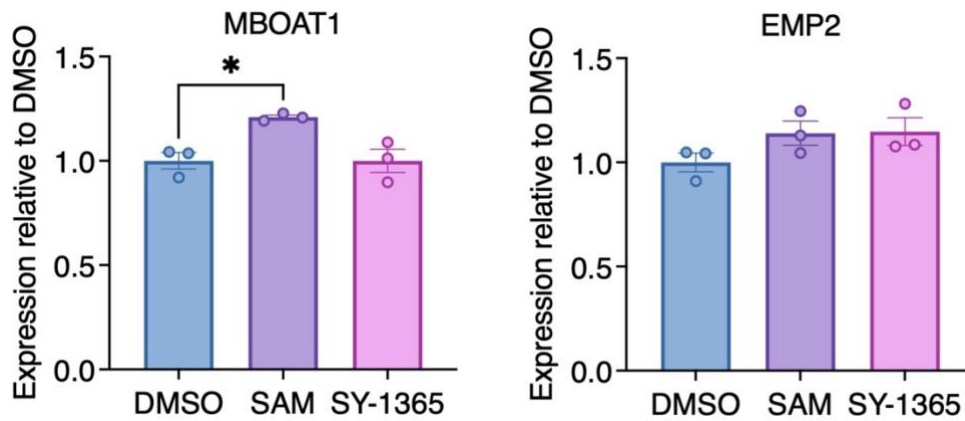


Figure 3.32 Samuraciclib increases MBOAT1 expression in MCF7 cells.

MCF7 cells were treated with the CDK7 inhibitors samuraciclib (SAM; 0.15 μ M) or SY-1365 (5 nM) for 24 hours. MBOAT1 and EMP2 expression was measured using reverse transcription quantitative PCR (RT-qPCR). Gene expression is shown relative to DMSO and normalized to the housekeeping gene GAPDH. Bars represent mean expression of three independent experiments with n=3 internal replicates. Error bars show the SEM.

4. Discussion

Breast cancer is a complex disease, with multiple molecularly and pathologically distinct subtypes. Understanding of this complexity has led to the development of subtype-specific targeted therapies, principally hormone therapies for ER+ breast cancer and drugs/antibodies for targeting HER2+ breast cancer. However, *de novo* resistance, as well as acquired resistance, often several decades following initial diagnosis, surgery and adjuvant treatment, mean that there remains a need for the development of new targeted therapies. The development of effective personalized treatments requires a detailed understanding of both the mechanisms of action and molecular factors associated with response or lack of response to the therapeutic agent. Samuraciclib is the most clinically advanced, orally bioavailable selective CDK7 inhibitor in clinical development today. It has been shown to be effective in inhibiting many cancer cell types (Patel *et al.*, 2018a). Importantly, samuraciclib is in phase I clinical trials (ClinicalTrials.gov: NCT03363893) and has recently been granted Fast Track designations from the US Food and Drug Administration (FDA) for phase 2 clinical trials for CDK4/6i-resistant HR+, HER2- advanced BC in combination with fulvestrant and for locally advanced or metastatic TNBC in combination with chemotherapy (Therapeutics, 2021). With samuraciclib, and other CDK7 inhibitors rapidly advancing through clinical trials, it is imperative to develop a more fundamental understanding of what drives sensitivity and resistance to CDK7 inhibition to optimize treatment efficacy and guide patient stratification. My thesis focussed on the use of genome-wide screening approaches for identifying genes and signalling pathways that modulate the growth inhibitory action of samuraciclib. Towards this end, I focussed on the results of genome wide CRISPR-Cas9 knockout screening and performed quantitative proteomics profiling of cells treated with samuraciclib.

4.1 The role of mTOR in the cellular response to samuraciclib

4.1.1 Samuraciclib-induced growth arrest in breast cancer cells requires a functional mTOR pathway

The PI3K/Akt/mTOR pathway orchestrates a multifaceted network within cells, regulating essential processes spanning from growth and proliferation to metabolism and survival (Weichhart, 2018; Yang *et al.*, 2021a). mTOR regulation of cellular processes involves the strict control of upstream regulator phosphorylation, namely PI3K and AKT, as well as signal transduction to downstream effectors, such as eukaryotic translation initiation factor 4E-binding protein 1 (EIF4-EBP1) and ribosomal protein S6 kinase (S6K1). In addition to its role

in cell growth and metabolism, the PI3K/Akt/mTOR pathway also modulates the tumour microenvironment by promoting angiogenesis, and contributes to driving metastasis and drug resistance by regulating cell motility and tissue invasion (Conciatori *et al.*, 2018; Murugan, 2019). Further, the PI3K/Akt/mTOR pathway controls transcription and translation, protein synthesis, lipid metabolism, and autophagy. Importantly, the PI3K/Akt/mTOR pathway has been implicated in endocrine resistance, where Akt was shown to activate the ER independently of estrogen, as well as HER2-directed and cytotoxic therapies in BC (Nahta, 2012; Paplomata & O'Regan, 2014; Paplomata & O'Regan, 2013). With PI3K/Akt/mTOR pathway inhibitors rapidly progressing through clinical trials, an understanding of optimal combination therapies is imperative.

Due to the diverse roles of the PI3K/Akt/mTOR pathway in cancer, attributing the association between mTOR and the response to samuraciclib to a single mechanism can be difficult. My results, and those of others (Wilson *et al.*, 2023), demonstrated that an active PI3K/Akt/mTOR pathway is required for samuraciclib-induced growth-arrest. This result is somewhat unexpected as the canonical role of the PI3K/Akt/mTOR pathway suggests mTOR activity is expected to promote cell growth and increase gene transcription (Paplomata & O'Regan, 2014; Polivka & Janku, 2014; Porta *et al.*, 2014).

Intriguingly, our CRISPR-Cas9 genome-wide gene dropout screens in NALM-6 and MCF7 cells revealed that some of the most prominent genes associated with response to samuraciclib were involved in the mTOR pathway. Specifically, sgRNAs targeting genes directly involved in mTORC1/2 complexes, including mTOR itself, were significantly enriched, suggesting that the loss of mTOR activity leads to resistance to samuraciclib. Further, analysis of the NALM-6 gene dropout CRISPR screen results demonstrated the enrichment of mTOR signalling pathways (Wilson *et al.*, 2023). In my analysis, mTOR genes and proteins were dysregulated in the previously generated RNA-seq dataset as well as my quantitative proteomics data by samuraciclib. Furthermore, the mTOR pathway was significantly enriched in both RNA-sequencing and proteomics data.

Samuraciclib was shown to inhibit cell proliferation, which is in line with my EdU incorporation assay results, by inducing cell cycle arrest predominantly in G1. Wilson *et al.* suggested that by inhibiting CDK7 with samuraciclib, cancer cells irreversibly enter a senescent state which largely depends on active mTOR growth signalling (Wilson *et al.*, 2023).

To understand the role of mTOR in the cellular response to samuraciclib, I first determined if the growth-inhibitory response to samuraciclib is dependent on a functional mTOR pathway. Inhibiting the mTOR pathway prior to samuraciclib treatment prevented the samuraciclib-induced growth arrest in MCF7 cells. Furthermore, the extent to which growth arrest by samuraciclib was reduced differed depending on the mTOR pathway inhibitor. For example, the mTORC1/2 inhibitor torin1 prevented the growth arrest by samuraciclib most strikingly, followed by the mTORC1/2 and PI3K inhibitor samotolisib, and the PI3K α inhibitor alpelisib. Everolimus (mTORC1 inhibitor), sapanisertib (mTORC1/2 inhibitor), and MK-2206 (AKT inhibitor) reduced samuraciclib-induced growth-arrest to a lesser extent. The differences between the drugs targeting different components of the PI3K/Akt/mTOR pathway in modulating growth-arrest by samuraciclib are likely to be due to off-target effects and the involvement of other pathways. However, this conclusion should be tempered by the possibility that a more appropriate concentration of the drugs needs to be established. For a better understanding of which part of the PI3K/Akt/mTOR pathway is involved in samuraciclib response, similar experiments with optimised drug concentrations would have to be repeated, allowing for statistical analyses. Regardless, the observation that samuraciclib-induced growth-arrest was mitigated by inhibiting the PI3K/Akt/mTOR pathway at several different points, including by inhibiting PI3K, Akt, and mTORC1/2, suggests that PI3K/Akt/mTOR pathway activity is indeed important for cellular response to samuraciclib.

4.1.2 mTOR inhibition impairs samuraciclib-mediated downregulation of cell cycle CDK phosphorylation

In addition to showing that a loss of PI3K/Akt/mTOR pathway function impairs samuraciclib-induced growth arrest, I also demonstrated that increased PI3K/Akt/mTOR pathway activity enhances the response to samuraciclib. To this effect, I evaluated how PI3K/Akt/mTOR pathway-activating mutations of PIK3CA, the catalytic subunit of PI3K, influences samuraciclib-induced cell growth arrest. The PIK3CA-E545K and -H1047R mutations, which constitutively activate PIK3CA and thus the PI3K/Akt/mTOR pathway (Meyer *et al.*, 2013), prolong the growth-arrest induced by samuraciclib treatment, as indicated by long-term growth assays in isogenic MCF10A cells in which these PIK3CA mutations common in ER+ breast cancer, have been introduced with CRISPR-Cas9 genome engineering (Koundouros *et al.*, 2020).

To further profile the consequences of an overactive PI3K/Akt/mTOR pathway in the cellular response to samuraciclib, I performed immunoblotting of key mTOR pathway effectors and CDK7 substrates. I observed several differences in protein and phosphorylation levels that confirmed CDK7 inhibition by samuraciclib. For example, samuraciclib treatment decreased CDK1 and CDK2 phosphorylation in all three cell lines, irrespective of the PIK3CA mutational status. Interestingly, the inhibition of CDK1 and CDK2 phosphorylation by samuraciclib was abrogated in the presence of torin1 in PIK3CA-WT and -E545K cells, although the increased phosphorylation was accompanied by increased levels of CDK1 and CDK2 proteins. However, a similar increase in CDK1 and CDK2 levels in the combination of samuraciclib with torin1 was not observed in PIK3CA-H1047R cells. Whether this reflects a difference in the mode of action of the two PIK3CA mutations in cell cycle progression was not investigated but remains an intriguing observation. This difference in CDK1/2 levels was not translated to differences in growth inhibition by samuraciclib and/or torin1, when comparing the E545K and H1047R cells. Samuraciclib inhibited the growth of MCF10A with either PIK3CA mutation to a similar extent and much more potently than it inhibited PIK3CA-WT cells. A similar increase in CDK1/2 and their phosphorylation was not observed for the PI3K α inhibitor alpelisib, or the AKT inhibitor MK-2206. Overall, these results would indicate that an active mTORC1/2, but not PI3K α or Akt, is necessary for samuraciclib to inhibit CDK1 and CDK2 phosphorylation. These results further suggest that the involvement of the PI3K/Akt/mTOR pathway in cell growth arrest by samuraciclib may be by facilitating reductions in the phosphorylation and/or levels of cell cycle CDKs by CDK7 inhibitors. However, it is important to consider that my results show only a single replicate, and confirmation of this observation would be required.

4.1.3 Patients with PIK3CA mutations may benefit from samuraciclib treatment

The correlation between PI3K/Akt/mTOR pathway activity and samuraciclib-induced growth arrest could provide a novel therapeutic opportunity for a subset of BC patients. PIK3CA mutations are among the most common alterations in BC and occur in 20-40% of patients, with E545K and H1047R accounting for 35% and 17% of PIK3CA mutations in BC patients, respectively (Martinez-Saez *et al.*, 2020). PIK3CA mutations are especially relevant in ER+ BC. Moreover PIK3CA mutations have been associated with a lower complete therapeutic responses to hormone therapies, due to the PI3K/Akt/mTOR pathway driving BC growth and progression independently from hormone receptor pathways (Bosch *et al.*, 2015; Guo *et al.*, 2020). Improving response rates in patients with activating PIK3CA mutations, for example by

combining samuraciclib with hormone therapies, would represent an important clinical advance.

My results suggest that a novel therapeutic strategy for PIK3CA-mutant BC patients could be samuraciclib treatment. I demonstrated that MCF10A cells with activating mutations in PIK3CA, namely E545K and H1047R, show enhanced growth inhibition by samuraciclib. Thus, the substantial subset of BC patients with PIK3CA driver mutations could especially benefit from samuraciclib treatment. The recently reported phase I clinical trial did not note any correlation between PIK3CA mutational status and progression-free survival in ER+ patients co-treated with samuraciclib and fulvestrant (Coombes *et al.*, 2023). Importantly, these patients had already progressed on prior hormone therapy combined with CDK4/6 inhibitors, which may have led to altered CDK1 and/or CDK2 levels or activities. Indeed, *in vitro* studies identify elevated CDK2 activity as a mechanism of resistance to CDK4/6i (Al-Qasem *et al.*, 2022; Herrera-Abreu *et al.*, 2016). Moreover, the combination with fulvestrant may mask differences in response to samuraciclib. In this context, cyclin E1 and cyclin E2, which are the cyclins that bind to CDK2 during the G1/S transition, have been proposed as biomarkers of response to CDK4/6i, implying that their elevated levels increase CDK2 activity and possible substitution for CDK4/6 in the G1 phase of the cell cycle (Milioli *et al.*, 2020). Both cyclin E1 and cyclin E2 are ER target genes and fulvestrant would reduce their levels and thus reduce CDK2 activity. It would undoubtedly be interesting to determine the influence of combining samuraciclib with fulvestrant in the growth response of PIK3CA-WT and mutant cells, although I note that MCF10A is a transformed, but non-tumourigenic, ER-negative breast cell line. More relevant breast cancer cell lines would, therefore, need to be assessed. Patient numbers with detectable PIK3CA mutations in the published phase I study were limited in number (five patients with detected PIK3CA mutations out of a cohort of 25). Comparison of larger patient cohorts treated with samuraciclib and fulvestrant would also aid in determining the influence of PIK3CA mutations on response to samuraciclib. The larger phase 2 studies for ER+ BC patients now underway will compare fulvestrant alone with fulvestrant with samuraciclib, which should facilitate more detailed analysis of the impact of PIK3CA mutations on response to samuraciclib.

Importantly, aside from activating PIK3CA mutations, other genomic alterations of PI3K/Akt/mTOR pathway components lead to an overactive pathway and are common in BC. For example, in addition to the commonality of PIK3CA mutations in BC, genomic

amplifications of PIK3CA has been noted in 8.7% of BC cases (Wu *et al*, 2005). Loss of the tumour suppressor PTEN or activating mutations in Akt are also notable in BC and would contribute to enhancing the activity of the PI3K/Akt/mTOR pathway (LoPiccolo *et al*, 2008; Mabuchi *et al*, 2015; Polivka & Janku, 2014; Porta *et al.*, 2014). Considering the results of my and others' work (Wilson *et al.*, 2023) in illuminating the role of an active PI3K/Akt/mTOR pathway in driving samuraciclib response, patients with any of the mentioned alterations that upregulate the pathway activity may benefit from treatment with the CDK7i.

In addition to identifying a subgroup of BC patients who are more responsive to samuraciclib, it is also important consider patients who would not respond to CDK7i. My results directly demonstrate that patients receiving therapies against PI3K/Akt/mTOR may be less responsive to samuraciclib. mTORC1/2 inhibitors, such as everolimus and sapanisertib (Gokmen-Polar *et al.*, 2012; Hasskarl, 2018), pan-Akt inhibitors, such as GDC-0068 (Ippen *et al.*, 2019), the PI3K α inhibitor, alpelisib (Narayan *et al*, 2021), and dual PI3K/mTOR inhibitors, such as dactolisib (NVP-BEZ235) (Serra *et al*, 2008), are all clinically-approved or in development for targeting PI3K/Akt/mTOR pathway-activated cancers. Most recently, the AKT inhibitor capavertisib in combination with fulvestrant was shown to significantly prolong progression-free survival, compared to fulvestrant alone (Turner *et al*, 2023). On this basis, capivasertib, in combination with fulvestrant, has been granted FDA approval for patients with locally advanced or metastatic ER+, HER2- BC with one or more alterations in PIK3CA/Akt1/PTEN, following progression on prior endocrine therapy. Given the clinical use of everolimus, capivasertib, and alpelisib, caution would need to be exercised in considering any combination with CDK7i. It would also be interesting to test if prior treatment with inhibitors of PI3K/Akt/mTOR reduces responsiveness to samuraciclib or if cessation of PI3K/Akt/mTOR inhibition prior to challenge with CDK7i would be effective.

4.2 CDK7i and the ferroptosis cell death pathway

4.2.1 Samuraciclib pre-treatment sensitizes breast cancer cells to ferroptosis

Ferroptosis is a novel form of iron-dependent regulated cell death (RCD), which is distinct from other types of regulated cell death and is characterized by oxidative damage to phospholipids. Ferroptosis can be induced by inactivating ferroptosis-suppressing proteins. As a result, RSL3 and ML210 have become the standard small molecule inhibitors extensively used *in vitro* to study mechanisms of ferroptosis (Sui *et al.*, 2018; Wang *et al.*, 2023). Both

compounds covalently link to the selenocysteine, the nucleophilic amino acid residue at the active site of GPX4, and inhibit the enzymatic function of GPX4, the main intracellular antioxidant that prevents cells from undergoing ferroptosis (Forcina & Dixon, 2019; Yang *et al.*, 2016).

Our CRISPR-Cas9 genome-wide gene dropout screens in NALM-6 and MCF7 cells notably revealed the major ferroptosis suppressor, *GPX4*, whose guide RNA was significantly downregulated following 150 nM and 500 nM samuraciclib treatment in MCF7 and NALM-6 cell lines, respectively, indicating that GPX4 promotes resistance to this CDK7i (Forcina & Dixon, 2019; Ingold *et al.*, 2018) and indicated that ferroptosis is associated with the cellular response to samuraciclib. As part of my aim to explore the role of ferroptosis, I have demonstrated that pre-treatment with samuraciclib sensitizes ER+ and triple negative breast cancer cells to ferroptosis induction by RSL3 and ML210. Pre-treatment with samuraciclib resulted in RSL3-induced cell death of MCF7 cells, which are otherwise reported to be insensitive to RSL3-directed ferroptosis (Park *et al.*, 2023). The same effect was seen in another ER+ breast cancer cell line, T47D, as well as the triple-negative MDA-MB-453. On its own RSL3 did not inhibit MCF7 or MDA-MB-453 cell growth and only modestly reduced growth of T47D cells.

The loss in cell numbers with RSL3 or ML210 treatment, after CDK7i pre-treatment, unequivocally demonstrates that the form of cell death is ferroptosis because this cell death was completely reversed by Ferrostatin-1 (F1). F1 is a ferroptosis-specific inhibitor that has no effect on cells undergoing apoptosis or any other form of cell death (Dixon *et al.*, 2012; Zilka *et al.*, 2017). In MCF7 cells, sensitization to RSL3-induced cell death was also seen following a nine-day pre-treatment with the covalent CDK7i, SY-1365, albeit with a shorter growth inhibition compared to pre-treatment with the non-covalent CDK7i, samuraciclib. These results may indicate that non-covalent CDK7 inhibition results in a more pronounced sensitization of cancer cells to ferroptosis. Although, it is important to note that the SY-1365 pre-treatment was only done once and requires confirmation. Use of additional covalent CDK7i (THZ1, YKL-5-124) and non-covalent CDK7i (SY-5609, LDC4297) (Sava *et al.*, 2020; Song *et al.*, 2024) would determine if there are indeed differences in ferroptosis sensitisation among the two main classes of CDK7i.

While these results indicate that both ER+ and triple-negative breast cancer cell lines become sensitized to ferroptosis by CDK7i several of my experiments must be repeated for greater confidence. This work consisted of only one biological replicate for several experiments, including the covalent CDK7i SY-1365, the GPX4 inhibitor ML210, and the TNBC cell line MDA-MB-453. Further, this study should be expanded to more cell lines to elucidate the full scope of samuraciclib sensitization to ferroptosis, including HER2+ cell lines; HER2+ BC makes up 15-20% of all BC cancers and has not been evaluated for response to CDK7i. Nonetheless, my data suggest that ER+ and possibly triple-negative BC patients may benefit from treatment with ferroptosis-inducers after or in combination with CDK7i treatment. While GPX4 inhibitors, such as RSL3 and ML210, are not suitable for studies *in vivo*, the emergence of orally bioavailable ferroptosis-inducing drugs, such as the GPX4 inhibitor compound 28 (Rodencal *et al*, 2024), enables future clinical testing to improve patient outcomes possibly in combination with samuraciclib. To gain a greater understanding of the clinical relevance of combining GPX4i with samuraciclib, this work could be expanded to patient-derived organoid or mouse xenograft models, where patient tumours following a CDK7i treatment regimen could be tested for sensitivity to ferroptosis through the experimental ferroptosis inducers, RSL3, ML210, or the oral candidate, compound 28.

4.2.2 Samuraciclib disrupts iron homeostasis in MCF7 cells

The proteomics analysis following 24-hour treatment with samuraciclib offers an analysis of protein changes following CDK7 inhibition. I confirmed several of the most significant protein changes observed in the samuraciclib proteome analysis by Western blotting, corroborating the robustness of the proteomic dataset. These changes included significant alterations in proteins associated with intracellular iron homeostasis, with FTH1, NCOA4, and HMOX1 showing clear upregulation in the proteomics analysis with 0.5 μ M samuraciclib. While the role of HMOX1 in ferroptosis induction remains context-dependent (Kwon *et al.*, 2015; Sun *et al*, 2016), FTH1 oxidises ferrous iron (Fe^{2+}) into ferric iron (Fe^{3+}), storing it for later use, and protecting cells from ferrous iron-induced ferroptosis (Tian *et al.*, 2020). On the other hand, NCOA4 is a ferritin cargo receptor that regulates ferritinophagy, which is a form of autophagic degradation of ferritin that increases intracellular iron pools and contributes to ferroptosis (Hou *et al.*, 2016; Mancias *et al*, 2015). Disruption of these proteins by CDK7i may lead to increased vulnerability to ferroptosis, as iron accumulation within cells is a hallmark of ferroptosis (Dixon & Stockwell, 2019). Thus, the availability of redox-active iron, because of altered levels of iron-regulating genes, such as FTH1, HMOX1, or NCOA4, may potentiate the GPX4i

sensitising effects of samuraciclib. To test this, ferrous (Fe^{2+}) iron-specific fluorescent probes, as previously described (Ni *et al.*, 2022), could be used to determine levels of Fe^{2+} after samuraciclib treatment, where an accumulation of ferrous iron would explain cell sensitization to GPX4i. Further, cell growth assays could be repeated using an iron chelator, deferoxamine (DFO) (Dixon *et al.*, 2012), in combination with samuraciclib during the nine day-pre-treatment period to determine whether iron accumulation is sufficient to sensitize cells to GPX4i-mediated cell death following CDK7i pre-treatment. While deeper mechanistic insights remain to be elucidated, my data strongly indicate that iron homeostasis is disrupted by samuraciclib.

4.2.3 Short-term treatment with CDK7 inhibitors upregulates an anti-ferroptotic response

Interestingly, treatment with SY-1365 and samuraciclib for 48 hours showed upregulation of proteins involved in anti-ferroptotic response, such as NRF2, FSP1, FTH1, GCLM, and GPX4, indicating that this is a general effect of CDK7 inhibition. Proteomic analysis of MCF7 cells treated for 24 hours with samuraciclib revealed a general upregulation of ferroptosis-associated proteins with 0.5 μM , and the exclusive upregulation of ferroptosis suppressors with 2.0 μM samuraciclib. Furthermore, the increase in the major antioxidant transcription factor NRF2 activity, as measured in AREc32 NRF2 reporter cells after a 24- and 48-hour treatment with samuraciclib, confirms that samuraciclib upregulates an anti-ferroptotic response within a short timeframe, albeit the response diminishes with higher samuraciclib concentrations (*i.e.*, 1 μM and 2 μM). Altogether, my findings indicate that treatment with a CDK7i initially promotes an anti-ferroptotic response in cancer cells. However, following longer-term treatment, as seen with the 33-day growth assays described earlier, cancer cells may exhaust their anti-ferroptotic defence systems and eventually succumb to ferroptosis induction by GPX4 inhibition.

4.2.4 CDK7 inhibition sensitizes cells to ferroptosis through a different mechanism than CDK4/6 inhibitors

Whereas GPX4 inhibitors caused ferroptosis following samuraciclib treatment, drugs inhibiting xCT (SLC7A11) and GCL did not cause ferroptosis. Specifically, the system xCT inhibitors sorafenib and sulfasalazine (Dixon *et al.*, 2012; Louandre *et al.*, 2013), and BSO, which inhibits GCL, the rate-limiting enzyme responsible for GSH production (Yang *et al.*, 2014), did not sensitize samuraciclib-pretreated MCF7 cells to ferroptosis. The reasons for this difference are unclear, given that inhibition of SLC7A11 and GCL has been shown in many

studies to cause ferroptosis (Dixon *et al.*, 2012; Dixon *et al.*, 2014; Yang *et al.*, 2016). By causing cell cycle arrest, the CDK4/6 inhibitor, palbociclib, has been shown to sensitize cancer cells to ferroptosis via GPX4 inhibition by RSL3 and ML210, and not erastin2 (Rodencal *et al.*, 2024), a potent and selective inhibitor of system xCT (Dixon *et al.*, 2014). Considering that CDK7 inhibition will reduce CDK4/6 activity and arrest cells in G1 (Bisteau *et al.*, 2013; Garrett *et al.*, 2001; Schachter *et al.*, 2013), CDK7 inhibitors may sensitize cancer cells to ferroptosis via GPX4 inhibition through the same mechanisms as CDK4/6 inhibitors. To test this hypothesis, I looked at the expression of the two proteins identified by Rodencal *et al.* that are downregulated following CDK4/6 inhibition, *MBOAT1* and *EMP2*. These proteins were associated with cell cycle arrest and have been correlated with sensitization to ferroptosis (Rodencal *et al.*, 2024). Downregulation of *MBOAT1* and *EMP2* expression, after treatment with palbociclib and the MEK1/2 inhibitor pimasertib, which also arrests cells at the same cell cycle phase as palbociclib (G1), was shown to associate with RSL3 and ML210 sensitivity, as defined by correlation analysis of GPX4i sensitivity data from the cancer therapeutics response portal (CTRP) (Rodencal *et al.*, 2024). Pimasertib treatment alone was shown to downregulate *MBOAT1*, indicating it is a cell cycle arrest associated effect. Interestingly, *MBOAT1* overexpression in HT-1080^N cells was sufficient to revert sensitization to GPX4 inhibition upon cell cycle arrest. Furthermore, *EMP2* expression was reduced following overexpression of p21 (Rodencal *et al.*, 2024). These results lead to the hypothesis that CDK7 inhibition by samuraciclib, which sensitizes cells to GPX4i and causes cell cycle arrest and p21 upregulation (Nguyen *et al.*, unpublished), would also reduce *MBOAT1* and *EMP2*. However, a 24-hour treatment with samuraciclib or SY-1365 did not show reductions in *MBOAT1* and *EMP2* expression. Alongside *MBOAT1* and *EMP2*, Rodencal *et al.* identified two more genes associated with CDK4/6i-induced sensitivity to GPX4i, namely *MYO19* and *GPD2*. Expression of *MYO19* was not significantly altered by samuraciclib in our RNA-seq dataset for 0.5 μ M samuraciclib, while *GPD2* was significantly, but not extensively, downregulated (P-adjusted = 5.01E-5, log2FC = -0.46). In the proteomics study, *MBOAT1* and *EMP2* proteins were not detected, while *GPD2* and *MYO19* were detected, but their levels were not altered by 0.5 μ M or 2.0 μ M samuraciclib. Altogether, my data suggest that CDK7i does not sensitize cells to ferroptosis by the same cell cycle arrest-directed mechanism as described for CDK4/6i. Of note, Rodencal *et al.* prioritized the analysis of genes involved in cell cycle arrest, by overlapping the palbociclib RNA-seq dataset with RNA-seq following pimasertib treatment (Rodencal *et al.*, 2024). Therefore, while cell cycle arrest in G1 may sensitize cells to ferroptosis through *MBOAT1* and *EMP2*, CDK7 inhibition also arrests BC cells in G2/M (Patel

et al., 2018a; Sun *et al.*, 2020) and inhibits transcription in addition to its role in inhibiting the activities of cell cycle CDKs. Moreover, the results presented by Rodencal *et al.* (2024) relate to the a HT-1080 fibrosarcoma cell line, in which CDK4/6i may work through mechanisms distinct from those in ER+ BC cells. This potentially important mechanism for improving the anti-cancer action of CDK7i clearly requires further investigation.

Evidence that CDK7 inhibition sensitizes cells to a different mechanism than CDK4/6i is also evident in my CTRP data analysis, performed similarly to Rodencal *et al.* To identify which ferroptosis-associated proteins are altered following samuraciclib treatment, I overlapped the proteins from the FerrDb database with significant proteins from the 24-hour proteomics dataset. Next, these ferroptosis-associated proteins from the 24-hour proteomics were tested for sensitivity to at least one GPX4i (RSL3, ML210, or ML162). Of the 129 proteins in the “prioritized gene/protein set”, 43 genes/proteins were found to modulate GPX4i sensitivity, 11 of which were also significantly differentially expressed at the RNA level following samuraciclib treatment (9 altered in the same direction as the proteome). Interestingly, the prioritized gene set revealed four proteins that were previously reported as high-confidence CDK7 targets (Rimel *et al.*, 2020), namely SMG9, NDRG1, TFRC, and ELAVL1. Of these, only SMG9, which is part of the translation termination SURF complex and is involved in non-sense mediated decay (Kashima *et al.*, 2006; Yamashita *et al.*, 2009), was also significantly differentially regulated at the RNA level, indicating that samuraciclib primarily alters its transcription, which subsequently results in changes at the protein level. Considering the role of NDRG1 in lipid metabolism (Fang *et al.*, 2014) and the transferrin receptor (TFRC) in iron homeostasis (Fillebeen *et al.*, 2019; Gammella *et al.*, 2017), it brings to the fore the idea that samuraciclib could be sensitizing cells to ferroptosis by altering lipid composition or iron homeostasis mechanisms. Indeed, lipid peroxidation analysis via FACS, using the BODIPY C11 lipid peroxidation probe which localizes to lipid membranes, revealed a significant increase in lipid peroxidation after RSL3 and ML210 treatment in cells that were pre-treated with samuraciclib compared to the vehicle control. Therefore, pre-treatment with a CDK7i enhances membrane lipid peroxidation by RSL3 or ML210, sensitizing cells previously deemed to be insensitive to ferroptosis induction.

4.2.5 CDK7i sensitization to ferroptosis offers a new therapeutic strategy for breast cancer

The discovery that CDK7 inhibition sensitizing cells to ferroptosis has clinical implications for cancer treatment. Ferroptosis-inducing agents have received FDA approval. For example, altretamine, an anti-cancer drug that induces ferroptosis by inhibiting GPX4, is approved for treatment of ovarian cancer (Woo *et al*, 2015). Metformin, a diabetes drug that has gained attention for a cancer treatment, induces ferroptosis by inhibiting xCT (Yang *et al*, 2021b). Sorafenib, which is an approved therapy for kidney and liver cancer, also induces ferroptosis by inhibiting xCT (Lachaier *et al.*, 2014). Finally, FDA approval of sulfasalazine for the treatment of rheumatoid arthritis and ulcerative colitis facilitates its use in other clinical studies (Plosker & Croom, 2005). Of course, my data reveal that direct inhibition of GPX4 would be necessary for induction of ferroptosis in cells treated with CDK7i. Whether inhibition of drugs other than those targeting GPX4, such as xCT inhibitors, could work in other cancer types, remains to be determined. Notwithstanding, the sensitisation to ferroptosis provides a potential route to potentiating the therapeutic response to CDK7i.

The value of using ferroptosis induction in the context of cancer treatment is further emphasized by the recent notion that persister cells, or cancers that have become drug tolerant and escape cancer therapies, are selectively sensitive to ferroptosis (Hangauer *et al.*, 2017; Viswanathan *et al.*, 2017). Drug-tolerant persister cells represent a population of cells that have survived anti-cancer treatment and may eventually emerge as cells that cause tumour relapse. Targeting these cell populations using an intrinsic vulnerability emphasizes the importance of using ferroptosis inducers as a novel therapeutic strategy. Thus, the combination of samuraciclib with GPX4 inhibitors may prove to be an effective combination strategy for patients, especially those whose cancer has recurred and are now faced with limited treatment options or in adjuvant settings to prevent recurrence. Collectively, my results suggest that a cancer therapy that combines samuraciclib with ferroptosis inducers, such as altretamine, could be a novel therapeutic strategy that is especially effective for patients at-risk of tumour recurrence.

5. Conclusions

Previous research conducted in our lab implicated GPX4 and the PI3K/Akt/mTOR pathway in the cellular response to samuraciclib. The aim of my work was to provide more understanding on the role of ferroptosis and the PI3K/Akt/mTOR pathway in modulating the response to CDK7i. My results shows that active mTOR signalling promotes the cell growth arrest induced by samuraciclib and that specific inhibition of the mTORC1/2 complexes reverts the anti-proliferative effects of samuraciclib by impairing the downregulation of cell cycle CDK phosphorylation. In addition, I found that samuraciclib sensitizes HR+ and triple-negative BC cells to ferroptosis by increasing lipid peroxidation following GPX4 inhibition via RSL3 and ML210 and by altering key iron-regulatory proteins. While the precise molecular mechanisms remain to be further defined, these data present compelling evidence that could help to guide samuraciclib treatment strategies. My results indicate that the administration of samuraciclib in combination with mTOR inhibitors should be avoided, while ferroptosis inducers may help promote the anti-cancer action of the CDK7 inhibitor. My research has enhanced our understanding of the molecular responses to samuraciclib that may help guide future BC therapeutic strategies.

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Appendix

Supplementary tables

Supplementary table 1 Significantly enriched pathways of the Reactome gene sets from 24-hour proteomics data treated with 0.5 μ M and 2 μ M samuraciclib.

Pathway name	P-adjusted	Gene set size	Source of enrichment
REACTOME_RRNA_PROCESSING	5.95E-44	205	500nM-DMSO 2000nM-DMSO
REACTOME_INFLUENZA_INFECTION	4.46E-38	157	500nM-DMSO 2000nM-DMSO
REACTOME_EUKARYOTIC_TRANSLATION_INITIATION	1.67E-30	120	500nM-DMSO 2000nM-DMSO
REACTOME_REGULATION_OF_EXPRESSION_OF_SLITS_AND_ROBOS	1.83E-29	172	500nM-DMSO 2000nM-DMSO
REACTOME_SELENOAMINO_ACID_METABOLISM	9.38E-28	118	500nM-DMSO 2000nM-DMSO
REACTOME_SIGNALING_BY_ROBO_RECEPTORS	1.18E-27	218	500nM-DMSO 2000nM-DMSO
REACTOME_NONSENSE_MEDIATED_DECAY_NMD	1.28E-27	116	500nM-DMSO 2000nM-DMSO
REACTOME_EUKARYOTIC_TRANSLATION_ELONGATION	2.04E-27	94	500nM-DMSO 2000nM-DMSO
REACTOME_SRP_DEPENDENT_COTRANSLATIONAL_PROTEIN_TARGETING_TO_MEMBRANE	4.17E-27	113	500nM-DMSO 2000nM-DMSO
REACTOME_HIV_INFECTION	1.63E-24	231	500nM-DMSO 2000nM-DMSO
REACTOME_RESPONSE_OF_EIF2AK4_GCN2_TO_AMINO_ACID_DEFICIENCY	3.49E-24	102	500nM-DMSO 2000nM-DMSO
REACTOME_MRNA_SPLICING	2.92E-23	212	500nM-DMSO 2000nM-DMSO
REACTOME_MITOTIC_METAPHASE_AND_ANAPHASE	2.65E-22	236	500nM-DMSO 2000nM-DMSO
REACTOME_HOST_INTERACTIONS_OF_HIV_FACTORS	2.08E-21	131	500nM-DMSO 2000nM-DMSO
REACTOME_CELLULAR_RESPONSE_TO_STARVATION	1.19E-19	157	500nM-DMSO 2000nM-DMSO
REACTOME_RRNA_MODIFICATION_IN_THE_NUCLEUS_AND_CYTOSOL	1.46E-16	60	500nM-DMSO 2000nM-DMSO
REACTOME_SARS_COV_2_HOST_INTERACTIONS	3.59E-16	204	500nM-DMSO 2000nM-DMSO
REACTOME_TRANSPORT_OF_MATURE_TRANSCRIPT_TO_CYTOPLASM	3.87E-16	84	500nM-DMSO 2000nM-DMSO
REACTOME_SEPARATION_OF_SISTER_CHROMATIDS	5.34E-16	191	500nM-DMSO 2000nM-DMSO
REACTOME_GLYCOSE_METABOLISM	1.49E-14	94	500nM-DMSO 2000nM-DMSO
REACTOME_S_PHASE	1.78E-14	163	500nM-DMSO 2000nM-DMSO
REACTOME_ACTIVATION_OF_THE_MRNA_UPON_BINDING_OF_THE_CAP_BINDING_COMPLEX_AND_EIFS_AND_SUBSEQUENT_BINDING_TO_4	1.31E-12	60	500nM-DMSO 2000nM-DMSO
REACTOME_CELLULAR_RESPONSE_TO_CHEMICAL_STRESS	1.48E-12	214	500nM-DMSO 2000nM-DMSO
REACTOME_CELLULAR_RESPONSE_TO_HEAT_STRESS	2.11E-12	101	500nM-DMSO 2000nM-DMSO
REACTOME_REGULATION_OF_HSF1_MEDIATED_HEAT_SHOCK_RESPONSE	3.38E-12	82	500nM-DMSO 2000nM-DMSO
REACTOME_HIV_LIFE_CYCLE	5.04E-12	149	500nM-DMSO
REACTOME_SARS_COV_1_INFECTION	5.53E-12	143	500nM-DMSO 2000nM-DMSO
REACTOME_CYTOSOLIC_TRNA_AMINOACYLATION	7.04E-12	24	2000nM-DMSO
REACTOME_SYNTHESIS_OF_DNA	7.81E-12	121	500nM-DMSO 2000nM-DMSO
REACTOME_INTERACTIONS_OF_REV_WITH_HOST_CELLULAR_PROTEINS	8.61E-12	37	500nM-DMSO 2000nM-DMSO
REACTOME_THE_CITRIC_ACID_TCA_CYCLE_AND_RESPIRATORY_ELECTRON_TRANSPORT	2.44E-11	178	500nM-DMSO 2000nM-DMSO
REACTOME_SNRNP_ASSEMBLY	3.60E-11	54	500nM-DMSO 2000nM-DMSO
REACTOME_NUCLEAR_IMPORT_OF_REV_PROTEIN	1.11E-10	34	500nM-DMSO 2000nM-DMSO
REACTOME_RNA_POLYMERASE_II_TRANSCRIPTION_TERMINATION	1.25E-10	67	500nM-DMSO 2000nM-DMSO
REACTOME_NUCLEAR_ENVELOPE_BREAKDOWN	1.56E-10	53	500nM-DMSO 2000nM-DMSO
REACTOME_VIRAL_MESSENGER_RNA_SYNTHESIS	2.72E-10	44	500nM-DMSO 2000nM-DMSO
REACTOME_SARS_COV_1_HOST_INTERACTIONS	5.29E-10	98	500nM-DMSO 2000nM-DMSO
REACTOME_TRANSPORT_OF_THE_SLRP_DEPENDANT_MATURE_MRNA	7.10E-10	36	500nM-DMSO
REACTOME_ER_TO_GOLGI_ANTEROGRADE_TRANSPORT	7.34E-10	155	500nM-DMSO 2000nM-DMSO
REACTOME_TRNA_AMINOACYLATION	7.82E-10	42	500nM-DMSO 2000nM-DMSO
REACTOME_GLYCOLYSIS	8.42E-10	74	500nM-DMSO 2000nM-DMSO
REACTOME_REGULATION_OF_MRNA_STABILITY_BY_PROTEINS_THAT_BIND_AU_RICH_ELEMENTS	9.91E-10	87	500nM-DMSO 2000nM-DMSO
REACTOME_TRANSPORT_OF_MATURE_MRNAS_DERIVED_FROM_INTRONLESS_TRANSCRIPTS	1.39E-09	43	500nM-DMSO
REACTOME_PROTEIN_LOCALIZATION	1.49E-09	164	500nM-DMSO 2000nM-DMSO
REACTOME_INTERACTIONS_OF_VPR_WITH_HOST_CELLULAR_PROTEINS	1.66E-09	37	500nM-DMSO 2000nM-DMSO
REACTOME_INTRA_GOLGI_AND_RETROGRADE_GOLGI_TO_ER_TRAFFIC	2.23E-09	203	500nM-DMSO 2000nM-DMSO
REACTOME_TRNA_PROCESSING_IN_THE_NUCLEUS	5.80E-09	59	500nM-DMSO
REACTOME_KEAP1_NFE2L2_PATHWAY	6.41E-09	125	500nM-DMSO 2000nM-DMSO
REACTOME_ISG15_ANTIVIRAL_MECHANISM	8.05E-09	75	500nM-DMSO 2000nM-DMSO
REACTOME_MITOTIC_G1_PHASE_AND_G1_S_TRANSITION	8.11E-09	149	500nM-DMSO 2000nM-DMSO
REACTOME_APC_C_MEDIATED_DEGRADATION_OF_CELL_CYCLE_PROTEINS	8.43E-09	88	500nM-DMSO 2000nM-DMSO
REACTOME_NUCLEAR_PORE_COMPLEX_NPC_DISASSEMBLY	8.90E-09	36	500nM-DMSO 2000nM-DMSO
REACTOME_EXPORT_OF_VIRAL_RIBONUCLEOPROTEINS_FROM_NUCLEUS	9.08E-09	33	500nM-DMSO
REACTOME_RESOLUTION_OF_SISTER_CHROMATID_COHESION	1.45E-08	126	500nM-DMSO 2000nM-DMSO
REACTOME_PROGRAMMED_CELL_DEATH	2.34E-08	211	500nM-DMSO 2000nM-DMSO
REACTOME_APC_C_CDH1_MEDIATED_DEGRADATION_OF_CDC20_AND_OTHER_APC_C_CDH1_TARGETED_PROTEINS_IN_LATE_MITOSIS_EARLY	2.35E-08	74	2000nM-DMSO
REACTOME_SUMOYLATION	2.85E-08	189	500nM-DMSO 2000nM-DMSO
REACTOME_NS1_MEDIATED_EFFECTS_ON_HOST_PATHWAYS	3.42E-08	41	500nM-DMSO 2000nM-DMSO
REACTOME_G2_M_CHECKPOINTS	3.66E-08	167	500nM-DMSO 2000nM-DMSO
REACTOME_SWITCHING_OF_ORIGINS_TO_A_POST_REPLICATIVE_STATE	4.00E-08	92	500nM-DMSO 2000nM-DMSO
REACTOME_REGULATION_OF_GLUKINASE_BY_GLUKINASE_REGULATORY_PROTEIN	4.97E-08	32	500nM-DMSO
REACTOME_NUCLEAR_EVENTS_MEDIATED_BY_NFE2L2	5.31E-08	96	500nM-DMSO 2000nM-DMSO
REACTOME_APOPTOSIS	6.28E-08	179	2000nM-DMSO
REACTOME_SUMOYLATION_OF_UBIQUITINYLIATION_PROTEINS	8.37E-08	39	500nM-DMSO
REACTOME_SUMOYLATION_OF_DNA_REPLICATION_PROTEINS	9.61E-08	46	combined
REACTOME_ANTIVIRAL_MECHANISM_BY_IFN_STIMULATED_GENES	2.15E-07	152	500nM-DMSO 2000nM-DMSO
REACTOME_MITOCHONDRIAL_PROTEIN_IMPORT	2.28E-07	65	500nM-DMSO 2000nM-DMSO
REACTOME_MITOTIC_PROMETAPHASE	2.66E-07	203	500nM-DMSO
REACTOME_MITOTIC_G2_G2_M_PHASES	4.49E-07	200	2000nM-DMSO
REACTOME_RESPIRATORY_ELECTRON_TRANSPORT	4.69E-07	103	2000nM-DMSO
REACTOME_SUMOYLATION_OF_SUMOYLATION_PROTEINS	5.01E-07	35	500nM-DMSO
REACTOME_SARS_COV_2_MODULATES_HOST_TRANSLATION_MACHINERY	5.54E-07	51	500nM-DMSO 2000nM-DMSO
REACTOME_TRANSPORT_TO_THE_GOLGI_AND_SUBSEQUENT_MODIFICATION	5.83E-07	186	500nM-DMSO 2000nM-DMSO
REACTOME_MITOTIC_SPINDLE_CHECKPOINT	8.19E-07	111	500nM-DMSO
REACTOME_ORC1_REMOVAL_FROM_CHROMATIN	1.21E-06	71	2000nM-DMSO
REACTOME_AUF1_HNRNP_D0_BINDS_AND_DESTABILIZES_MRNA	1.30E-06	55	2000nM-DMSO
REACTOME_RHOG_GTPASE_CYCLE	1.38E-06	74	500nM-DMSO 2000nM-DMSO
REACTOME_NUCLEAR_ENVELOPE_NE_REASSEMBLY	2.17E-06	76	500nM-DMSO
REACTOME_SUMOYLATION_OF_DNA_DAMAGE_RESPONSE_AND_REPAIR_PROTEINS	3.12E-06	77	500nM-DMSO
REACTOME_RESPIRATORY_ELECTRON_TRANSPORT_ATP_SYNTHESIS_BY_CHEMIOSMOTIC_COUPLING_AND_HEAT_PRODUCTION_BY_UNCOUPL	3.18E-06	127	2000nM-DMSO
REACTOME_THE_ROLE_OF_GTSE1_IN_G2_M_PROGRESSION_AFTER_G2_CHECKPOINT	7.38E-06	79	2000nM-DMSO
REACTOME_COPI_MEDIATED_ANTEROGRADE_TRANSPORT	8.03E-06	102	500nM-DMSO 2000nM-DMSO
REACTOME_SUMOYLATION_OF_RNA_BINDING_PROTEINS	9.45E-06	47	combined
REACTOME_GOLGI_TO_ER_RETROGRADE_TRANSPORT	1.05E-05	134	2000nM-DMSO
REACTOME_MHC_CLASS_II_ANTIGEN_PRESENTATION	1.77E-05	126	500nM-DMSO 2000nM-DMSO
REACTOME_RHO_GTPASES_ACTIVATE_FORMINS	1.77E-05	140	2000nM-DMSO
REACTOME_GENE_AND_PROTEIN_EXPRESSION_BY_JAK_STAT_SIGNALING_AFTER_INTERLEUKIN_12_STIMULATION	2.25E-05	38	2000nM-DMSO
REACTOME_FORMATION_OF_RNA_POL_II_ELONGATION_COMPLEX	3.35E-05	58	combined
REACTOME_INTERCONVERSION_OF_NUCLEOTIDE_DI_AND_TRIPHOSPHATES	3.76E-05	29	500nM-DMSO 2000nM-DMSO
REACTOME_SARS_COV_1_MODULATES_HOST_TRANSLATION_MACHINERY	4.19E-05	37	500nM-DMSO 2000nM-DMSO
REACTOME_METABOLISM_OF_POLYAMINES	4.45E-05	59	2000nM-DMSO
REACTOME_INTERLEUKIN_12_SIGNALING	4.62E-05	47	2000nM-DMSO
REACTOME_SARS_COV_2_ACTIVATES_MODULATES_INNATE_AND_ADAPTIVE_IMMUNE_RESPONSES	4.73E-05	126	500nM-DMSO 2000nM-DMSO

Pathway name	P-adjusted	Gene set size	Source of enrichment
REACTOME_SCF_BETA_TRCP_MEDIATED_DEGRADATION_OF_EMI1	4.77E-05	55	2000nM-DMSO
REACTOME_TRANS_GOLGI_NETWORK_VESICLE_BUDDING	7.47E-05	72	500nM-DMSO 2000nM-DMSO
REACTOME_DNA_REPLICATION	7.53E-05	187	2000nM-DMSO
REACTOME_NEGATIVE_REGULATION_OF_NOTCH4_SIGNALING	8.38E-05	54	2000nM-DMSO
REACTOME_TRANSCRIPTION_COUPLED_NUCLEOTIDE_EXCISION_REPAIR_TC_NER	8.93E-05	78	combined
REACTOME_SCF_SKP2_MEDIATED_DEGRADATION_OF_P27_P21	9.22E-05	60	2000nM-DMSO
REACTOME_DEGRADATION_OF_GLI1_BY_THE_PROTEASOME	9.98E-05	60	2000nM-DMSO
REACTOME_INTERLEUKIN_12_FAMILY_SIGNALING	0.000100133	57	2000nM-DMSO
REACTOME_DUAL_INCISION_IN_TC_NER	0.000136301	65	combined
REACTOME_TPS3_REGULATES_METABOLIC_GENES	0.000140566	87	2000nM-DMSO
REACTOME_FGFR2_ALTERNATIVE_SPLICING	0.000145195	27	500nM-DMSO
REACTOME_DISORDERS_OF_TRANSMEMBRANE_TRANSPORTERS	0.000170513	177	2000nM-DMSO
REACTOME_POSTMITOTIC_NUCLEAR_PORE_COMPLEX_NPC_REFORMATION	0.000179666	27	combined
REACTOME_CROSS_PRESENTATION_OF_SOLUBLE_EXOGENOUS_ANTIGENS_ENDOSOMES	0.000186535	50	2000nM-DMSO
REACTOME_DNA_STRAND_ELONGATION	0.000210086	32	500nM-DMSO
REACTOME_RNA_POLYMERASE_II_PRE_TRANSCRIPTION_EVENTS	0.000214534	81	combined
REACTOME_FATTY_ACID_METABOLISM	0.000223371	177	2000nM-DMSO
REACTOME_DNA_REPLICATION_PRE_INITIATION	0.000237441	159	2000nM-DMSO
REACTOME_PCP_CE_PATHWAY	0.00028518	92	2000nM-DMSO
REACTOME_MITOCHONDRIAL_TRANSLATION	0.000298285	96	2000nM-DMSO
REACTOME_HIV_TRANSCRIPTION_ELONGATION	0.000332974	43	combined
REACTOME_TRNA_PROCESSING	0.000371859	111	combined
REACTOME_GLUONEOGENESIS	0.000426138	35	500nM-DMSO 2000nM-DMSO
REACTOME_DEFECTIVE_CFTR_CAUSES_CYSTIC_FIBROSIS	0.000474303	61	2000nM-DMSO
REACTOME_CELLULAR_RESPONSE_TO_HYPOXIA	0.000525129	75	2000nM-DMSO
REACTOME_COPII_MEDIATED_VESICLE_TRANSPORT	0.000543725	68	500nM-DMSO 2000nM-DMSO
REACTOME_STABILIZATION_OF_P53	0.000547177	57	2000nM-DMSO
REACTOME_DEGRADATION_OF_DVL	0.000551445	57	2000nM-DMSO
REACTOME_REGULATION_OF_RUNX3_EXPRESSION_AND_ACTIVITY	0.000654708	55	2000nM-DMSO
REACTOME_DECTIN_1_MEDIATED_NONCANONICAL_NF_KB_SIGNALING	0.000669966	62	2000nM-DMSO
REACTOME_DOWNSTREAM_SIGNALING_EVENTS_OF_B_CELL_RECEPTOR_BCR	0.000759337	81	2000nM-DMSO
REACTOME_GOLGI_ASSOCIATED_VESICLE_BIOGENESIS	0.000807026	56	500nM-DMSO 2000nM-DMSO
REACTOME_MITOTIC_PROPHASE	0.00089508	142	combined
REACTOME_PROCESSING_OF_INTRONLESS_PRE_MRNAS	0.000963876	20	combined
REACTOME_PYRUVATE_METABOLISM_AND_CITRIC_ACID_TCA_CYCLE	0.00098891	55	500nM-DMSO
REACTOME_DEGRADATION_OF_AXIN	0.001294814	55	2000nM-DMSO
REACTOME_REGULATION_OF_RUNX2_EXPRESSION_AND_ACTIVITY	0.001389394	73	2000nM-DMSO
REACTOME_INTERLEUKIN_1_SIGNALING	0.001526204	114	2000nM-DMSO
REACTOME_METABOLISM_OF_NUCLEOTIDES	0.001578012	97	2000nM-DMSO
REACTOME_HEDGEHOG_LIGAND_BIOGENESIS	0.001793872	65	2000nM-DMSO
REACTOME_FORMATION_OF_THE_EARLY_ELONGATION_COMPLEX	0.001803121	33	combined
REACTOME_NUCLEOTIDE_EXCISION_REPAIR	0.001866294	110	combined
REACTOME_RHOD_GTPASE_CYCLE	0.001910937	53	2000nM-DMSO
REACTOME_CYTOPROTECTION_BY_HMOX1	0.002089209	64	2000nM-DMSO
REACTOME_CLEC7A_DECTIN_1_SIGNALING	0.002189634	100	2000nM-DMSO
REACTOME_SIGNALING_BY_BRAF_AND_RAF1_FUSIONS	0.002389663	65	2000nM-DMSO
REACTOME_CYCLIN_A_CDK2_ASSOCIATED_EVENTS_AT_S_PHASE_ENTRY	0.00317455	85	2000nM-DMSO
REACTOME_SOMITOGENESIS	0.003191794	55	2000nM-DMSO
REACTOME_PROCESSING_OF_CAPPED_INTRONLESS_PRE_MRNA	0.00344574	29	combined
REACTOME_CITRIC_ACID_CYCLE_TCA_CYCLE	0.00392339	22	combined
REACTOME_ABORTIVE_ELONGATION_OF_HIV_1_TRANSCRIPT_IN_THE_ABSENCE_OF_TAT	0.00404359	23	combined
REACTOME_RAC2_GTPASE_CYCLE	0.00443902	88	500nM-DMSO
REACTOME_REGULATION_OF_RAS_BY_GAPS	0.004496237	68	2000nM-DMSO
REACTOME_G1_S_DNA_DAMAGE_CHECKPOINTS	0.006004023	68	2000nM-DMSO
REACTOME_PROTEIN_UBIQUITINATION	0.00659572	79	500nM-DMSO
REACTOME_COPI_DEPENDENT_GOLGI_TO_ER_RETROGRADE_TRAFFIC	0.007139991	100	2000nM-DMSO
REACTOME_SYNTHESIS_OF_ACTIVE_UBIQUITIN_ROLES_OF_E1_AND_E2_ENZYMES	0.007315544	30	500nM-DMSO
REACTOME_TRANSCRIPTIONAL_REGULATION_BY_RUNX2	0.007933152	120	combined
REACTOME_DEGRADATION_OF_BETA_CATENIN_BY_THE_DESTRUCTION_COMPLEX	0.008069697	85	combined
REACTOME_TRANSCRIPTION_OF_THE_HIV_GENOME	0.008729858	70	combined
REACTOME_ANTIGEN_PRESENTATION_FOLDING_ASSEMBLY_AND_PEPTIDE_LOADING_OF_CLASS_I_MHC	0.009145475	29	2000nM-DMSO
REACTOME_RHOF_GTPASE_CYCLE	0.009504131	42	combined

Highlighted rows are shown in **Figure 3.13**.

Supplementary table 2 Significantly enriched pathways of the Gene Ontology Biological Processes (GOBP) gene sets from 24-hour proteomics data treated with 0.5 μ M and 2 μ M samuraciclib.

Pathway name	P-adjusted	Gene set size	Source of enrichment
GOBP_CYTOPLASMIC_TRANSLATION	4.54E-34	156	500nM-DMSO 2000nM-DMSO
GOBP_RIBOSOMAL_SMALL_SUBUNIT_BIOGENESIS	2.42E-24	104	500nM-DMSO 2000nM-DMSO
GOBP_PROTEIN_FOLDING	8.21E-20	223	500nM-DMSO 2000nM-DMSO
GOBP_REGULATION_OF_RNA_SPLICING	5.28E-18	180	500nM-DMSO 2000nM-DMSO
GOBP_RNA_LOCALIZATION	6.27E-18	197	500nM-DMSO 2000nM-DMSO
GOBP_REGULATION_OF_MRNA_PROCESSING	5.01E-17	128	500nM-DMSO 2000nM-DMSO
GOBP_NUCLEAR_EXPORT	5.52E-15	168	500nM-DMSO 2000nM-DMSO
GOBP_ESTABLISHMENT_OF_RNA_LOCALIZATION	7.03E-15	160	500nM-DMSO 2000nM-DMSO
GOBP_RNA_EXPORT_FROM_NUCLEUS	5.03E-14	92	500nM-DMSO 2000nM-DMSO
GOBP_CELLULAR_RESPIRATION	7.03E-13	243	500nM-DMSO 2000nM-DMSO
GOBP_AEROBIC_RESPIRATION	1.52E-12	197	500nM-DMSO 2000nM-DMSO
GOBP_SISTER_CHROMATID_SEGREGATION	1.90E-12	228	500nM-DMSO
GOBP_MITOTIC_SISTER_CHROMATID_SEGREGATION	4.13E-12	187	500nM-DMSO 2000nM-DMSO
GOBP_MRNA_TRANSPORT	4.57E-12	126	500nM-DMSO 2000nM-DMSO
GOBP_REGULATION_OF_RNA_SPLICING_VIA_SPLICEOSOME	5.13E-12	106	500nM-DMSO 2000nM-DMSO
GOBP_MATURATION_OF_SSU_RRNA	9.87E-11	53	500nM-DMSO 2000nM-DMSO
GOBP_NUCLEOBASE_CONTAINING_COMPOUND_TRANSPORT	1.08E-10	225	500nM-DMSO 2000nM-DMSO
GOBP_ENDOPLASMIC_RETICULUM_TO_GOLGI_VESICLE_MEDIATED_TRANSPORT	2.92E-10	138	500nM-DMSO 2000nM-DMSO
GOBP_PROTEIN_RNA_COMPLEX_ORGANIZATION	3.40E-10	241	500nM-DMSO 2000nM-DMSO
GOBP_MITOCHONDRIAL_TRANSPORT	8.13E-10	189	500nM-DMSO 2000nM-DMSO
GOBP_MRNA_EXPORT_FROM_NUCLEUS	9.63E-10	70	500nM-DMSO 2000nM-DMSO
GOBP_TRANSLATIONAL_INITIATION	1.51E-09	123	500nM-DMSO 2000nM-DMSO
GOBP_ALTERNATIVE_MRNA_SPLICING_VIA_SPLICEOSOME	3.03E-09	71	500nM-DMSO 2000nM-DMSO
GOBP_PROTEIN_LOCALIZATION_TO_MITOCHONDRION	3.89E-09	131	500nM-DMSO 2000nM-DMSO
GOBP_MRNA_3_END_PROCESSING	4.18E-09	56	500nM-DMSO 2000nM-DMSO
GOBP_AMINO_ACID_ACTIVATION	5.81E-09	45	2000nM-DMSO
GOBP_MATURATION_OF_SSU_RRNA_FROM_TRICISTRONIC_RRNA_TRANSCRIPT_SSU_RRNA_5_8S_RRNA_LSU_RR	6.23E-09	36	500nM-DMSO 2000nM-DMSO
GOBP_MICROTUBULE_CYTOSKELETON_ORGANIZATION_INVOLVED_IN_MITOSIS	6.34E-09	165	500nM-DMSO 2000nM-DMSO
GOBP_POSITIVE_REGULATION_OF_TRANSLATION	6.65E-09	141	500nM-DMSO 2000nM-DMSO
GOBP_RIBOSOMAL_LARGE_SUBUNIT_BIOGENESIS	1.13E-08	76	500nM-DMSO 2000nM-DMSO
GOBP_RESPIRATORY_ELECTRON_TRANSPORT_CHAIN	2.78E-08	124	2000nM-DMSO
GOBP_MITOTIC_SPINDLE_ORGANIZATION	3.05E-08	132	500nM-DMSO 2000nM-DMSO
GOBP_SPINDLE_ORGANIZATION	3.35E-08	218	500nM-DMSO 2000nM-DMSO
GOBP_ELECTRON_TRANSPORT_CHAIN	3.68E-08	176	2000nM-DMSO
GOBP_RNA_3_END_PROCESSING	5.25E-08	102	combined
GOBP_CHAPERONE_MEDIATED_PROTEIN_FOLDING	5.51E-08	75	2000nM-DMSO
GOBP_POSITIVE_REGULATION_OF_AMIDE_METABOLIC_PROCESS	7.76E-08	169	500nM-DMSO 2000nM-DMSO
GOBP_REGULATION_OF_CHROMOSOME_ORGANIZATION	7.77E-08	247	500nM-DMSO 2000nM-DMSO
GOBP_TRNA_METABOLIC_PROCESS	7.77E-08	197	500nM-DMSO 2000nM-DMSO
GOBP_IMPORT_INTO_NUCLEUS	2.03E-07	166	500nM-DMSO 2000nM-DMSO
GOBP_ATP_SYNTHESIS_COUPLED_ELECTRON_TRANSPORT	4.88E-07	102	2000nM-DMSO
GOBP_MITOCHONDRIAL_MEMBRANE_ORGANIZATION	5.43E-07	119	2000nM-DMSO
GOBP_ENDOPLASMIC_RETICULUM_ORGANIZATION	6.07E-07	101	500nM-DMSO 2000nM-DMSO
GOBP_NUCLEUS_ORGANIZATION	7.57E-07	147	500nM-DMSO 2000nM-DMSO
GOBP_CYTOPLASMIC_TRANSLATIONAL_INITIATION	1.06E-06	40	500nM-DMSO 2000nM-DMSO
GOBP_REGULATION_OF_CHROMOSOME_SEGREGATION	1.39E-06	131	combined
GOBP_REGULATION_OF_ALTERNATIVE_MRNA_SPLICING_VIA_SPLICEOSOME	1.80E-06	54	500nM-DMSO
GOBP_PROTEIN_TARGETING_TO_MITOCHONDRION	1.82E-06	106	500nM-DMSO 2000nM-DMSO
GOBP_OXIDATIVE_PHOSPHORYLATION	2.73E-06	148	2000nM-DMSO
GOBP_PROTEIN_STABILIZATION	4.52E-06	217	2000nM-DMSO
GOBP_DICARBOXYLIC_ACID_METABOLIC_PROCESS	4.89E-06	95	2000nM-DMSO
GOBP_MITOCHONDRIAL_TRANSLATION	5.64E-06	130	2000nM-DMSO
GOBP_REGULATION_OF_TRANSLATIONAL_INITIATION	9.71E-06	83	2000nM-DMSO
GOBP_RIBOSE_PHOSPHATE_BIOSYNTHETIC_PROCESS	9.88E-06	235	2000nM-DMSO
GOBP_ORGANELLE_DISASSEMBLY	1.01E-05	146	2000nM-DMSO
GOBP_MITOCHONDRIAL_GENE_EXPRESSION	1.01E-05	163	2000nM-DMSO
GOBP_NEGATIVE_REGULATION_OF_MRNA_METABOLIC_PROCESS	1.14E-05	98	2000nM-DMSO
GOBP_NEGATIVE_REGULATION_OF_RNA_SPLICING	1.59E-05	27	2000nM-DMSO
GOBP_DNA_CONFORMATION_CHANGE	1.65E-05	92	2000nM-DMSO
GOBP_GLUCCOSE_METABOLIC_PROCESS	1.72E-05	193	500nM-DMSO 2000nM-DMSO
GOBP_REGULATION_OF_NUCLEOCYTOPLASMIC_TRANSPORT	1.82E-05	109	500nM-DMSO
GOBP_MONOSACCHARIDE_BIOSYNTHETIC_PROCESS	2.59E-05	102	500nM-DMSO 2000nM-DMSO
GOBP_SPINDLE_ASSEMBLY	3.12E-05	149	500nM-DMSO
GOBP_REGULATION_OF_PROTEIN_LOCALIZATION_TO_NUCLEUS	3.90E-05	143	2000nM-DMSO
GOBP_PROTEIN_INSERTION_INTO_MEMBRANE	4.15E-05	77	2000nM-DMSO
GOBP_MATURATION_OF_5_8S_RRNA	5.81E-05	36	2000nM-DMSO
GOBP_TRICARBOXYLIC_ACID_CYCLE	7.35E-05	33	500nM-DMSO 2000nM-DMSO
GOBP_MITOTIC_CYTOKINESIS	9.03E-05	88	2000nM-DMSO
GOBP_METAPHASE_CHROMOSOME_ALIGNMENT	0.000103505	98	500nM-DMSO
GOBP_ESTABLISHMENT_OF_PROTEIN_LOCALIZATION_TO_MITOCHONDRIAL_MEMBRANE	0.000123749	38	2000nM-DMSO
GOBP_REGULATION_OF_SISTER_CHROMATID_SEGREGATION	0.000138803	104	combined
GOBP_RETROGRADE_TRANSPORT_ENDOSOME_TO_GOLGI	0.00016983	102	500nM-DMSO 2000nM-DMSO
GOBP_NUCLEOSIDE_TRIPHOSPHATE_METABOLIC_PROCESS	0.000179261	184	2000nM-DMSO
GOBP_CHROMOSOME_SEPARATION	0.000190491	81	combined
GOBP_NEGATIVE_REGULATION_OF_MRNA_PROCESSING	0.000200147	24	2000nM-DMSO
GOBP_PTERIDINE_CONTAINING_COMPOUND_METABOLIC_PROCESS	0.000203173	33	combined
GOBP_VESICLE_TARGETING	0.000234086	67	2000nM-DMSO
GOBP_NADP_METABOLIC_PROCESS	0.000243751	44	500nM-DMSO 2000nM-DMSO
GOBP_MITOTIC_SPINDLE_ASSEMBLY	0.000279143	73	combined
GOBP_CYTOSOLIC_TRANSPORT	0.000290958	186	500nM-DMSO
GOBP_REGULATION_OF_PROTEASOMAL_PROTEIN_CATABOLIC_PROCESS	0.000310915	196	combined
GOBP_MATURATION_OF_5_8S_RRNA_FROM_TRICISTRONIC_RRNA_TRANSCRIPT_SSU_RRNA_5_8S_RRNA_LSU_RR	0.000370131	25	combined
GOBP_CELL_REDOX_HOMEOSTASIS	0.000390387	41	2000nM-DMSO
GOBP_FIBROBLAST_PROLIFERATION	0.000393384	107	2000nM-DMSO
GOBP_STRESS_GRANULE_ASSEMBLY	0.000413588	31	500nM-DMSO
GOBP_VESICLE_BUDDING_FROM_MEMBRANE	0.000459392	84	500nM-DMSO 2000nM-DMSO
GOBP_FOLIC_ACID_CONTAINING_COMPOUND_METABOLIC_PROCESS	0.00047666	27	combined
GOBP_PYRIDINE_CONTAINING_COMPOUND_METABOLIC_PROCESS	0.00048459	86	2000nM-DMSO
GOBP_DNA_TEMPLATED_TRANSCRIPTION_ELONGATION	0.000547177	135	combined
GOBP_POSITIVE_REGULATION_OF_MRNA_PROCESSING	0.000554714	36	2000nM-DMSO

Pathway name	P-adjusted	Gene set size	Source of enrichment
GOBP_POSITIVE_REGULATION_OF_NUCLEOCYTOPLASMIC_TRANSPORT	0.000581038	62	combined
GOBP_MITOTIC_SISTER_CHROMATID_SEPARATION	0.000675521	62	combined
GOBP_SELECTIVE_AUTOPHAGY	0.000717927	97	2000nM-DMSO
GOBP_REGULATION_OF_PROTEOLYSIS_INVOLVED_IN_PROTEIN_CATABOLIC_PROCESS	0.000754436	236	combined
GOBP_MRNA_POLYADENYLATION	0.000781116	30	combined
GOBP_TRANSLATIONAL_ELONGATION	0.000825455	71	combined
GOBP_CYTOSKELETON_DEPENDENT_CYTOKINESIS	0.000915452	117	2000nM-DMSO
GOBP_POSITIVE_REGULATION_OF_RNA_SPLICING	0.000931825	45	2000nM-DMSO
GOBP_RESPONSE_TO_TOPOLOGICALLY_INCORRECT_PROTEIN	0.000987092	158	combined
GOBP_CHROMOSOME_LOCALIZATION	0.001168593	118	500nM-DMSO
GOBP_GLUCOSE_CATABOLIC_PROCESS	0.001208049	27	2000nM-DMSO
GOBP_REGULATION_OF_INTRINSIC_APOPTOTIC_SIGNALING_PATHWAY	0.00165947	190	combined
GOBP_DE_NOVO_PROTEIN_FOLDING	0.002275673	41	combined
GOBP_AUTOPHAGY_OF_MITOCHONDRION	0.002279975	98	combined
GOBP_MATURATION_OF_LSU_RRNA	0.002365866	28	500nM-DMSO 2000nM-DMSO
GOBP_GLUCOSE_6_PHOSPHATE_METABOLIC_PROCESS	0.002530806	29	2000nM-DMSO
GOBP_POSITIVE_REGULATION_OF_PROTEIN_LOCALIZATION_TO_NUCLEUS	0.002613909	93	2000nM-DMSO
GOBP_COPII_COATED_VESICLE_BUDDING	0.002696648	44	500nM-DMSO 2000nM-DMSO
GOBP_ENDOPLASMIC_RETICULUM_TUBULAR_NETWORK_ORGANIZATION	0.002808406	20	2000nM-DMSO
GOBP_GLYCOLYTIC_PROCESS_THROUGH_GLUCOSE_6_PHOSPHATE	0.003069815	20	2000nM-DMSO
GOBP_POSITIVE_REGULATION_OF_INTRACELLULAR_TRANSPORT	0.004722309	200	500nM-DMSO
GOBP_MITOTIC_METAPHASE_CHROMOSOME_ALIGNMENT	0.005044686	59	combined
GOBP_NUCLEAR_ENVELOPE_ORGANIZATION	0.005225613	54	2000nM-DMSO
GOBP_MONOSACCHARIDE_CATABOLIC_PROCESS	0.005287474	52	2000nM-DMSO
GOBP_APOPTOTIC_MITOCHONDRIAL_CHANGES	0.005996901	109	combined
GOBP_CYTOKINESIS	0.006060982	188	combined
GOBP_GOLGI_ORGANIZATION	0.006334283	158	2000nM-DMSO
GOBP_CHAPERONE_COFACTOR_DEPENDENT_PROTEIN_REFOLDING	0.006388369	32	combined
GOBP_MITOTIC_CELL_CYCLE_CHECKPOINT_SIGNALING	0.006442273	142	combined
GOBP_REGULATION_OF_MITOCHONDRION_ORGANIZATION	0.006469878	152	combined
GOBP_CELLULAR_MODIFIED_AMINO_ACID_METABOLIC_PROCESS	0.006517934	127	2000nM-DMSO
GOBP_MITOCHONDRIAL_ELECTRON_TRANSPORT_CYTOCHROME_C_TO_OXYGEN	0.006690109	25	2000nM-DMSO
GOBP_MITOCHONDRIAL_RESPIRATORY_CHAIN_COMPLEX_ASSEMBLY	0.006690109	101	2000nM-DMSO
GOBP_MITOCHONDRIAL_TRANSMEMBRANE_TRANSPORT	0.006693904	60	combined
GOBP_NADPH_REGENERATION	0.006809945	23	combined
GOBP_INNER_MITOCHONDRIAL_MEMBRANE_ORGANIZATION	0.007019414	40	2000nM-DMSO
GOBP_RIBOSOME_ASSEMBLY	0.007265462	64	500nM-DMSO 2000nM-DMSO
GOBP_CELLULAR_ALDEHYDE_METABOLIC_PROCESS	0.008804903	70	2000nM-DMSO
GOBP_REGULATION_OF_INTRACELLULAR_PROTEIN_TRANSPORT	0.009508035	225	500nM-DMSO

Highlighted rows are shown in **Figure 3.13**.

Supplementary table 3 Significantly enriched pathways of the Hallmarks gene sets from 24-hour proteomics data treated with 0.5 μ M and 2 μ M samuraciclib.

Pathway name	P-adjusted	Gene set size	Source of enrichment
HALLMARK_MYC_TARGETS_V1	4.00E-53	200	500nM-DMSO 2000nM-DMSO
HALLMARK_OXIDATIVE_PHOSPHORYLATION	1.65E-37	200	500nM-DMSO 2000nM-DMSO
HALLMARK_MTORC1_SIGNALING	1.20E-33	200	500nM-DMSO 2000nM-DMSO
HALLMARK_E2F_TARGETS	6.74E-32	200	500nM-DMSO 2000nM-DMSO
HALLMARK_DNA_REPAIR	5.15E-14	150	500nM-DMSO 2000nM-DMSO
HALLMARK_ADIPOGENESIS	7.44E-14	200	500nM-DMSO 2000nM-DMSO
HALLMARK_FATTY_ACID_METABOLISM	2.20E-12	158	500nM-DMSO 2000nM-DMSO
HALLMARK_G2M_CHECKPOINT	2.77E-12	200	500nM-DMSO 2000nM-DMSO
HALLMARK_MYC_TARGETS_V2	2.84E-10	58	2000nM-DMSO
HALLMARK_GLYCOLYSIS	7.10E-09	200	500nM-DMSO 2000nM-DMSO
HALLMARK_XENOBIOTIC_METABOLISM	2.07E-07	200	500nM-DMSO 2000nM-DMSO
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	2.08E-07	113	500nM-DMSO 2000nM-DMSO
HALLMARK_PROTEIN_SECRETION	1.73E-05	96	500nM-DMSO 2000nM-DMSO
HALLMARK_PEROXISOME	0.000229163	104	combined
HALLMARK_MITOTIC_SPINDLE	0.00029114	199	combined

Highlighted rows are shown in **Figure 3.13**.

Supplementary table 4 Significantly enriched pathways of the Reactome gene sets from 6-hour RNA-sequencing and 24-hour proteomics data treated with 0.5 μ M samuraciclib.

Pathway name	P-adjusted	Gene set size	Source of enrichment
REACTOME_EUKARYOTIC_TRANSLATION_INITIATION	1.93E-72	120	proteomics rnaseq
REACTOME_SRP_DEPENDENT_COTRANSLATIONAL_PROTEIN_TARGETING_TO_MEMBRANE	3.86E-70	113	proteomics rnaseq
REACTOME_EUKARYOTIC_TRANSLATION_ELONGATION	1.90E-68	94	proteomics rnaseq
REACTOME_RESPONSE_OF_EIF2AK4_GCN2_TO_AMINO_ACID_DEFICIENCY	1.90E-65	102	proteomics rnaseq
REACTOME_RRNA_PROCESSING	1.32E-64	205	proteomics rnaseq
REACTOME_NONSENSE_MEDIATED_DECAY_NMD	9.96E-62	116	proteomics rnaseq
REACTOME_INFLUENZA_INFECTION	9.48E-59	157	proteomics rnaseq
REACTOME_SELENOAMINO_ACID_METABOLISM	1.86E-56	118	proteomics rnaseq
REACTOME_REGULATION_OF_EXPRESSION_OF_SLITS_AND_ROBOS	1.40E-54	172	proteomics rnaseq
REACTOME_SIGNALING_BY_ROBO_RECEPTORS	1.02E-51	218	proteomics rnaseq
REACTOME_CELLULAR_RESPONSE_TO_STARVATION	2.01E-51	157	proteomics rnaseq
REACTOME_ACTIVATION_OF_THE_MRNA_UPON_BINDING_OF_THE_CAP_BINDING_COMPLEX_AND_EIFS_AND_SUBSEQUENT_BINDING_TO_43S	1.19E-35	60	proteomics rnaseq
REACTOME_SARS_COV_1_MODULATES_HOST_TRANSLATION_MACHINERY	5.01E-33	37	proteomics rnaseq
REACTOME_SARS_COV_2_MODULATES_HOST_TRANSLATION_MACHINERY	2.47E-29	51	proteomics rnaseq
REACTOME_SARS_COV_1_INFECTION	7.56E-26	143	proteomics rnaseq
REACTOME_SARS_COV_1_HOST_INTERACTIONS	3.02E-21	98	proteomics rnaseq
REACTOME_SARS_COV_2_HOST_INTERACTIONS	9.15E-21	204	proteomics rnaseq
REACTOME_THE_CITRIC_ACID_TCA_CYCLE_AND_RESPIRATORY_ELECTRON_TRANSPORT	3.93E-20	178	proteomics rnaseq
REACTOME_MITOTIC_METAPHASE_AND_ANAPHASE	1.19E-19	236	proteomics rnaseq
REACTOME_CELLULAR_RESPONSE_TO_CHEMICAL_STRESS	4.48E-17	214	proteomics rnaseq
REACTOME_RESPIRATORY_ELECTRON_TRANSPORT	5.56E-15	103	rnaseq
REACTOME_RRNA_MODIFICATION_IN_THE_NUCLEUS_AND_CYTOSOL	2.77E-13	60	proteomics rnaseq
REACTOME_HIV_INFECTION	6.86E-13	231	proteomics rnaseq
REACTOME_RESPIRATORY_ELECTRON_TRANSPORT_ATP_SYNTHESIS_BY_CHEMIOSMOTIC_COUPLING_AND_HEAT_PRODUCTION_BY_UNCOUPLING_PROTEINS	9.50E-13	127	rnaseq
REACTOME_SEPARATION_OF_SISTER_CHROMATIDS	2.46E-12	191	proteomics rnaseq
REACTOME_MRNA_SPLICING	1.11E-11	212	proteomics rnaseq
REACTOME_HOST_INTERACTIONS_OF_HIV_FACTORS	2.62E-10	131	proteomics rnaseq
REACTOME_REGULATION_OF_MRNA_STABILITY_BY_PROTEINS_THAT_BIND_AU_RICH_ELEMENTS	3.44E-10	87	proteomics rnaseq
REACTOME_S_PHASE	5.01E-10	163	proteomics rnaseq
REACTOME_MITOTIC_PROMETAPHASE	2.64E-09	203	proteomics rnaseq
REACTOME_TRANSPORT_OF_MATURE_TRANSCRIPT_TO_CYTOPLASM	3.47E-08	84	proteomics rnaseq
REACTOME_RESOLUTION_OF_SISTER_CHROMATID_COHESION	4.53E-08	126	proteomics rnaseq
REACTOME_KEAP1_NFE2L2_PATHWAY	5.35E-08	125	proteomics rnaseq
REACTOME_MITOTIC_G1_PHASE_AND_G1_S_TRANSITION	8.08E-08	149	proteomics rnaseq
REACTOME_SYNTHESIS_OF_DNA	1.35E-07	121	proteomics rnaseq
REACTOME_NUCLEAR_EVENTS_MEDIATED_BY_NFE2L2	2.06E-07	96	proteomics rnaseq
REACTOME_GLUCCOSE_METABOLISM	2.21E-07	94	proteomics rnaseq
REACTOME_SUMOYLATION	2.37E-07	189	proteomics rnaseq
REACTOME_MITOTIC_SPINDLE_CHECKPOINT	2.62E-07	111	proteomics rnaseq
REACTOME_RHO_GTPASES_ACTIVATE_FORMINS	3.54E-07	140	rnaseq
REACTOME_PCP_CE_PATHWAY	6.71E-07	92	rnaseq
REACTOME_COMPLEX_I_BIOGENESIS	8.57E-07	57	rnaseq
REACTOME_HIV_LIFE_CYCLE	2.00E-06	149	proteomics rnaseq
REACTOME_RNA_POLYMERASE_II_TRANSCRIPTION_TERMINATION	2.52E-06	67	proteomics rnaseq
REACTOME_G2_M_CHECKPOINTS	4.12E-06	167	proteomics rnaseq
REACTOME_APC_C_MEDIATED_DEGRADATION_OF_CELL_CYCLE_PROTEINS	4.20E-06	88	proteomics rnaseq
REACTOME_MITOTIC_G2_G2_M_PHASES	8.65E-06	200	rnaseq
REACTOME_ER_TO_GOLGI_ANTEROGRADE_TRANSPORT	9.60E-06	155	proteomics
REACTOME_PROTEIN_LOCALIZATION	1.05E-05	164	proteomics
REACTOME_SUMOYLATION_OF_DNA_DAMAGE_RESPONSE_AND_REPAIR_PROTEINS	1.44E-05	77	proteomics
REACTOME_INTERLEUKIN_12_SIGNALING	3.00E-05	47	rnaseq
REACTOME_ISG15_ANTIVIRAL_MECHANISM	4.01E-05	75	proteomics
REACTOME_GLYCOLYSIS	4.68E-05	74	proteomics rnaseq
REACTOME_INTRA_GOLGI_AND_RETROGRADE_GOLGI_TO_ER_TRAFFIC	5.53E-05	203	proteomics
REACTOME_COPII_MEDIATED_VESICLE_TRANSPORT	5.64E-05	68	proteomics
REACTOME_APC_C_CDH1_MEDIATED_DEGRADATION_OF_CDC20_AND_OTHER_APC_C_CDH1_TARGETED_PROTEINS_IN_LATE_MITOSIS_EARLY_G1	6.03E-05	74	proteomics rnaseq
REACTOME_SWITCHING_OF_ORIGINS_TO_A_POST_REPLICATIVE_STATE	6.54E-05	92	proteomics rnaseq
REACTOME_PYRUVATE_METABOLISM_AND_CITRIC_ACID_TCA_CYCLE	6.59E-05	55	proteomics rnaseq
REACTOME_ORC1_REMOVAL_FROM_CHROMATIN	6.81E-05	71	rnaseq
REACTOME_CYTOPROTECTION_BY_HMOX1	8.01E-05	64	rnaseq
REACTOME_GENE_AND_PROTEIN_EXPRESSION_BY_JAK_STAT_SIGNALING_AFTER_INTERLEUKIN_12_STIMULATION	8.11E-05	38	rnaseq
REACTOME_SCF_SKP2_MEDIATED_DEGRADATION_OF_P27_P21	8.85E-05	60	rnaseq
REACTOME_NUCLEAR_ENVELOPE_NE_REASSEMBLY	0.000101193	76	combined
REACTOME_INTERLEUKIN_12_FAMILY_SIGNALING	0.000118746	57	rnaseq
REACTOME_AUF1_HNRNP_D0_BINDS_AND_DESTABILIZES_MRNA	0.000132294	55	rnaseq
REACTOME_ANTIVIRAL_MECHANISM_BY_IFN_STIMULATED_GENES	0.000214044	152	proteomics
REACTOME_NUCLEAR_ENVELOPE_BREAKDOWN	0.000249717	53	proteomics
REACTOME_GLUCCONEOGENESIS	0.000387954	35	proteomics rnaseq
REACTOME_THE_ROLE_OF_GTSE1_IN_G2_M_PROGRESSION_AFTER_G2_CHECKPOINT	0.000463801	79	rnaseq
REACTOME_TRANS_GOLGI_NETWORK_VESICLE_BUDDING	0.000478466	72	proteomics
REACTOME_TRANSCRIPTIONAL_REGULATION_BY_RUNX2	0.000639335	120	rnaseq
REACTOME_TRANSPORT_TO_THE_GOLGI_AND_SUBSEQUENT_MODIFICATION	0.000736839	186	proteomics
REACTOME_SCF_BETA_TRCP_MEDIATED_DEGRADATION_OF_EMI1	0.000854469	55	rnaseq
REACTOME_PROGRAMMED_CELL_DEATH	0.001366399	211	proteomics
REACTOME_BETA_CATENIN_INDEPENDENT_WNT_SIGNALING	0.001388905	146	rnaseq
REACTOME_PROCESSING_OF_CAPPED_INTRONLESS_PRE_MRNA	0.001650057	29	combined
REACTOME_DEGRADATION_OF_DVL	0.001867087	57	rnaseq
REACTOME_COPI_MEDIATED_ANTEROGRADE_TRANSPORT	0.002095096	102	combined
REACTOME_TP53_REGULATES_METABOLIC_GENES	0.002484631	87	rnaseq
REACTOME_CITRIC_ACID_CYCLE_TCA_CYCLE	0.002861735	22	combined
REACTOME_CYCLIN_A_CDK2_ASSOCIATED_EVENTS_AT_S_PHASE_ENTRY	0.002901694	85	combined
REACTOME_DEGRADATION_OF_GLI1_BY_THE_PROTEASOME	0.003147427	60	combined
REACTOME_TRANSPORT_OF_MATURE_MRNAS_DERIVED_FROM_INTRONLESS_TRANSCRIPTS	0.003307436	43	proteomics
REACTOME_DNA_REPLICATION	0.003355335	187	combined
REACTOME_NEGATIVE_REGULATION_OF_NOTCH4_SIGNALING	0.003917326	54	rnaseq
REACTOME_REGULATION_OF_RUNX2_EXPRESSION_AND_ACTIVITY	0.004118917	73	rnaseq
REACTOME_CLATHRIN_MEDIATED_ENDOCYTOSIS	0.004816812	145	combined
REACTOME_CLEC7A_DECTIN_1_SIGNALING	0.004870099	100	rnaseq
REACTOME_DEGRADATION_OF_AXIN	0.005056786	55	rnaseq
REACTOME_REGULATION_OF_RUNX3_EXPRESSION_AND_ACTIVITY	0.005056786	55	rnaseq

Pathway name	P-adjusted	Gene set size	Source of enrichment
REACTOME_DECTIN_1_MEDIATED_NONCANONICAL_NF_KB_SIGNALING	0.00506771	62	rnaseq
REACTOME_MITOCHONDRIAL_PROTEIN_IMPORT	0.005071142	65	combined
REACTOME_SARS_COV_2_ACTIVATES_MODULATES_INNATE_AND_ADAPTIVE_IMMUNE_RESPONSES	0.005911451	126	combined
REACTOME_SYNTHESIS_OF_ACTIVE_UBIQUITIN_ROLES_OF_E1_AND_E2_ENZYMES	0.006093704	30	proteomics
REACTOME_INTERACTIONS_OF_REV_WITH_HOST_CELLULAR_PROTEINS	0.006337198	37	proteomics
REACTOME_PROCESSING_OF_INTRONLESS_PRE_MRNAS	0.006558452	20	combined
REACTOME_REGULATION_OF_PTEN_STABILITY_AND_ACTIVITY	0.007568208	69	rnaseq
REACTOME_DNA_REPLICATION_PRE_INITIATION	0.008169265	159	combined
REACTOME_SNRNP_ASSEMBLY	0.009815007	54	proteomics

Highlighted rows are shown in **Figure 3.17**.

Supplementary table 5 Significantly enriched pathways of the gene ontology biological processes (GOBP) gene sets from 6-hour RNA-sequencing and 24-hour proteomics data treated with 0.5 μ M samuraciclib.

Pathway name	P-adjusted	Gene set size	Source of enrichment
GOBP_CYTOPLASMIC_TRANSLATION	4.72E-73	156	proteomics rnaeq
GOBP_RIBOSOMAL_SMALL_SUBUNIT_BIOGENESIS	6.02E-21	104	proteomics rnaeq
GOBP_AEROBIC_RESPIRATION	4.32E-18	197	rnaeq
GOBP_CELLULAR_RESPIRATION	4.06E-17	243	proteomics rnaeq
GOBP_PROTEIN_RNA_COMPLEX_ORGANIZATION	7.43E-17	241	proteomics rnaeq
GOBP_MITOTIC_SISTER_CHROMATID_SEGREGATION	8.84E-16	187	proteomics rnaeq
GOBP_ATP_SYNTHESIS_COUPLED_ELECTRON_TRANSPORT	8.05E-15	102	rnaeq
GOBP_RESPIRATORY_ELECTRON_TRANSPORT_CHAIN	7.31E-14	124	rnaeq
GOBP_RIBOSOMAL_LARGE_SUBUNIT_BIOGENESIS	1.70E-13	76	proteomics rnaeq
GOBP_OXIDATIVE_PHOSPHORYLATION	5.75E-13	148	rnaeq
GOBP_ELECTRON_TRANSPORT_CHAIN	1.18E-12	176	rnaeq
GOBP_SISTER_CHROMATID_SEGREGATION	1.89E-12	228	proteomics rnaeq
GOBP_PROTEIN_FOLDING	4.25E-12	223	proteomics rnaeq
GOBP_REGULATION_OF_RNA_SPLICING	4.27E-12	180	proteomics rnaeq
GOBP_RIBOSOME_ASSEMBLY	3.88E-11	64	rnaeq
GOBP_REGULATION_OF_MRNA_PROCESSING	1.40E-10	128	proteomics rnaeq
GOBP_RNA_LOCALIZATION	2.28E-10	197	proteomics rnaeq
GOBP_MICROTUBULE_CYTOSKELETON_ORGANIZATION_INVOLVED_IN_MITOSIS	6.01E-10	165	proteomics rnaeq
GOBP_SPINDLE_ORGANIZATION	1.22E-09	218	proteomics rnaeq
GOBP_SPINDLE_ASSEMBLY	2.45E-09	149	proteomics rnaeq
GOBP_METAPHASE_CHROMOSOME_ALIGNMENT	1.51E-08	98	proteomics rnaeq
GOBP_MITOTIC_CYTOKINESIS	1.51E-08	88	proteomics rnaeq
GOBP_RNA_3_END_PROCESSING	1.95E-08	102	rnaeq
GOBP_MITOTIC_SPINDLE_ORGANIZATION	2.01E-08	132	proteomics rnaeq
GOBP_VESICLE_BUDDING_FROM_MEMBRANE	6.76E-08	84	proteomics rnaeq
GOBP_NUCLEOSIDE_TRIPHOSPHATE_METABOLIC_PROCESS	7.16E-08	184	rnaeq
GOBP_CELL_CYCLE_CHECKPOINT_SIGNALING	7.57E-08	192	rnaeq
GOBP_TRANSLATIONAL_INITIATION	8.58E-08	123	proteomics rnaeq
GOBP_MATURATION_OF_LSU_RRNA	1.34E-07	28	proteomics rnaeq
GOBP_ESTABLISHMENT_OF_RNA_LOCALIZATION	1.68E-07	160	proteomics rnaeq
GOBP_MITOTIC_CELL_CYCLE_CHECKPOINT_SIGNALING	1.83E-07	142	rnaeq
GOBP_ENDOPLASMIC_RETICULUM_TO_GOLGI_VESICLE_MEDIATED_TRANSPORT	1.91E-07	138	proteomics
GOBP_CHROMOSOME_LOCALIZATION	2.46E-07	118	proteomics
GOBP_REGULATION_OF_MRNA_SPLICING_VIA_SPLICEOSOME	2.80E-07	106	proteomics rnaeq
GOBP_REGULATION_OF_CHROMOSOME_ORGANIZATION	2.88E-07	247	rnaeq
GOBP_MITOCHONDRIAL_ELECTRON_TRANSPORT_NADH_TO_UBIQUINONE	5.23E-07	51	rnaeq
GOBP_MITOTIC_SPINDLE_ASSEMBLY	7.47E-07	73	rnaeq
GOBP_POSITIVE_REGULATION_OF_TRANSLATION	7.77E-07	141	proteomics rnaeq
GOBP_MRNA_TRANSPORT	9.94E-07	126	proteomics rnaeq
GOBP_STRESS_GRANULE_ASSEMBLY	1.07E-06	31	proteomics
GOBP_POSITIVE_REGULATION_OF_AMIDE_METABOLIC_PROCESS	1.40E-06	169	proteomics
GOBP_TRICARBOXYLIC_ACID_CYCLE	1.41E-06	33	proteomics
GOBP_MRNA_3_END_PROCESSING	1.44E-06	56	rnaeq
GOBP_NUCLEOSIDE_TRIPHOSPHATE_BIOSYNTHETIC_PROCESS	1.59E-06	127	rnaeq
GOBP_ATP_METABOLIC_PROCESS	1.71E-06	137	rnaeq
GOBP_MATURATION_OF_SSU_RRNA	2.31E-06	53	proteomics
GOBP_CHROMOSOME_SEPARATION	2.64E-06	81	rnaeq
GOBP_ALTERNATIVE_MRNA_SPLICING_VIA_SPLICEOSOME	3.58E-06	71	proteomics rnaeq
GOBP_VESICLE_TARGETING	7.70E-06	67	rnaeq
GOBP_NUCLEAR_EXPORT	9.44E-06	168	proteomics rnaeq
GOBP_CYTOSKELETON_DEPENDENT_CYTOKINESIS	1.43E-05	117	rnaeq
GOBP_NUCLEUS_ORGANIZATION	1.43E-05	147	proteomics rnaeq
GOBP_REGULATION_OF_NUCLEOCYTOPLASMIC_TRANSPORT	1.88E-05	109	proteomics rnaeq
GOBP_REGULATION_OF_INTRINSIC_APOPTOTIC_SIGNALING_PATHWAY	1.93E-05	190	rnaeq
GOBP_RNA_EXPORT_FROM_NUCLEUS	2.03E-05	92	proteomics rnaeq
GOBP_DNA_TEMPLATED_TRANSCRIPTION_ELONGATION	2.37E-05	135	rnaeq
GOBP_CYTOKINESIS	3.30E-05	188	rnaeq
GOBP_RIBOSE_PHOSPHATE_BIOSYNTHETIC_PROCESS	3.31E-05	235	rnaeq
GOBP_PROTEIN_STABILIZATION	3.54E-05	217	rnaeq
GOBP_RIBOSOMAL_LARGE_SUBUNIT_ASSEMBLY	3.71E-05	26	rnaeq
GOBP_DNA_TEMPLATED_DNA_REPLICATION	4.15E-05	162	proteomics
GOBP_MEMBRANE_DOCKING	4.46E-05	92	combined
GOBP_REGULATION_OF_CYTOPLASMIC_TRANSLATION	5.48E-05	33	rnaeq
GOBP_REGULATION_OF_SPINDLE_ASSEMBLY	6.01E-05	33	rnaeq
GOBP_IMPORT_INTO_NUCLEUS	6.18E-05	166	proteomics rnaeq
GOBP_NEGATIVE_REGULATION_OF_MITOTIC_CELL_CYCLE	6.35E-05	248	rnaeq
GOBP_EPIGENETIC_REGULATION_OF_GENE_EXPRESSION	7.45E-05	229	proteomics rnaeq
GOBP_REGULATION_OF_CHROMOSOME_SEGREGATION	0.000105405	131	rnaeq
GOBP_NADH_DEHYDROGENASE_COMPLEX_ASSEMBLY	0.000106456	60	rnaeq
GOBP_CELL_CYCLE_G2_M_PHASE_TRANSITION	0.000140965	155	rnaeq
GOBP_MACROMOLECULE_METHYLATION	0.000144223	210	rnaeq
GOBP_NUCLEOBASE_CONTAINING_COMPOUND_TRANSPORT	0.000160942	225	proteomics rnaeq
GOBP_HETEROCHROMATIN_ORGANIZATION	0.000201701	101	proteomics rnaeq
GOBP_REGULATION_OF_MITOTIC_SPINDLE_ASSEMBLY	0.000244966	23	rnaeq
GOBP_POSITIVE_REGULATION_OF_CHROMOSOME_ORGANIZATION	0.000302789	109	rnaeq
GOBP_REGULATION_OF_DNA_TEMPLATED_TRANSCRIPTION_ELONGATION	0.000304243	105	rnaeq
GOBP_NUCLEAR_ENVELOPE_ORGANIZATION	0.000305357	54	rnaeq
GOBP_PROTON_MOTIVE_FORCE_DRIVEN_ATP_SYNTHESIS	0.000386607	76	rnaeq
GOBP_REGULATION_OF_SPINDLE_ORGANIZATION	0.000403607	47	rnaeq
GOBP_MRNA_EXPORT_FROM_NUCLEUS	0.000462353	70	proteomics rnaeq
GOBP_CYTOPLASMIC_TRANSLATIONAL_INITIATION	0.000469997	40	proteomics
GOBP_DNA_INTEGRITY_CHECKPOINT_SIGNALING	0.000559192	135	rnaeq
GOBP_REGULATION_OF_UBIQUITIN_PROTEIN_TRANSFERASE_ACTIVITY	0.000560415	39	rnaeq
GOBP_MATURATION_OF_SSU_RRNA_FROM_TRICISTRONIC_RRNA_TRANSCRIPT_SSU_RRNA_5_8S_RRNA_LSU_RRNA	0.000568919	36	proteomics
GOBP_MITOTIC_METAPHASE_CHROMOSOME_ALIGNMENT	0.000623728	59	combined
GOBP_PLASMA_MEMBRANE_REPAIR	0.000677001	29	rnaeq
GOBP_MITOTIC_CYTOKINETIC_PROCESS	0.000814344	27	combined
GOBP_BIOLOGICAL_PROCESS_INVOLVED_IN_INTERACTION_WITH_HOST	0.000946115	213	rnaeq
GOBP_REGULATION_OF_TRANSLATIONAL_INITIATION	0.001010015	83	proteomics rnaeq
GOBP_MEMBRANE_FISSION	0.001423416	42	combined

Pathway name	P-adjusted	Gene set size	Source of enrichment
GOBP_MITOTIC_SISTER_CHROMATID_SEPARATION	0.001559526	62	combined
GOBP_MITOCHONDRIAL_ELECTRON_TRANSPORT_CYTOCHROME_C_TO_OXYGEN	0.001697027	25	rnaseq
GOBP_ATP_BIOSYNTHETIC_PROCESS	0.001769761	102	rnaseq
GOBP_COPII_COATED_VESICLE_BUDDING	0.002005423	44	proteomics
GOBP_RECOMBINATIONAL_REPAIR	0.002172055	179	combined
GOBP_MITOTIC_DNA_INTEGRITY_CHECKPOINT_SIGNALING	0.00227396	87	rnaseq
GOBP_VIRAL_GENE_EXPRESSION	0.002712361	101	combined
GOBP_POSITIVE_REGULATION_OF_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR	0.002804146	30	combined
GOBP_DNA_CONFORMATION_CHANGE	0.002852481	92	combined
GOBP_CYTOKINETIC_PROCESS	0.00290254	43	combined
GOBP_NEGATIVE_REGULATION_OF_MITOTIC_CELL_CYCLE_PHASE_TRANSITION	0.003055878	196	rnaseq
GOBP_VESICLE_DOCKING	0.003353336	64	combined
GOBP_SIGNAL_TRANSDUCTION_IN_RESPONSE_TO_DNA_DAMAGE	0.003654185	189	rnaseq
GOBP_REGULATION_OF_MITOTIC_SISTER_CHROMATID_SEGREGATION	0.003670778	54	rnaseq
GOBP_REGULATION_OF_TRANSCRIPTION_ELONGATION_BY_RNA_POLYMERASE_II	0.004004441	87	rnaseq
GOBP_NEGATIVE_REGULATION_OF_GENE_EXPRESSION_EPIGENETIC	0.004297651	120	combined
GOBP_NEGATIVE_REGULATION_OF_CHROMOSOME_ORGANIZATION	0.004610798	94	rnaseq
GOBP_CYTOSOLIC_TRANSPORT	0.004912511	186	proteomics
GOBP_PROTEIN_LOCALIZATION_TO_MITOCHONDRION	0.005017603	131	proteomics
GOBP_DNA_REPLICATION_INITIATION	0.005565537	37	combined
GOBP_MITOCHONDRIAL_RESPIRATORY_CHAIN_COMPLEX_ASSEMBLY	0.005731857	101	combined
GOBP_REGULATION_OF_SISTER_CHROMATID_SEGREGATION	0.006280205	104	combined
GOBP_PROTEIN_REFOLDING	0.00632645	27	combined
GOBP_REGULATION_OF_CELL_CYCLE_G2_M_PHASE_TRANSITION	0.006647912	116	combined
GOBP_CELL_REDOX_HOMEOSTASIS	0.009140163	41	combined
GOBP_VESICLE_LOCALIZATION	0.00974598	218	rnaseq

Highlighted rows are shown in **Figure 3.17**.

Supplementary table 6 Significantly enriched pathways of the Hallmarks gene sets from 6-hour RNA-sequencing and 24-hour proteomics data treated with 0.5 μ M samuraciclib.

Pathway name	P-adjusted	Gene set size	Source of enrichment
HALLMARK_OXIDATIVE_PHOSPHORYLATION	9.45E-35	200	proteomics rnaseq
HALLMARK_MYC_TARGETS_V1	2.44E-28	200	proteomics rnaseq
HALLMARK_E2F_TARGETS	3.16E-23	200	proteomics rnaseq
HALLMARK_MTORC1_SIGNALING	4.13E-15	200	proteomics
HALLMARK_PROTEIN_SECRETION	1.82E-13	96	proteomics rnaseq
HALLMARK_FATTY_ACID_METABOLISM	4.45E-13	158	proteomics rnaseq
HALLMARK_G2M_CHECKPOINT	4.20E-12	200	proteomics rnaseq
HALLMARK_ADIPOGENESIS	6.77E-12	200	proteomics rnaseq
HALLMARK_GLYCOLYSIS	3.58E-07	200	proteomics rnaseq
HALLMARK_DNA_REPAIR	1.77E-06	150	proteomics rnaseq
HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY	5.32E-05	49	combined
HALLMARK_MITOTIC_SPINDLE	7.40E-05	199	rnaseq
HALLMARK_MYC_TARGETS_V2	0.00011407	58	combined
HALLMARK_P53_PATHWAY	0.000191135	200	proteomics rnaseq
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	0.000496543	113	combined

Highlighted rows are shown in **Figure 3.17**.

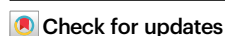
Publication

High-resolution cryo-EM of the human CDK-activating kinase for structure-based drug design

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Rational design of next-generation therapeutics can be facilitated by high-resolution structures of drug targets bound to small-molecule inhibitors. However, application of structure-based methods to macromolecules refractory to crystallization has been hampered by the often-limiting resolution and throughput of cryogenic electron microscopy (cryo-EM). Here, we use high-resolution cryo-EM to determine structures of the CDK-activating kinase, a master regulator of cell growth and division, in its free and nucleotide-bound states and in complex with 15 inhibitors at up to 1.8 Å resolution. Our structures provide detailed insight into inhibitor interactions and networks of water molecules in the active site of cyclin-dependent kinase 7 and provide insights into the mechanisms contributing to inhibitor selectivity, thereby providing the basis for rational design of next-generation therapeutics. These results establish a methodological framework for the use of high-resolution cryo-EM in structure-based drug design.

The human CDK-activating kinase (CAK) is a heterotrimeric protein complex formed by CDK7, cyclin H, and MAT1 and acts as a master regulator of cell growth and division^{1,2}. It regulates transcription initiation and the cell cycle by phosphorylating RNA polymerase II and cyclin-dependent kinases, respectively. Due to this central role in cellular physiology, the CAK has been identified as a promising target for cancer therapeutics³ and it is a possible target for antivirals⁴. Several groups have discovered specific inhibitors of the CAK^{4–9}, including high-affinity inhibitors that occupy the active site to compete with adenosine-nucleotide binding, and covalent inhibitors that modify a cysteine residue in the vicinity of the active site. We have developed a CDK7 inhibitor series and advanced one of these compounds, ICEC0942 (CT7001, samuraciclib)⁵, into clinical trials for treatment of

advanced-stage solid cancers (clinicaltrials.gov NCT04802759, NCT03363893). To date, five other CDK7 inhibitors have entered clinical trials: SY-1365 (NCT03134638), SY-5609 (NCT04247126, NCT04929223), LY-3405105 (NCT03770494), Q901 (NCT05394103) and XL-102 (NCT04726332). One of the major challenges in the development of CDK-targeting compounds is specificity¹⁰ because 20 different enzymes in human cells are classified as CDKs and often exhibit high sequence identity near their catalytic sites^{11,12}. Furthermore, CDKs are characterized by large conformational changes upon association with their activatory cyclins and phosphorylation of their regulatory T-loops, which can change their compound-binding properties¹³. To enable the discovery and rational design of next-generation therapeutics with increased potency and reduced off-

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target effects, structural data permitting the application of structure-based drug design approaches are instrumental.

Working towards the goal of making the human CAK accessible to structure-based drug design, we previously determined its cryo-EM structure bound to nucleotide analogs and the inhibitors ICEC0942 and THZ1^{14,15}. Our ICEC0942-bound structure revealed interesting conformational differences between the inhibitor bound to its clinical target, CAK, and bound to CDK2, an off-target complex with the potential to cause side effects in patients, highlighting the potential of using structural data to guide the development of next-generation inhibitors in this system^{14,16}. Our published cryo-EM structures used the human proteins and have reached higher resolution than X-ray crystal structures of a homologous complex from a thermophilic fungus¹⁷. Furthermore, one advantage of cryo-EM is the ability to capture a series of different states from a single sample, which can facilitate the structural analysis of dynamic systems. This may be particularly beneficial for CDKs due to their conformation-dependent compound-binding properties¹³. These considerations indicate that cryo-EM is the method of choice in this system if throughput and resolution reach the levels required for drug discovery applications.

In this work, we structurally characterize complexes of CAK bound to a range of both commercially available molecules and the series of compounds developed and characterized alongside ICEC0942, aiming to uncover the structural basis of CDK7 inhibitor selectivity to pave the way towards next-generation therapeutics. The nature of this endeavor requires high resolution for this small, asymmetric complex as well as high throughput, creating a technical challenge and situating this effort at the edge of what is currently feasible using cryo-EM¹⁸. To address this challenge, we use rapid screening on a 200 kV cryo-transmission electron microscope (cryo-TEM) to obtain initial structures and identify promising specimens, and we progress suitable specimens to high-end data collection on an energy-filtered 300 kV cryo-TEM with a cold field emission gun (cold-FEG). We obtain 18 structures of the 85 kDa CDK-cyclin-module of the human CAK in its nucleotide-bound, free, and inhibitor-bound states at up to 1.8 Å resolution, with the inhibitors comprising pyrazolopyrimidine, pyrazolotriazine, and phenylaminopyrimidine-class compounds. In addition to achieving high resolution from large datasets, we establish a routine ~4 Å and ~3 Å-resolution structure determination workflow for ligand-bound complexes using the 200 kV setup combined with on-the-fly data processing from 1 h and 4 h of data collection. This work provides a workflow for the application of cryo-EM to structure-based drug discovery, expands our understanding of how structurally diverse inhibitors interact with the active site of CDK7, and thereby provides a basis for the design of next-generation cancer therapeutics.

Results

High-resolution structures of the human CAK

Structure-based drug design greatly benefits from high-resolution data that provide molecular models with atomic accuracy and information on networks of water molecules present in the active sites of target complexes. Knowledge of ordered water positions is crucial for understanding how inhibitors bind¹⁹ and can guide efforts in inhibitor optimization^{20,21}. Our previous structure of nucleotide-bound human CAK reached 2.8 Å resolution¹⁵, limiting its ability to resolve many ordered waters, and an apo-structure of the fully assembled human CAK is lacking altogether. We therefore set out to determine the high-resolution structures of apo- and nucleotide-bound human CAK using the latest generation Krios cryo-TEM²². Furthermore, as a proof of concept for high-resolution structure determination of inhibitor-bound CAK, we re-determined the structure of CAK modified by the covalent inhibitor THZ1 (Supplementary Fig. 1a), which has been shown to disrupt super-enhancer transcription in human cancer cell lines⁸, and we determined the structure of CAK in complex with the high-affinity inhibitor LDC4297 (Supplementary Fig. 1b), a

pyrazolotriazine-class compound that exhibits anti-viral activity⁴, a property that is of particular interest given current global health challenges.

These experiments resulted in cryo-EM reconstructions of the ligand-bound human CAK at 1.9–2.1 Å resolution (Fig. 1a–c, Supplementary Fig. 1c–e, Supplementary Table 1). The maps show protein side chain and main chain features consistent with the reported resolution (Fig. 1d, e), allow direct identification of post-translational modifications, such as N-terminal protein acetylation (Fig. 1f), and reveal the locations of many water molecules (Fig. 1d–f). At high resolution, density for the regulatory CDK7 T-loop (residues 155–182 between the conserved DFG and APE motifs) becomes fragmented, indicating structural heterogeneity.

The structure of CAK bound to the nucleotide analog ATPγS at 1.9 Å resolution allows the accurate placement of the bound nucleotide and a water-coordinated Mg²⁺ ion in the active site (Supplementary Fig. 1f). Compared to the X-ray crystal structure of the homologous complex from *Chaetomium thermophilum*, the orientation of the adenosine base in the bound nucleotides is different, adopting a *syn*-conformation in the fungal complex (Supplementary Fig. 1g)¹⁷. This conformation is rare but has been observed previously in the structure of homoserine kinase²³. It is currently unclear if the *syn*-conformation in the fungal complex is the preferred conformation in catalytically activated complexes, which could point to subtle differences in the nucleotide binding pockets between the fungal and human enzyme that impact their small molecule-binding properties, or if the *syn*-conformation in the *C. thermophilum* structure is favored only in the context of the protein conformation compatible with the crystal lattice. The structure of apo-CAK shows great similarity to the nucleotide- and inhibitor-bound structures, even for most of the active site pocket, except for the β-sheet in the N-terminal kinase lobe, which is substantially more flexible in the apo state than in all structures that have an occupied active site (Supplementary Fig. 1h–j). Flexibility limits the overall resolution of this reconstruction to 2.3 Å (Supplementary Fig. 1k) and is greatest at the outermost two β-strands, connected by the G-rich loop (residues 19–24, sequence GEGQFA), which are barely visible in the density. Ligand-dependent conformational changes of the N-terminal kinase lobe have been observed previously in cryo-EM structures of the human CAK¹⁵, X-ray crystal structures of the homologous *C. thermophilum* complex¹⁷, and X-ray crystal structures of a range of other kinases, including CDK2²⁴ and cAMP-dependent protein kinase, where parts of this domain are displaced by almost 10 Å²⁵. However, unlike X-ray crystal structures, where mobile domains can be trapped in specific conformations due to interactions within the crystal lattice and thereby manifest as defined conformations, cryo-EM structures can capture the full range of conformations accessible to molecular complexes in solution. This can explain the fragmented density observed in our cryo-EM map of apo-CAK.

The structure of THZ1-bound CAK at 1.9 Å resolution (Fig. 1a–c, Supplementary Fig. 1c) agrees with our previous interpretation at lower resolution¹⁵. Notably, even in our high-resolution map, the extended arm that harbors the reactive acrylamide group that covalently modifies CDK7 residue C312 is resolved far less well than the indole and chloropyrimidine groups of the inhibitor that are embedded in the CDK7 active site pocket (Supplementary Fig. 2a, b). This is probably linked to continuous flexibility of this part of the inhibitor, a notion that is supported by the observation that the equivalent cysteine-reactive functional groups of two copies of the related inhibitor THZ531 are positioned 12 Å apart in the X-ray crystal structure of this compound bound to CDK12²⁶. We quantified these differences in local atom resolvability using Q-scores (Supplementary Fig. 2c)²⁷ because the local map quality in the presence of inhibitor heterogeneity does not necessarily closely correlate with the overall resolution of the reconstruction.

In contrast to THZ1, which acts via a covalent mechanism, LDC4297 is a highly selective reversible inhibitor of CDK7⁴. The map

quality of our structure of CAK in complex with LDC4297 at 2.1 Å resolution (Supplementary Fig. 1d) was improved by locally refining only the approximately 40 kDa CDK7 density (Supplementary Fig. 2d). The structure reveals that the substituted phenyl ring assumes a similar conformation to the equivalent group in ICEC0942, while the ether-linked piperidine group was refined into a boat-like conformation, though structural flexibility and sampling of other conformations cannot be excluded (Supplementary Fig. 2e).

Knowledge of the positions of bound water molecules in macromolecular complexes is essential to understand inhibitor binding and to explore possibilities for inhibitor optimization^{19–21}. Therefore, the visualization of water molecules is an important feature of our high-resolution cryo-EM maps. A systematic validation of our cryo-EM-based assignments by comparing them to the locations of water densities identified with X-ray crystal structures is challenging due to the lack of structures of CDK7 or cyclin H at resolutions of 2 Å or better. However, we find that many of the water locations identified in our cryo-EM maps correspond to those found in CDK7 and cyclin H at lower resolution or in related CDKs at high resolution (Supplementary Fig. 2f, g), supporting the reliability of our assignments. Nevertheless, when compared to X-ray crystallographic structures at similar resolutions, we observe that our cryo-EM structures appear to resolve a smaller total number of water molecules (approx. 160 as compared to nearly 400 in PDB ID 6ATH at 1.8 Å resolution²⁸). This has been observed previously for cryo-EM reconstructions at atomic resolution, but the reasons for this discrepancy are currently not known²⁹.

Design of a cryo-EM workflow for high-throughput structure determination of inhibitor-bound CAK complexes

ICEC0942 is an orally bioavailable high-affinity inhibitor of CDK7 that has entered clinical trials for cancer treatment⁵. ICEC0942 was developed as a part of a series of pyrazolo[1,5-a]pyrimidine compounds.

In-vitro kinase inhibition data show that inhibitor selectivity spans almost 4 orders of magnitude, ranging from approximately 100× selectivity for CDK7 over CDK2 (ICEC0829) to 100× selectivity for CDK2 over CDK7 (BS-194) (Fig. 2a, Supplementary Table 2)^{5,16,30–33}, with low selectivity conferring a risk of inducing undesirable off-target effects in patients. Given the importance of understanding selectivity, and considering our previous observation of conformational differences of ICEC0942 bound to CDK7 and CDK2¹⁴, which suggests that structural analysis may aid in rationalizing inhibitor selectivity and provide insights that may be applied to next-generation inhibitors, we sought to undertake the structural analysis of 12 pyrazolopyrimidine-type CDK inhibitors (Fig. 2a) to explore the molecular mechanisms of CDK7 selectivity.

To enable high-resolution and high-throughput cryo-EM data collection suitable for structure determination of small-molecule-bound CAK complexes, we devised and implemented a three-stage cryo-EM workflow comprising rapid initial grid and sample screening, intermediate-resolution structure determination, and finally high-resolution structure determination of the best grids (Fig. 2b–e). We applied this workflow to 12 compounds (Fig. 2a, Supplementary Tables 1, 3, 4), but the approach can be scaled to even larger numbers of small-molecule ligands in the future.

To aid in the design of this workflow, we first benchmarked the performance of a 300 kV Krios G4 cryo-TEM equipped with a Falcon 4i detector, a Selectris X energy filter, and a cold-FEG against a 200 kV Glacios 2 cryo-TEM equipped with an X-FEG, a Falcon 4i detector, and a Selectris X energy filter. We found that for the CDK-activating kinase, the 300 kV-cryo-TEM system provided an approximately 0.3 Å resolution advantage (Fig. 3a–e), with resolutions of 2.0 Å and 2.3 Å achieved for data collected from the same grid, notably in a shorter time and from approximately 50% fewer electron micrograph movies for the 300 kV system compared to the 200 kV system (see

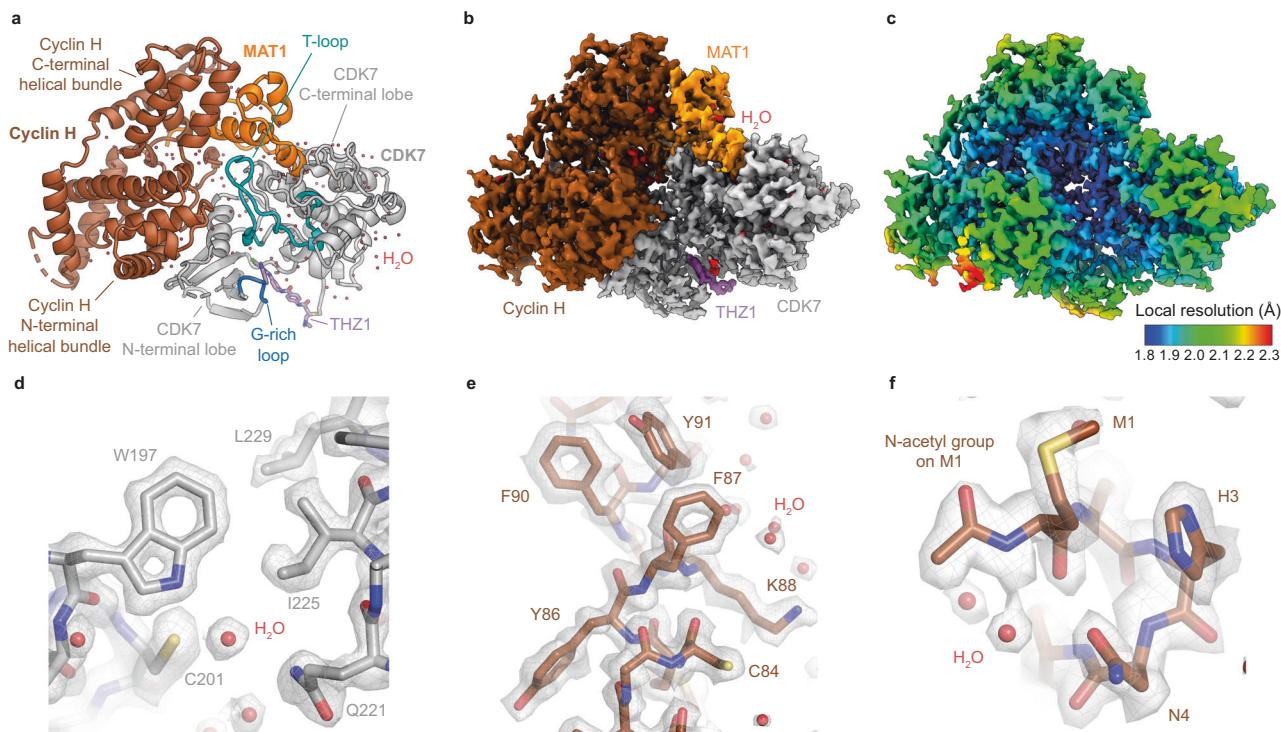


Fig. 1 | High-resolution structures of the human CDK-activating kinase.

a Atomic model of the CAK-THZ1 adduct with distinctly colored subunits (cyclin H brown, MAT1 orange, CDK7 gray, THZ1 violet). The domains of CDK7 and cyclin H are indicated and the T-loop and G-rich loop of CDK7 are shown in cyan and blue, respectively. Water molecules are shown in red. **b** Cryo-EM map of the CAK-THZ1

adduct. **c** Local resolution plot for the cryo-EM map shown in (b). **d**, **e** Examples of high-quality density at 1.9 Å resolution (CAK-THZ1 map). **f** Direct identification of post-translational N-terminal acetyl modification on cyclin H in the cryo-EM map (CAK-ATPyS map).

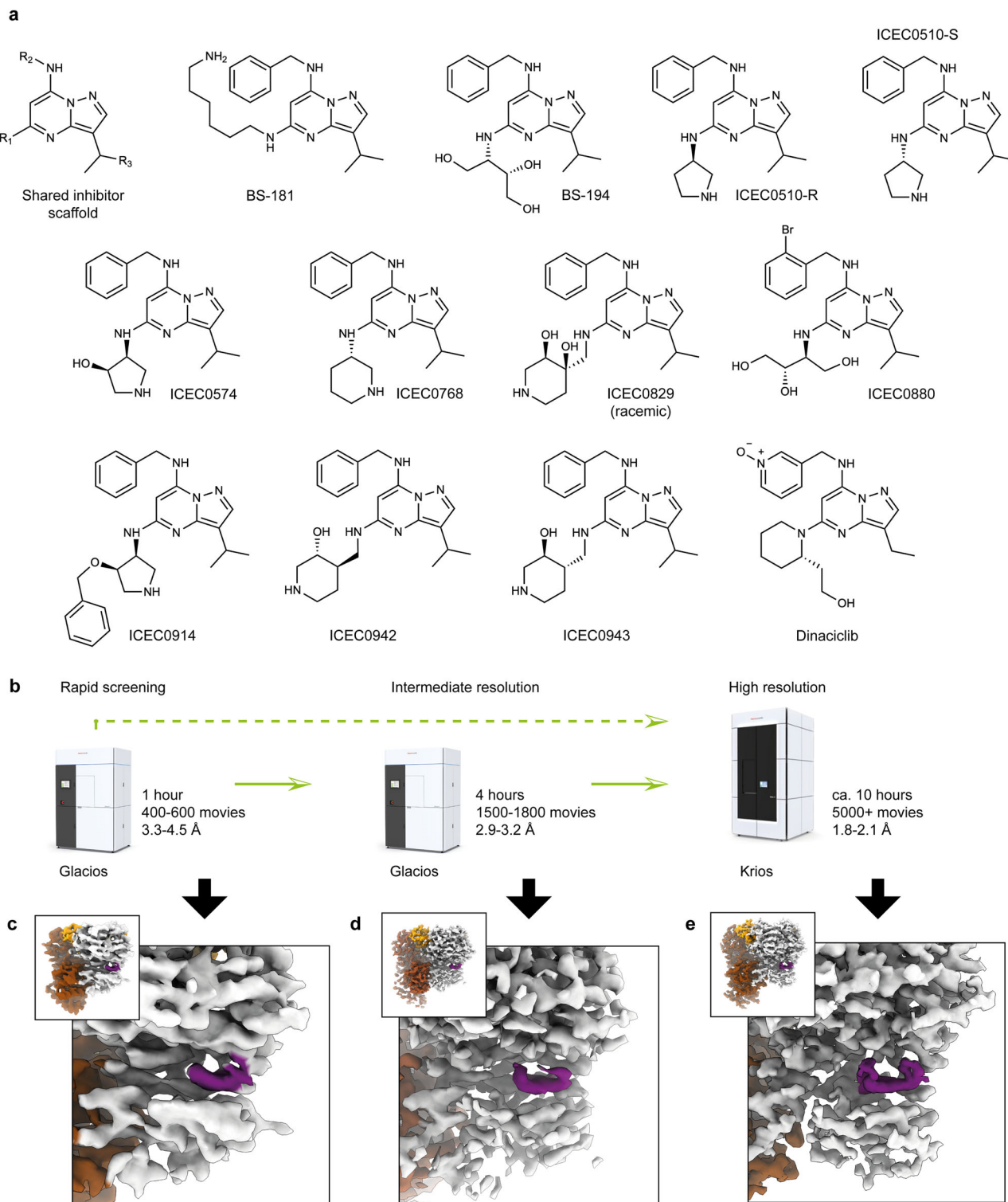


Fig. 2 | Cryo-EM workflow for inhibitor series structure determination.

a Chemical structures of pyrazolopyrimidine-type inhibitors used for cryo-EM structure determination. The chemical core shared by all inhibitors is shown in the first position, along with definition of the R_1 - R_3 substituent positions that will be used in the text. **b** Schematic of tripartite cryo-EM workflow. The possibility of using

a two-stage workflow is indicated by a dashed green arrow. **c** Example result from 1-h Glacios screening with visible ligand density (purple). **d** Example result from 4-h Glacios screening with visible ligand density (purple). **e** Example high-resolution cryo-EM map from Krios G4 collection with visible ligand density (purple).

Supplementary Note 1). We also investigated the contribution of the energy filter to high-resolution structure determination of our 85 kDa target complex. We found a 0.3 Å resolution loss in the absence of energy filtration under the conditions tested (Fig. 3a, f, g,

Supplementary Note 2). Given these results, we decided to use the 200 kV Glacios 2 setup for rapid sample screening and intermediate-resolution structure determination, and to leverage the 300 kV Krios G4 setup for collection of high-quality datasets on selected specimens.

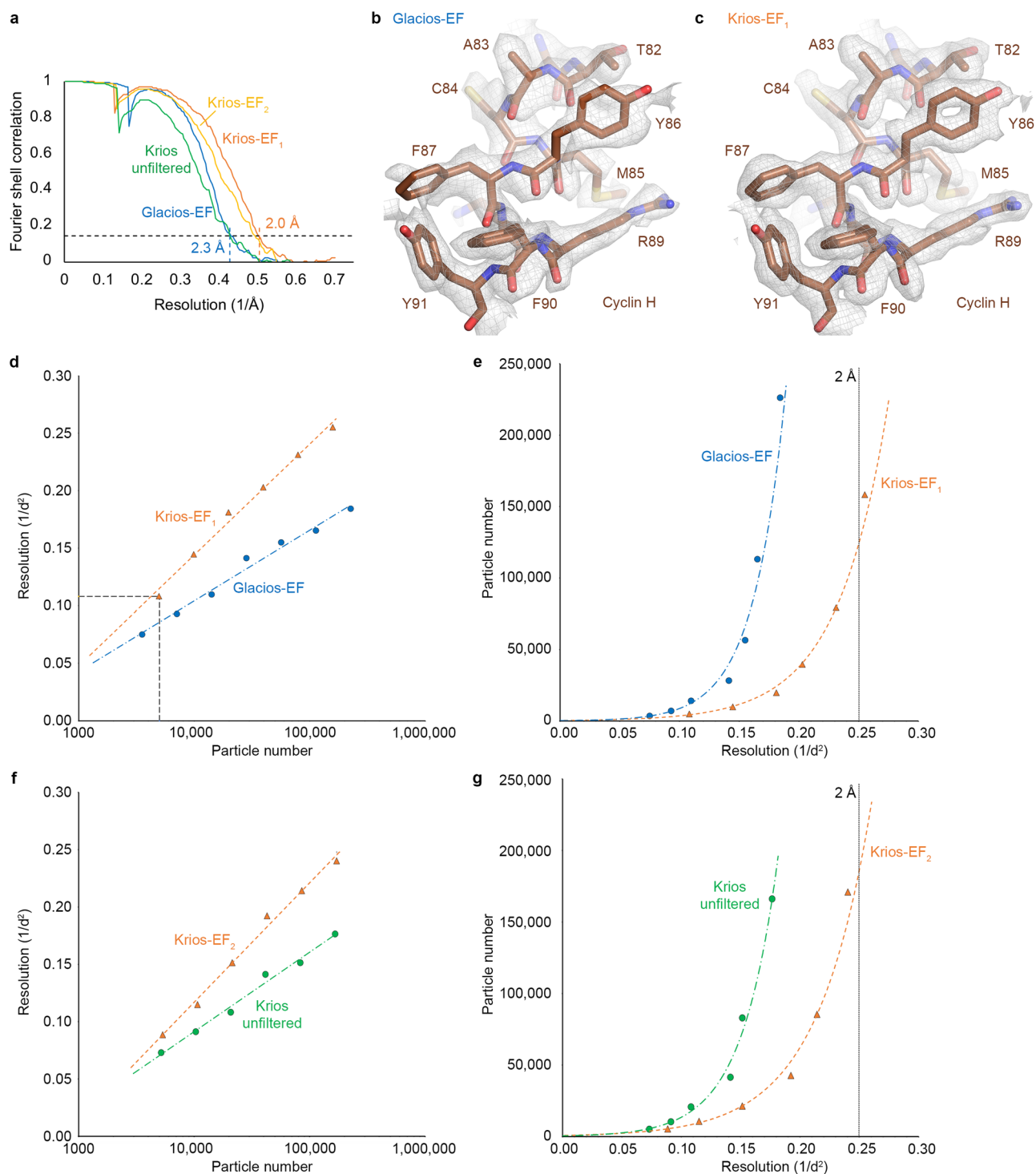


Fig. 3 | Glacios vs. Krios resolution comparison and impact of energy filtration on the resolution obtained. **a** FSC curves for the Glacios and Krios cryo-EM reconstructions (EF = energy filtered). Comparison between cryo-EM maps from Glacios-EF (**b**) and Krios-EF₁ (**c**) data. A segment of cyclin H is shown as an example. **d** Henderson-Rosenthal plot comparing Krios-EF₁ and Glacios-EF data. The inverse squared resolution of data subsets is plotted against the logarithm of the particle

number, resulting in a linear relationship. **e** Particle number plotted against inverse squared resolution. It is apparent that the Glacios data may not cross the 2 Å line even for very large amounts of data. **f**, **g** As **d** and **e**, but comparing the reconstructions obtained from the energy-filtered Krios setup (dataset Krios-EF₂) against the results obtained after retracting the slit from the beam path (Krios unfiltered). Source data are provided as a Source Data file.

Application of our cryo-EM workflow to structure determination of CAK-bound pyrazolopyrimidine-type inhibitors

The goal of the initial rapid sample screening phase was the identification of specimens exhibiting (i) good ice thickness, (ii) suitable particle density, (iii) suitable particle orientation distribution, (iv) presence of inhibitor, and (v) promising resolution from on-the-fly

processing of a dataset of a defined size during collection. Using our CAK-inhibitor complexes, we were able to satisfy these requirements using only 1 h of data collection time per dataset (Fig. 2b, c), thereby determining 26 structures confirming the presence of 12 different bound inhibitors from 13 samples, most of them at approximately 3.5–4.0 Å resolution (Supplementary Figs. 3, 4, 5a, Supplementary

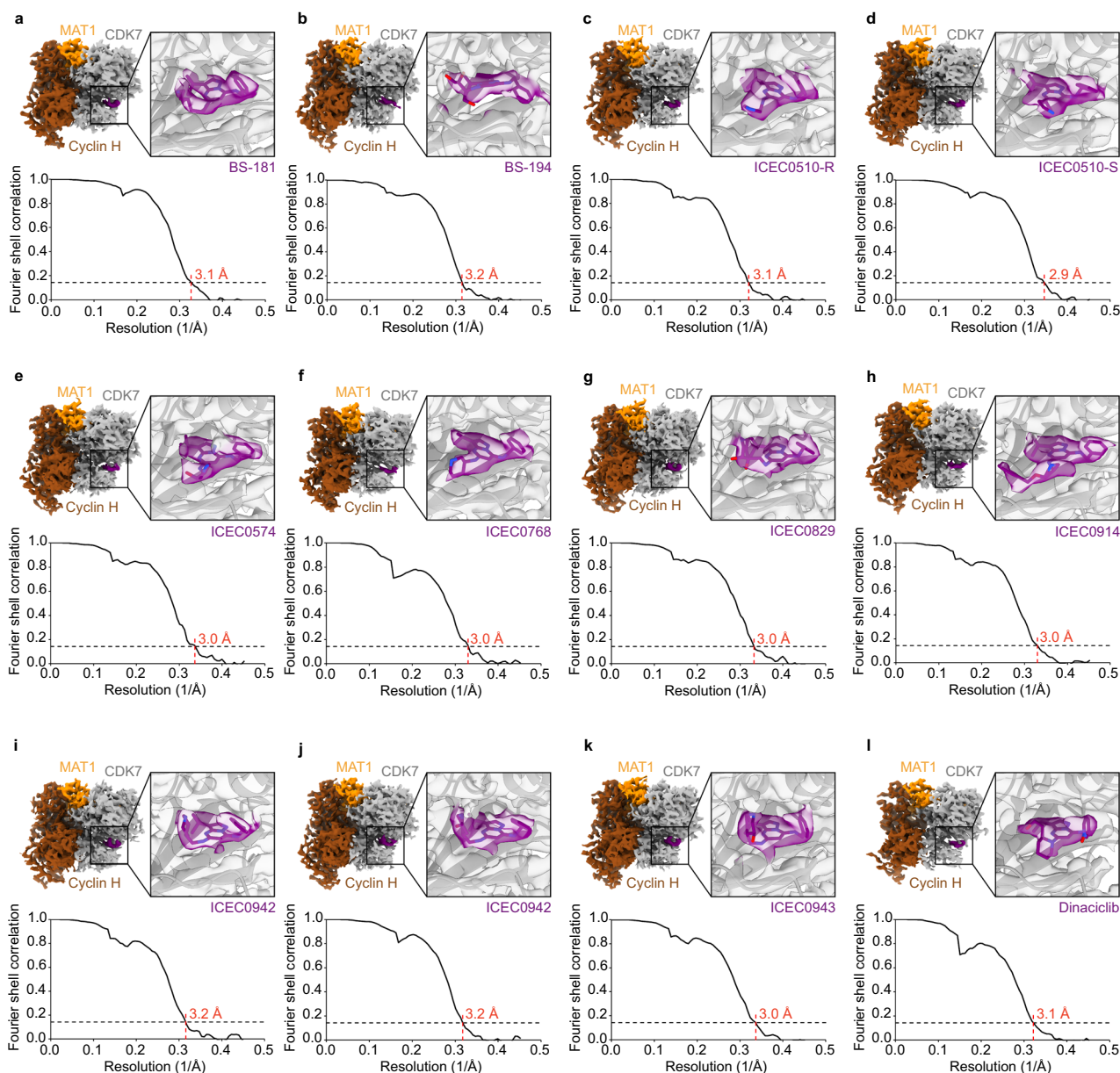


Fig. 4 | Results of 4-h Glacios screening. Overview of 4-h Glacios dataset results. Top panels show a view of the 3D reconstruction (CDK7 gray, cyclin H brown, MAT1 orange) and a close-up view of the model and density for bound inhibitors (purple). Bottom panels show the resolution according to the FSC = 0.143 threshold.

a BS-181. **b** BS-194, a compound selective for CDK2. **c** ICEC510-R. **d** ICEC510-S.

e ICEC0574. **f** ICEC0768. **g** ICEC0829. **h** ICEC0914. **i, j** The clinical inhibitor ICEC0942. **k** ICEC0943, the enantiomer of ICEC0942. **l** Dinaciclib, a compound selective for CDKs 1, 2, 5, and 9. Source data are provided as a Source Data file.

Table 3). All data processing at this stage was performed using cryoSPARC live³⁴, enabling rapid visualization of the results during data collection. To enable a quantitative assessment of map and model quality and allow comparisons with higher-resolution structures (see below), we plotted the Q-scores of map-model fits for these datasets (Supplementary Fig. 5b–d).

We subsequently performed intermediate-resolution structure determination using the 200 kV Glacios 2 setup on selected grids, aiming to produce cryo-EM maps suitable for preliminary interpretation and approximate inhibitor docking. To this end, we extended the data collection time to 4 h and achieved approximately 3 Å resolution for most datasets (Figs. 2d, 4, Supplementary Fig. 5a, Supplementary Table 4), which led to a substantial increase in the average Q-score for both the fitted protein and ligands (Supplementary Fig. 5b–d). We applied a combination of cryoSPARC and RELION for processing of

these datasets (see “Methods” for details). The complete processing of more than a dozen datasets was feasible in approximately one day after the end of data collection on a single 4-GPU server. Parallelization of this step across multiple servers to reduce this time further will be straightforward if required. These acquisition sessions yielded 12 structures at around 3 Å resolution (Fig. 4), in which inhibitors can be oriented and refined into the density and major conformational differences in large substituents are visible. Models derived from these reconstructions may also be used as starting points for molecular dynamics simulations or other computational methods, an option that we did not further pursue at this stage because we opted to collect high-resolution data for these complexes.

Aiming to resolve CAK-bound inhibitors at high resolution to provide highly accurate molecular models and identify water molecules that may contribute to inhibitor binding and specificity, we

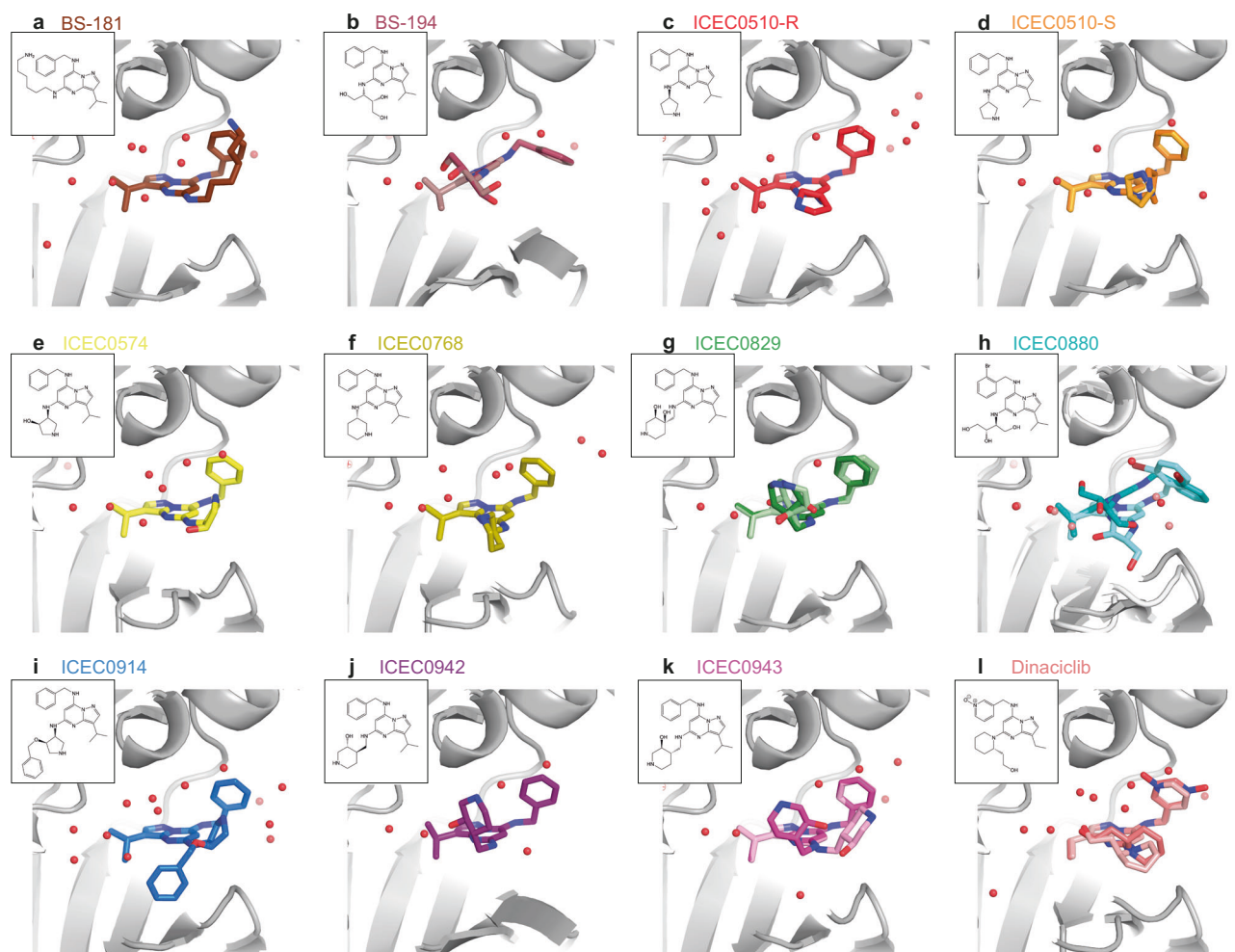


Fig. 5 | Structures of pyrazolopyrimidine-type inhibitors bound to CDK7. CDK7 is shown in gray, waters in red, ligands in color. Insets show the chemical structures of the corresponding inhibitor. **a** BS-181. **b** BS-194, a compound selective for CDK2 (two conformers with minor differences). **c** ICEC510-R. **d** ICEC510-S (two

conformers). **e** ICEC0574 (two conformers). **f** ICEC0768. **g** ICEC0829 (two enantiomers). **h** ICEC0880 (two different positions correlated with conformational changes in CDK7). **i** ICEC0914. **j** The clinical inhibitor ICEC0942. **k** ICEC0943, the enantiomer of ICEC0942 (two conformers). **l** Dinaciclib (two conformers).

used the 300 kV Krios G4 setup for high-resolution data collection (Fig. 2b, e). Data collections lasted for approximately 10 h and yielded roughly 5,000 micrographs for each sample. These data were initially processed using cryoSPARC live and cryoSPARC³⁴ and final classifications, Bayesian polishing³⁵, CTF refinement³⁶ and 3D refinement were performed in RELION³⁷. In contrast to the rapid screening and intermediate-resolution structure determination phases, the data processing effort for high-resolution reconstruction was substantial and extended substantially beyond the end of the data collection.

The large volume of CAK data acquired using this workflow (approximately 5000 micrographs per dataset for 12 inhibitors) allowed us to explore the resolution limits for our specimen and instrument by combining 6 fully processed datasets that had previously reached 1.8–1.9 Å resolution individually (see below). From 2,060,503 particles, we obtained a CAK reconstruction with averaged inhibitor density at a resolution of 1.7 Å after CTF refinement and an additional round of particle polishing (Supplementary Fig. 6a–c). Given the large volume of data that entered these computations, further progress towards atomic-resolution structure determination of the CAK is likely to depend on new specimen preparation methods, such as the introduction of nanobody derivatives to improve alignability of the particle images³⁸, or on new instrumentation (Supplementary Fig. 6d, e).

High-resolution structures of CAK-bound pyrazolopyrimidine inhibitor complexes

Our high-throughput screening and collection workflow enabled us to efficiently visualize 12 CAK-inhibitor complexes at 1.8–2.2 Å resolution (Figs. 5a–l, 6a–f, Supplementary Figs. 5a, 7–10, Supplementary Tables 1, 5–11). The cryo-EM maps typically show well-resolved density for the pyrazolopyrimidine core and the benzylamine-derived substituents (R_2 position) of the bound inhibitors, and less-well resolved density for the variable substituents at the R_1 position, indicating structural heterogeneity in the bound compounds (e.g. Figs. 5g, h, k, l, 6c, d, f, Supplementary Figs. 8–10). The Q-scores for the protein and bound ligands are again improved compared to the 4-hour screening datasets, with average Q-scores reaching a value of approximately 0.8 in most structures (Supplementary Fig. 5b–d).

The bound ICEC-series compounds exploit hydrogen bonding interactions with the backbone amide and carbonyl of CDK7 residue M94 in the hinge region that are shared between all compounds, leading to a relatively well-preserved positioning of the pyrazolopyrimidine core within the CDK7 active site (Fig. 5a–l). These interactions are also exploited by other hinge-binding kinase inhibitors¹⁰, including the CDK7 inhibitors THZ1 and LDC4297 (see above). Further interactions shared among the analyzed pyrazolopyrimidines are formed by the aliphatic substituent (ethyl for R_3 = H in dinaciclib and isopropyl for R_3 = CH₃ in all other inhibitors) that protrudes into a hydrophobic

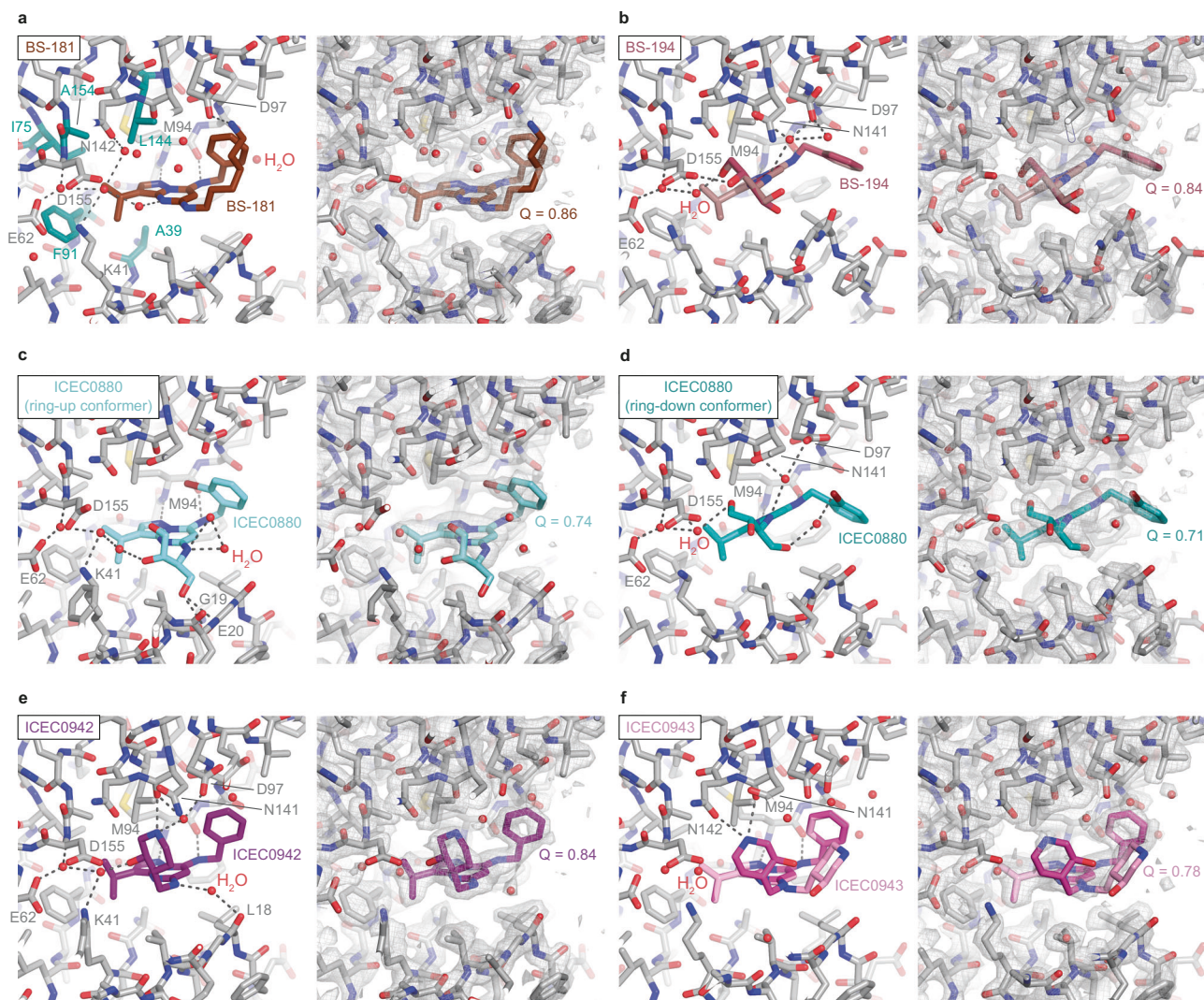


Fig. 6 | Detailed analysis of selected CDK7-inhibitor complexes. Hydrogen bonding interactions are indicated in the left sub-panel and the fit to the map including evaluation by Q-score is shown in the right sub-panel for each inhibitor. **a** BS-181, a highly CDK7-selective compound, assumes a ring-up conformation. Hydrophobic residues surrounding the R₃ substituent of the inhibitor (see text for details) are shown in teal. **b** BS-194, a highly CDK2-selective compound, assumes a

ring-down conformation. **c, d** ICEC0880, a BS-194 derivative with an additional bromine atom at the benzylamine group, assumes both ring-up (**c**) and ring-down (**d**) conformations. **e** ICEC0942 engages in an extended network of water interactions that may be responsible for its high selectivity. **f** ICEC0943, the enantiomer of ICEC0942, appears to be unable to engage in the same water-mediated interactions, and its hydroxypiperidine substituent is found in two conformations.

cavity within the CDK7 active site formed by A39, I75, L144, A154, and the gatekeeper residue F91 (Fig. 6a). The R₁ substituent is the structurally most diverse among the inhibitors tested and engages in a variety of interactions that are specific to each inhibitor. For example, the protonated primary amine in BS-181 (Fig. 6a) forms a salt bridge with CDK7 residue D97, while the trihydroxybutyl substituent in BS-194 engages in a hydrogen bonding network that involves bound water molecules in the CDK7 active site cavity as well as CDK7 residues E62 and D155 (Fig. 6b). A more detailed analysis of the interactions and conformational dynamics of the R₁ and R₂ substituents (Fig. 6c–f) and their contributions to inhibitor selectivity will be provided in the following paragraphs.

Structural insight into ICEC0942 selectivity

The high-resolution analysis of the CAK-ICEC0942 complex (Fig. 6e) extends and further defines our previous results, in which the orientation of the hydroxypiperidine substituent at the R₁ position was ambiguous¹⁴. Our high-resolution density supports an orientation of the six-membered piperidine ring in which the hydroxy group points

into the active site cavity (Fig. 6e). This allows the hydroxy group to participate in a network of hydrogen bonding interactions involving two water molecules, the side chains of CDK7 residues K41 and E62, and the backbone amide of residue D155 (Fig. 6e, Supplementary Fig. 11a). Two additional water molecules are within hydrogen bonding distance of the protonated secondary amine in the hydroxypiperidine substituent and the exocyclic amino group connecting the R₁ substituent to the pyrazolopyrimidine core, respectively, and mediate interactions between the inhibitor and residues lining the CDK7 active site pocket (Fig. 6e). The protonated secondary amine of the hydroxypiperidine moiety is additionally hydrogen bonded to the backbone carbonyl of CDK7 residue N141 (Fig. 6e). It is worth noting that the equivalent group in the structurally related inhibitor CT7030 (see below) is slightly repositioned, by less than 1 Å, which additionally brings it into hydrogen bonding distance with the side chain of CDK7 residue N142. It is therefore possible that some conformations of ICEC0942 can access this interaction as well.

The hydroxy group on the ICEC0942 hydroxypiperidine substituent appears to be an important modulator of inhibitor binding,

likely due to its ability to participate in the hydrogen bonding networks outlined above. In agreement with this rationale, the interactions of the ICEC0942 hydroxypiperidine substituent in the CDK2 off-target complex are different. Here, the hydroxy group points out of the active site cavity and interacts with a water molecule that is hydrogen bonded to a backbone amide and carbonyl (Supplementary Fig. 11b). The hydroxypiperidine secondary amine is hydrogen bonded to the side chain of CDK2 residue N132, but positioned more than 4 Å away from the carbonyl oxygen of residue Q131 (the equivalents of CDK7 N142 and N141, respectively). Additionally, the benzylamine substituent assumes the ring-down conformation, likely due to small conformational differences of the hinge region and a leucine (CDK7) to isoleucine (CDK2) amino acid substitution in the N-terminal kinase lobe (Supplementary Fig. 11c, d). The side chain of CDK2 K89 (Supplementary Fig. 11b, d) could potentially interfere with the ring-up conformation, but the density for this side chain in the X-ray crystal structure (PDB ID 5JQ5)¹⁶ is comparably weak, indicating that this side chain is partially flexible.

The importance of a specific configuration of the hydroxypiperidine group for CDK7 binding is further emphasized by the ICEC0942 enantiomer ICEC0943 (Fig. 2a). This compound exhibits poorly defined density for the entire hydroxypiperidine ring, suggestive of two different orientations (Fig. 6f). This inability to form the same stabilizing interactions in the CDK7 active site explains why ICEC0943 binds CDK7 with much reduced affinity, as reported previously¹⁶.

Further insights into CDK7 inhibitor selectivity

Our structural analysis yielded additional insight into inhibitor selectivity. BS-194 is the only inhibitor for which we observed a homogeneous ring-down conformation of the benzylamine substituent at the R₂ position (Figs. 5b, 6b). We observed previously that ICEC0942 assumes a ring-up conformation bound to CDK7 and a ring-down conformation bound to CDK2 (Fig. 7a), suggesting a link to target selectivity¹⁴. Comparisons of our data with existing structures show that dinaciclib³⁹ and ICEC0943¹⁶ show a similar conformational switch between the CDK7 and CDK2-bound complexes, while BS-194 assumes a homogeneous ring-down conformation in complex with both kinases (Fig. 7b–d). Interestingly, BS-194 is strongly selective for CDK2 over CDK7, while the related derivative BS-181, which is selective for CDK7 over CDK2 (Supplementary Table 2)^{30,31}, exhibits a ring-up conformation bound to CDK7 (Figs. 6a, 7e). These observations support our hypothesis that this conformational change is due to properties of the respective CDK active sites, and that the ability of an inhibitor to accommodate this change may be connected to target preference, providing a possible secondary mechanism of the CDK7 selectivity of ICEC0942, in addition to the interactions formed by the hydroxypiperidine group (see above). The conformational change in the benzylamine ring is coupled to a shift of the pyrazolopyrimidine core of the inhibitors, which is observed for multiple compounds in CDK7 and CDK2 (Fig. 7a–f).

In contrast to the other inhibitors, which exhibit clear ring-up or ring-down conformations, ICEC0880 exhibits both conformations when bound to CDK7 (Figs. 5h, 6c, d, 7e, f). Because these inhibitor binding modes coincide with movements of the β -sheet in the N-terminal kinase lobe of CDK7, it was possible to computationally separate the cryo-EM data into distinct classes and directly visualize and compare them (Figs. 5h, 6c, d). Given the small molecular weight of the inhibitor, which produces only a minute signal in the raw particle images, this would likely have been impossible in the absence of correlated motions in the protein.

It is worth noting that both BS-194 and its derivative ICEC0880 contain a 1,2,4-trihydroxybutyl substituent in the R₁ position. It is possible that this substituent contributes to the observed effect by promoting a conformation of the pyrazolopyrimidine core that is

conductive to the ring-down conformation and reduced CDK7 selectivity (or increased CDK2 selectivity), which is counteracted by the bulky exocyclic bromine at the benzylamine substituent of the ICEC0880. This added steric constraint might destabilize binding in the ring-down conformation, as exhibited by the shift of the entire pyrazolopyrimidine core in the two ICEC0880 structures. Notably, LDC4297, which is highly specific for CDK7, carries an even more bulky substituent at its ring-up benzylamine group (Supplementary Figs. 1b, 2e), and the recently reported CDK7-selective compound LGR6768⁴⁰ exploits a bulky biphenyl substituent at this position.

The inhibitor CT7030 shows increased CDK7 selectivity

To directly test the idea that substituents at the benzylamine group could impinge on target selectivity, we performed in vitro kinase assays and structure determination of an inhibitor that is based on the ICEC0942 scaffold but harbors an *ortho*-methoxy substituent at the benzylamine group (CT7030, Fig. 8a). The cryo-EM structure of the CAK-CT7030 complex confirms that the inhibitor assumes the expected ring-up conformation (Fig. 8b, Supplementary Fig. 12), and in vitro kinase inhibition assays confirmed an increase in selectivity for CDK7 over CDK2 to more than 200 $\times$ (Fig. 8c–e), supporting the validity of our cryo-EM-based mechanistic hypothesis.

Discussion

We report a series of structures of the human CAK in free, nucleotide-bound, and small-molecule inhibitor-bound states, 14 of them at 2 Å resolution or better. Notably, we obtained these results using a molecular complex that is the target of actively ongoing drug discovery programs and clinical trials. Before our efforts, the structure of only one <100 kDa protein complex had been determined at better than 2 Å resolution by cryo-EM—that of streptavidin (e.g. EMD-31083)—and none for a medically relevant target or a protein complex lacking symmetry in this size range. We have now demonstrated that current cryo-EM technology is suitable for routine 2 Å structure determination of small protein complexes from less than one day of data collection on a high-end system, opening the door to high-throughput, high-resolution cryo-EM even for nominally difficult targets¹⁸. Additionally, our screening approaches demonstrate the feasibility of the determination of more than a dozen 3.5–4.0 Å-structures a day, and several 3 Å structures a day, using a 200 kV microscope. Using a 300 kV instrument for screening harbors the potential for even higher sample throughput at the same resolution, or improved resolution at constant sample throughput. These results and the methodology outlined in this work will facilitate the application of cryo-EM to iterative structure-based drug design efforts, which are of critical importance to address global health challenges.

Our data show that the hydroxypiperidine substituent of the clinical inhibitor ICEC0942 engages in favorable interactions in the CDK7 active site, which results in the high affinity and selectivity of the compound. Our data additionally support a mechanistic hypothesis for a secondary mechanism of CDK7 selectivity in pyrazolopyrimidine-based CDK inhibitors, specifically that small structural differences in the nucleotide-binding pockets in CDK7 and CDK2 induce a preference for different conformations of the benzylamine substituent that forms part of the scaffold of the inhibitors we analyzed, and that chemical modifications that favor one conformation over the other may be used to improve kinase selectivity of these compounds. These findings will pave the way for future structure-guided CDK7 inhibitor design. Furthermore, they provide a proof of principle for the benefit of the application of cryo-EM in structure-based CDK7 inhibitor design.

Methods

Synthesis of compounds

All CDK inhibitors were synthesized through the course of prior studies using methods described^{30,31}. In brief, all compounds were

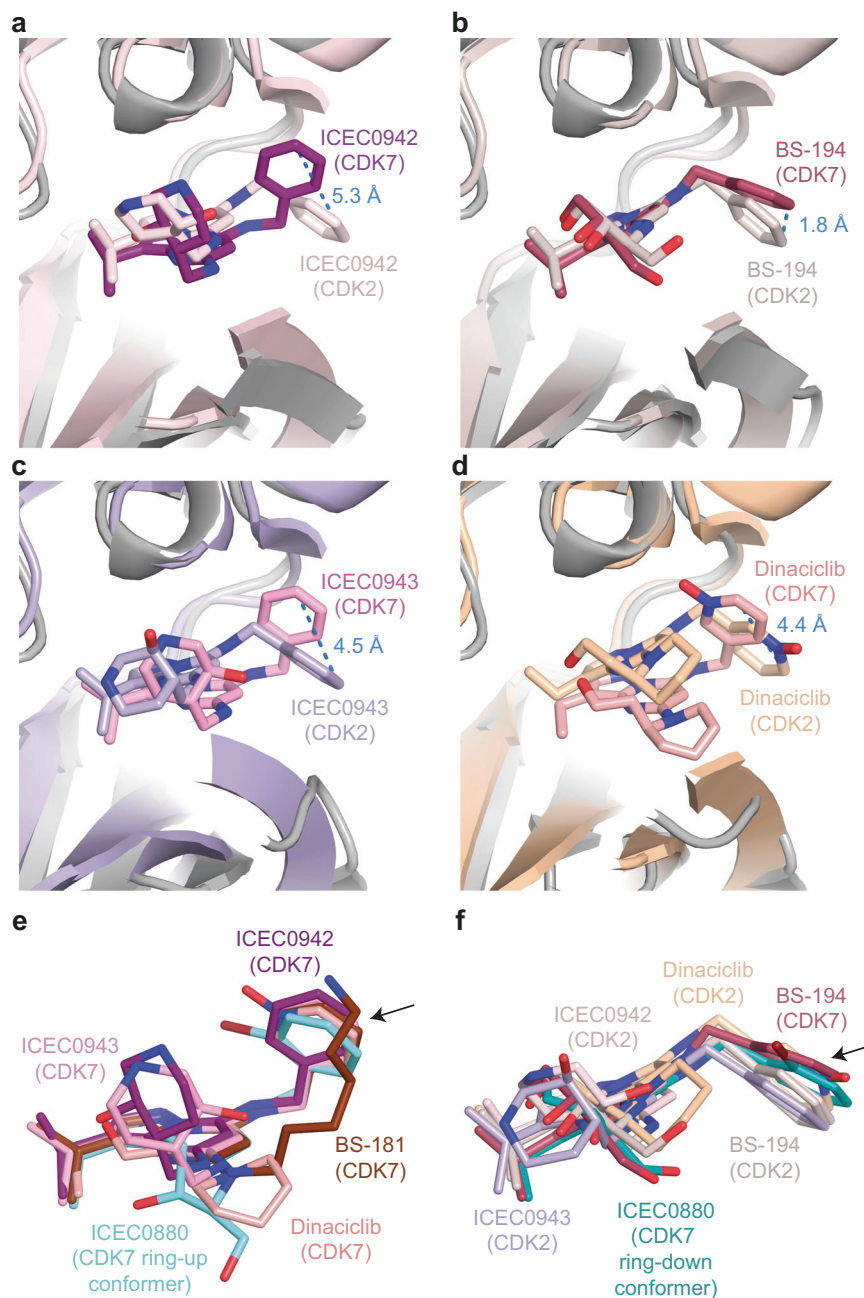


Fig. 7 | Analysis of high-resolution inhibitor structures. **a** Comparison of ICEC0942 bound to CDK7 (this work) and CDK2 (PDB ID 5JQ5)¹⁶. The conformation of the benzylamine group and the placement of the inhibitor core differ between the two structures. The displacement distance of the most distal carbon atom of the phenyl ring in the benzylamine substituent is indicated. **b** Comparison of BS-194 bound to CDK7 (this work) and CDK2 (PDB ID 3NS9)³¹. **c** Comparison of ICEC0943 bound to CDK7 (this work) and CDK2 (PDB ID 5JQ8)¹⁶. **d** Comparison of dinaciclib

bound to CDK7 (this work) and CDK2 (PDB ID 4KD1)³⁹. For clarity, only one conformer for the CDK7-bound compounds is shown in (**b–d**). **e** Superposition of ICEC0942, BS-181, ICEC0943, dinaciclib, and the ring-up ICEC0880 conformer, all bound to CDK7. **f** Superposition of ICEC0942, BS-194, ICEC0943, and dinaciclib, all bound to CDK2, with BS-194 and the ring-down conformer of ICEC0880 bound to CDK7. The flipping ring system is indicated with an arrow.

prepared from dichloropyrazolo[1,5-*a*]pyrimidine by sequential selective substitution of the chlorides by amines. Firstly, the R_2 substituent was added by nucleophilic aromatic substitution using General Procedure A, followed by protection of the NH using General Procedure B. Protection of the benzylic amine as a carbamate was essential for successful amination of the second (R_1) position. The R_1 substituent was added using a palladium-catalyzed Buchwald-Hartwig reaction, according to General Procedure C, followed by deprotection (General Procedure D).

General Procedure A. Dichloropyrazolo[1,5-*a*]pyrimidine (1.0 equiv) and the R_2 amine (2.0 equiv) in EtOH (0.13 M) were heated at reflux for 16 h. The reaction mixture was cooled to ambient temperature and concentrated *in vacuo*. Crystallization (EtOAc) or chromatography (EtOAc: hexanes) was used for purification.

General Procedure B. The relevant substrate (1.0 equiv), di-*tert*-butyl dicarbonate (1.3 equiv), and 4-dimethylaminopyridine (catalytic) in THF (0.14 M) were stirred for 20 h at ambient temperature. EtOAc was

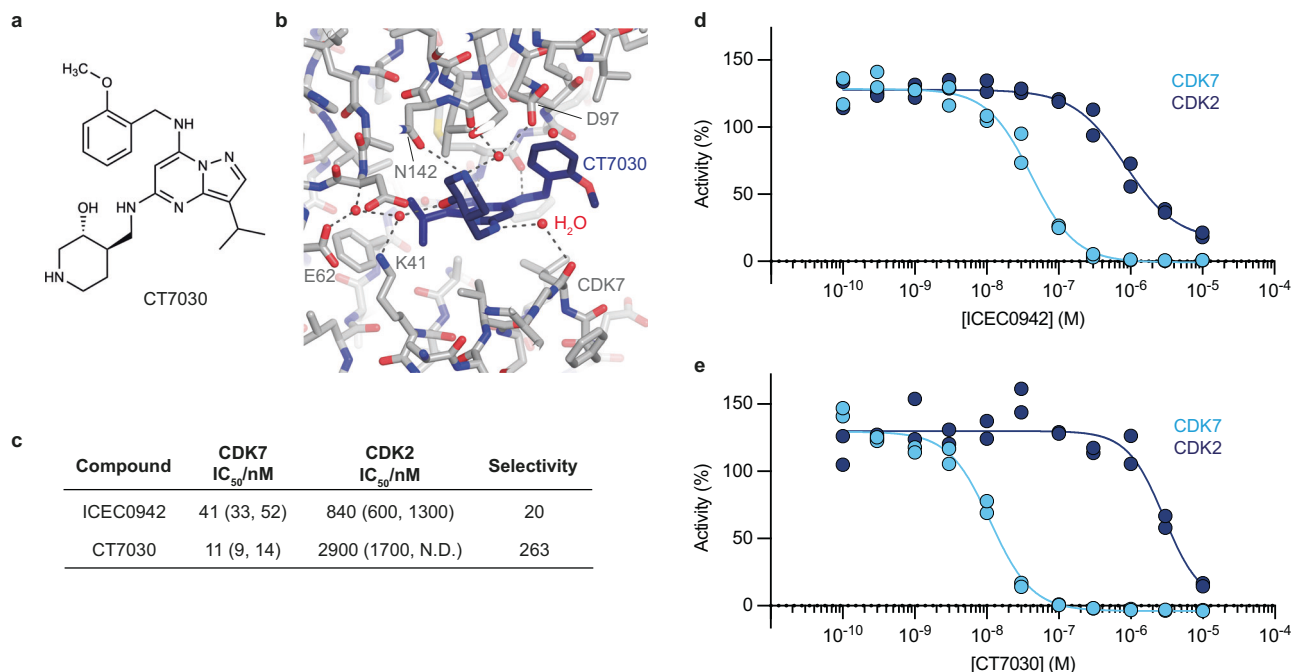


Fig. 8 | The CDK7 inhibitor CT7030. a Chemical structure of CT7030. **b** Cryo-EM structure of CAK-bound CT7030. **c** In vitro kinase inhibition data of CT7030 compared with ICEC0942. Numbers in brackets indicate 95% confidence intervals

for the IC₅₀ values (ND not determined). **d, e** Plots of the data underlying the values in **a** ($n = 2$). Source data are provided as a Source Data file.

added, and the organic layer was washed with water (2×), saturated aqueous NaHCO₃, and dried (Na₂SO₄). The organic solvent was removed *in vacuo*. Chromatography on silica (EtOAc: hexanes) was used for purification.

General Procedure C. The relevant substrate (1.0 equiv) was reacted with tris(dibenzylideneacetone)dipalladium (0.05 equiv), 2,2'-bis(diphenylphosphino)-1,1'-binaphthalene (0.15 equiv), and sodium *tert*-butoxide (1.1 equiv) in PhMe (0.30 M). The mixture was stirred for 15 min. The corresponding R₁ amine (1.1 equiv) was added, and the mixture heated to 95 °C for 16 h and cooled and diluted with EtOAc. The organic phase was washed with water (3×), brine, dried (MgSO₄), and concentrated *in vacuo*. Chromatography on silica (EtOAc: hexanes) was used for purification.

General Procedure D. AcCl was dissolved in MeOH (5.0 M) with stirring at 0 °C. After 15 min, the solution was warmed to room temperature and stirred for a further 15 min. The relevant substrate was added, and the mixture stirred at ambient temperature for 3 h and concentrated *in vacuo*. The residue was dissolved in CH₂Cl₂, washed with saturated aqueous sodium bicarbonate, dried (Na₂SO₄), and concentrated *in vacuo*. Crystallization (MeCN or MeOH) or chromatography (CH₂Cl₂: MeOH) was used for purification.

Characterization of ICEC0510 and ICEC0510-R/S

m.p. (MeOH) = 200–230 °C. IR (neat) $\nu_{\max}$ 3316, 2911, 2718, 2428, 1636, 1588, 1579, 1553, 1495, 1445, 1418, 1356, 1235, 1170, 765, 698 cm⁻¹. ¹H NMR (400 MHz, DMSO-*d*₆): δ 9.16 (s, 2H, NH⁺), 7.96 (t, $J = 6.5$ Hz, 1H, NH), 7.66 (s, 1H), 7.34–7.31 (m, 4H), 7.27–7.22 (m, 1H), 6.99 (d, $J = 5.2$ Hz, 1H, NH), 5.09 (s, 1H), 4.46 (d, $J = 6.5$ Hz, 2H), 4.41–4.34 (m, 1H), 3.42 (dd, $J = 11.8$ Hz and $J = 6.5$ Hz, 1H), 3.33–3.17 (m, 2H), 3.04 (dd, $J = 11.8$ Hz and $J = 4.8$ Hz, 1H), 2.98 (heptuplet, $J = 6.9$ Hz, 1H), 2.21–2.12 (m, 1H), 1.91–1.82 (m, 1H), 1.265 (d, $J = 6.9$ Hz, 3H), 1.262 (d, $J = 6.9$ Hz, 3H). ¹³C NMR (125 MHz, DMSO-*d*₆): δ 155.6 (s), 146.2 (s), 145.0 (s), 139.8 (d), 138.3 (s), 128.4 (d, 2C), 126.9 (d), 126.6 (d, 2C), 111.0 (s), 72.5 (d), 49.8 (t),

44.5 (t), 43.5 (t), 29.8 (t), 23.4 (d), 23.1 (q), 23.0 (q). Elemental Analysis: Calculated for C₂₀H₂₇ClN₆: C, 62.08; H, 7.03; N, 21.72. Found: C, 62.18; H, 6.98; N, 21.68.

Racemic ICEC0510 was separated into *R* and *S* enantiomers using chiral HPLC, with $[\alpha]_D = -6.0$ (c 0.110 in MeOH) and $[\alpha]_D = 8.5$ (c 0.095 in MeOH) for ICEC0510-*R* and -*S*, respectively.

Characterization of ICEC0914

¹H NMR (600 MHz, MeOD) δ 7.69 (s, 1H), 7.44–7.41 (m, 2H), 7.38–7.34 (m, 2H), 7.30–7.27 (m, 1H), 7.23 (dd, $J = 7.6, 1.8$ Hz, 2H), 7.19–7.13 (m, 3H), 4.72–4.67 (m, 1H), 4.64 (d, $J = 12.0$ Hz, 1H), 4.60 (br s, NH), 4.59 (s, 2H), 4.50 (d, $J = 12.0$ Hz, 1H), 4.30–4.38 (m, 1H), 3.68–3.58 (m, 1H), 3.55–3.49 (m, 1H), 3.45–3.38 (m, 1H), 3.25–3.18 (m, 1H), 3.04 (heptuplet, $J = 6.9$ Hz, 1H), 1.38–1.25 (m, 6H). ¹H obscured by solvent peak. LC/MS (ES⁺): 1.90 min. Found: 457.2716. Calculated for C₂₇H₃₂N₆O (M + H⁺): 457.2710.

The amount of ICEC0914 available was insufficient for further characterization. However, inhibitor characterization using LC–MS (see below and Supplementary Table 12) and the presence of all expected chemical groups in our cryo-EM map of the compound-bound complex further support the identity of the compound.

Characterization of ICEC0768

m.p. = 175–180 °C. $[\alpha]_D = -20.2$ (c 0.47, MeOH). IR (neat) $\nu_{\max}$ 3293, 1635, 1575, 1441, 1360, 1219, 1173, 1063, 748, 699 cm⁻¹. ¹H NMR (400 MHz, MeOD): δ 7.73 (s, 1H), 7.43–7.36 (m, 4H), 7.31–7.28 (m, 1H), 5.29 (br s, 1H), 4.61 (br s, 2H), 4.26–4.15 (m, 1H), 3.61–3.57 (m, 1H), 3.33–3.26 (m, 1H), 3.12 (heptuplet, $J = 6.9$ Hz, 1H), 3.07–3.00 (m, 2H), 2.12–2.05 (m, 2H), 1.92–1.81 (m, 1H), 1.75–1.64 (m, 1H), 1.340 (d, $J = 6.9$ Hz, 3H), 1.336 (d, $J = 6.9$ Hz, 3H). ¹³C NMR (125 MHz, MeOD): Due to low signal intensity, some carbons could not be seen. δ 141.8 (d), 138.9 (s), 129.8 (d, 2C), 128.5 (d, C), 128.0 (d, 2C), 113.6 (s), 48.5 (t), 46.6 (t), 46.2 (d, low signal intensity), 45.0 (t), 29.2 (t), 24.8 (d), 23.8 (q), 23.7 (q), 21.9 (t). LC/MS (ES⁺): 3.71 min. Found: 365.2442. Calculated for C₂₁H₂₉N₆ (M + H⁺): 365.2454.

Characterization of ICEC0880

IR ν_{max} /cm⁻¹ (film) 3275 (w), 1637 (s), 1576 (m), 1553 (s), 1424 (m); ¹H NMR δ H (400 MHz, MeOD) 7.68 (1H, s), 7.62 (1H, dd, J 7.9, 1.1 Hz), 7.38 (1H, dd, J 7.7, 1.5 Hz), 7.32 (1H, td, J 7.5, 1.1 Hz), 7.20 (1H, td, J 7.7, 1.9 Hz), 5.20 (1H, s), 4.60 (2H, s), 4.03 (1H, m), 3.84 (1H, dd, J 11.2, 3.8 Hz), 3.78 (1H, dd, J 11.1, 5.9 Hz), 3.54 (3H, m), 3.02 (1H, sp, J 6.9 Hz), 1.29 (3H, d, J 6.9 Hz),

1.26 (3H, d, J 6.9 Hz); ¹³C NMR δ C (100 MHz, MeOD) 159.0, 148.2, 146.0, 141.7, 137.6, 134.0, 130.3, 129.5, 129.0, 123.9, 113.6, 74.3, 73.5, 64.5, 63.1, 55.8, 47.0, 24.7, 24.0, 23.6; m/z HRMS (ES⁺): (M + H)⁺ 464.1295, C₂₀H₂₇BrN₅O₃ requires 464.1297.

Characterization of CT7030

¹H NMR (400 MHz, DMSO) δ 7.64 (s, 1H), 7.57 (t, J = 6.4 Hz, 1H), 7.26 (td, J = 7.8, 1.7 Hz, 1H), 7.16 (dd, J = 7.5, 1.7 Hz, 1H), 7.03 (dd, J = 8.4, 1.1 Hz, 1H), 6.90 (td, J = 7.5, 1.1 Hz, 1H), 6.71 (t, J = 6.1 Hz, 1H), 5.38 (s, 1H), 5.12 (s, 1H), 4.41 (d, J = 6.4 Hz, 2H), 3.87 (s, 3H), 3.52 (m, 1H), 3.20 (m, 1H), 3.06 (m, 1H), 2.95 (m, 2H), 2.83 (m, 1H), 2.35 (m, 1H), 2.21 (dd, J = 11.6, 10.0 Hz, 1H), 1.58 (m, 1H), 1.36 (m, 1H), 1.25 (d, J = 4.7 Hz, 3H), 1.23 (d, J = 4.7 Hz, 3H), 1.16 (m, 1H). 1H was not observed.

The amount of CT7030 available was insufficient for further characterization. However, inhibitor characterization using LC-MS (see below and Supplementary Table 12) and the presence of all expected chemical groups in our cryo-EM map of the compound-bound complex further support the identity of the compound.

Compounds used for structural and enzyme inhibition studies

ATPγS was purchased from Sigma Aldrich; THZ1 was purchased from EMD Millipore; LDC4297 and dinaciclib were purchased from MedChemExpress (distributed by Insight Biotechnology, Wembley, UK). ICEC-series pyrazolopyrimidine compounds were synthesized and characterized during the course of the drug discovery program that led to the discovery of ICEC0942^{5,30–33,41}. Further characterization data for ICEC0510-R and S enantiomers, ICEC0768, ICEC0880, ICEC0914, and CT7030 are provided above. After retrieval from cold storage, integrity of ICEC-series compounds was verified using UV-vis spectroscopy and liquid chromatography/mass spectrometry (see below for detailed methods). The results of this characterization are summarized in Supplementary Table 12.

Verification of inhibitor integrity and purity

LC/MS grade solvents, formic acid, or alternative eluent modifiers were purchased from VWR (Poole, UK) and Fisher (Loughborough, UK) unless otherwise stated. 10 μ L of 1 mM DMSO solution of each compound was plated in a 384 Greiner (781280) well plate. 0.1 μ L standard injections (with needle wash) of the sample were made onto a Phenomenex Kinetex C18 30 $\times$ 2.1 mm, 2.6 μ m, 100Å column (Phenomenex, Torrance, CA, USA). Chromatographic separation was carried out at 40 °C using an Agilent 1260 Infinity II series UPLC (Agilent, Santa Clara, USA) over a 4 min gradient elution (KNOWNS_AM190319.m) from 90:10 to 10:90 water:methanol (both modified with 0.1% formic acid) at a flow rate of 0.4 mL/min. UV-vis spectra were acquired at 254 nm on a 1260 Series diode array detector (Agilent, Santa Clara, USA).

The post column eluent flow from the diode array detector was split, with 90% sent to waste. The remainder was infused into a 6530 Series QToF mass spectrometer fitted with an Agilent Jet Stream ESI source (Agilent, Santa Clara, USA). LC eluent and nebulizing gas was introduced into the grounded nebulizer with spray direction orthogonal to the capillary axis. A nozzle voltage of 0 V was applied to the charging electrode to generate a charged aerosol. The aerosol was dried by heated drying gas (10 L/min of nitrogen at 350 °C, 35 psi), producing ions by ESI. Ions entered the transfer capillary along which a potential difference of 4 kV was applied. The fragmenter voltage was set at 175 V and skimmer at 65 V. Signal was optimized by SWARM autotune. Profile mass spectrometry data was acquired in positive

ionization mode over a scan range of m/z 190–650 (scan rate 4.0) with reference mass correction at m/z 622.02896 hexakis(2,2-difluoroethoxy)phosphazene.

Raw data were processed using Agilent MassHunter Qualitative Analysis B.07.00 (AutoQC.m). The “Find Compounds by Formula” algorithm was used to identify compounds and calculate the purity. The compound purity was calculated using the highest value of %UV (at 254 nm) or %TIC (total ion count from the mass spectrometer).

Enzyme inhibition assays

In-vitro kinase assays comparing CT7030 and ICEC0942 activity were performed by the ADP-Glo method by Sygnature Discovery (Nottingham, UK) using in-house enzymes. Raw data were normalized using DMSO (negative) and staurosporine (positive) controls on the same plates. Data were analyzed in PRISM (versions 9 and 10; GraphPad Software, Inc.) as inhibitor concentration vs. normalized response with variable slope. Asymmetric confidence intervals were computed at a significance level of 95%. In-vitro kinase assays of inhibitors in Supplementary Table 2 reported in the literature were performed in ProQinase format^{5,16}, and we used the same assay, performed by Reaction Biology GmbH (Freiburg, Germany) for enzyme inhibition analysis reported in Supplementary Table 2 and Supplementary Fig. 13. ATP concentrations were set to the apparent K_m of the respective enzyme.

CAK expression, purification, and cryo-EM specimen preparation

For expression of all CAK variants used in this work, 438-series vectors encoding the multi-protein expression constructs⁴² were transformed into EMBacY cells for preparation of bacmids⁴³ by isopropanol precipitation. Bacmid DNA was transfected into Sf9 (*Spodoptera frugiperda*) insect cells (Thermo Fisher Scientific, catalog number 11496015) using CellFectin II transfection reagent (Thermo Fisher Scientific). Baculoviruses were amplified for two rounds in Sf9 cells (one round in Sf9 and one round in Hi5 for CAK-MAT1Δ219). Subsequently, 15 mL of insect cell culture supernatant (50 mL of cell culture for CAK-MAT1Δ219) were used for infection of 1-L cultures of High5 (*Trichoplusia ni*) insect cells (Thermo Fisher Scientific, catalog number B85502). After 60–72 h of incubation, insect cells expressing protein complexes were harvested, frozen in liquid N₂, and stored at –80 °C for later use.

CAK was purified by nickel-affinity, strep-tactin, and gel filtration chromatography¹⁵. Cells were thawed and stirred during resuspension in lysis buffer (250 mM KCl, 40 mM HEPES-KOH pH 7.9, 5 mM MgCl₂, 5 mM β -mercaptoethanol, 10 mM imidazole and 10% (v/v) glycerol supplemented with protease inhibitors and DNaseI). After lysis by sonication, cell debris were pelleted by centrifugation at 18,000 rpm (approx. 26,000 $\times$ g) for 30 min at 4 °C in a JA-25.50 rotor (Beckman Coulter) and the cleared lysate was incubated with Ni-NTA superflow resin (Qiagen) for 30 min. The beads were washed using CAK purification buffer (250 mM KCl, 25 mM HEPES-KOH pH 7.9, 2 mM MgCl₂, 5 mM β -mercaptoethanol, and 10% (v/v) glycerol) supplemented with 25 mM imidazole. After elution using purification buffer supplemented with 300 mM imidazole, the eluted fractions were incubated with Strep-Tactin superflow plus resin (Qiagen) for 30–45 min. The beads were washed and purified protein was eluted using purification buffer supplemented with 10 mM desthiobiotin (Sigma-Aldrich). Affinity tags were cleaved using Tobacco Etch Virus (TEV) protease at room temperature for 2 h, and tags as well as His₆-tagged protease were removed using reverse-phase nickel affinity chromatography. Size exclusion chromatography of the fractions containing CAK was performed using a Superdex 200 10/300 GL column (GE Healthcare) immediately after the reverse-phase nickel affinity chromatography or on the next day in the case of CAK-MAT1Δ219, in which case the protein was snap-frozen in liquid nitrogen and stored at –80 °C over night. Peak fractions were pooled, concentrated to 2 mg/mL, and frozen in

liquid N₂ for storage at −80 °C. Most datasets used the CAK-MAT1Δ219 variant, in which the N-terminal 219 residues of MAT1 are replaced by an MBP-tag, which is cleaved by incubation with TEV protease to yield the catalytic module of CAK lacking the TFIIF-binding portion of MAT1¹⁵. The LDC4297 dataset additionally used a construct in which the N-terminus of CDK7 is fused to three YSPTSPS-repeats as a substrate mimic resembling the Pol II-CTD, which has however never been visualized in any density arising from data using this construct¹⁵. CAK covalently modified by THZ1 was prepared by incubating 2 μM of CAK-MAT1Δ219 with 5 μM THZ1 (EMD Millipore) in buffer devoid of reducing agent for 1.5 h at room temperature, followed by gel filtration on a Superdex 200 10/300 GL column (GE Healthcare) to remove excess inhibitor and residual DMSO.

Cryo-EM specimens were prepared on UltrAuFoil R1.2/1.3 holey gold grids (Quantifoil Microtools). For complex formation, CAK complex at approximately 2 mg/mL stock concentration was diluted 5–6× in sample buffer (20 mM HEPES-KOH pH 7.9, 200 mM KCl, 2 mM MgCl₂, 5 mM β-mercaptoethanol for most samples; no β-mercaptoethanol for CAK-ICEC0574, CAK-ICEC0829, CAK-ICEC0942, apo-CAK, and CAK-ATPyS) and incubated with 50 μM non-covalent inhibitor (dissolved at 50 mM concentration in 100% DMSO, except ICEC0829 and ICEC0942, which were dissolved in water; no added inhibitor in case of CAK-THZ1, which was prepared as a covalent adduct) or 2 mM ATPyS (Merck Life Science UK) at room temperature for 5 min. 4 μL of CAK complex were applied to a plasma cleaned grid (Tergeo plasma cleaner, PIE scientific) mounted in a Vitrobot Mark IV (Thermo Fisher Scientific) operated at 5 °C and 100% humidity, blotted for 1, 1.5, 1.5, or 2 sec (4 grids made for each complex) and plunged into liquid ethane at liquid N₂ temperature. After vitrification, grids were clipped into autogrid cartridges (Thermo Fisher Scientific) for use with Glacios and Krios autoloader systems.

Initial screening

Initially, grids were coarsely screened on a Glacios cryo-transmission electron microscope (cryo-TEM) operated at 200 kV acceleration voltage and equipped with a Falcon 4 (later Falcon 4i) direct electron detector. Data were acquired in EER format at a pixel size of 0.5675 Å/pixel using a flux of 4–6 electrons. pixel^{−1} s^{−1} using aberration-free image shift (AFIS) to accelerate the data collection. Approximately 300–600 micrographs were processed in real time using cryoSPARC live³⁴ using 2× binning and with exposures fractionated into 60 frames for motion correction, which provided results suitable to judge overall sample quality and particle orientation distribution. This approach was later supplanted by the rapid-screening approach on a Glacios 2 electron microscope equipped with a Falcon 4i detector, a Selectris X energy filter, and fringe-free imaging capability enabling collection of 2–3 movies per hole and increased data collection rates. The latter data are shown in the “Results” section.

Multigrid-screening and data processing

We used acquisition settings that were similar to our previously reported conditions for structure determination of the CAK^{14,15}, adjusted for the changes in hardware compared to prior experiments, resulting in 0.57 Å pixel size, 70 electrons. Å^{−2} total exposure at 7–8 electrons. pixel^{−1} s^{−1} and EER format fractionated into 50 frames for screening runs and 70 frames for high-resolution runs (see below).

Data were collected using EPU-Multigrid (EPU version 2.14) on a 200 kV Glacios 2 microscope equipped with a Selectris X energy filter coupled with a Falcon 4i detector (Thermo Fisher Scientific). Data were collected using fringe-free imaging (FFI) and AFIS at a throughput of roughly 500 images/h. 2 grids each of 5 different samples and a dry Quantifoil Cu200 R2/2 or S106 cross-grating (11 grids total) were loaded into the autoloader for each EPU-Multigrid run. Within EPU, a new session queue was created, followed by sequentially loading grids to the stage for session set-up and grid square selection to give

approximately 500 (for 1 h-screening runs) or 2000 (for 4-h data collection session) images after automatic ice-filter selection. Before starting data collection, two-fold astigmatism was corrected, and beam tilt was adjusted to the coma-free axis using the dry Quantifoil Cu200 R2/2 or S106 cross-grating. The Selectris X filter slit was then centered on the zero-loss peak with a slit width of 10 eV, and the filter tuned for isochromaticity, magnification and chromatic aberrations using Sherpa software (version 2.0; Thermo Fisher Scientific) over vacuum. The EPU Multigrid queue was then started, and each grid was automatically loaded sequentially onto the stage, whereupon grid squares previously selected were automatically brought to eucentric height and holes for data collection were automatically selected with ice filter settings. Images were collected at a nominal magnification of 205,000× with a resulting pixel size of 0.57 Å. Flux measured on the detector over vacuum at spot size 3 at parallel illumination conditions was 7.94 electrons. pixel^{−1} s^{−1} and each exposure was 2.85 s long to accumulate a total dose of 70 electrons. Å^{−2} per exposure. Images were collected at defocus values −0.8 μm, −1.0 μm and −1.2 μm. AFIS together with FFI and a 20 μm C2 aperture was employed to acquire 2 exposures per 1.2 μm ice hole and to accelerate data collection to approximately 500 per hour.

Rapid-screening and intermediate-resolution data were processed in cryoSPARC live (version 3.3.1)³⁴. Movies were binned 2× during motion correction. Particles were picked using blob picker (elliptical blob, 80–110 Å diameter) and extracted in 160 × 160-pixel boxes. On-the-fly 2D classification and 3D refinement were performed using default parameters, with a previous reconstruction of CAK serving as an initial reference. Using a workstation with 4 RTX3090Ti GPUs (Nvidia Corporation), the software was able to match data collection rates.

The rapid screening workflow used cryoSPARC live processing exclusively. To improve interpretability of the maps in the intermediate resolution workflow, we added two steps after cryoSPARC live processing: (i) We performed an additional 2D classification of all live-picked particles (except obvious non-particles and artifacts) which led to recovery of more particles and clear classes for additional particle views. Subsequently (ii), we performed a 3D refinement, an alignment-free 3D classification, and a final refinement of the best class in RELION 4.0³⁷, providing the final reconstruction. The resolutions were typically almost unchanged (within 0.1 Å) compared to the cryoSPARC live output, but the RELION maps were cleaner. The cryoSPARC 2D classification took approximately 2 h per dataset, and the downstream processing in RELION was completed in approximately 1.5 h.

High-resolution data collection

Data were collected on a Krios G4 cryo-TEM (Thermo Fisher Scientific) equipped with a cold-FEG operating at 300 kV acceleration voltage, a Selectris X energy filter, and a Falcon 4i direct electron detector. Data were acquired using EPU (version 3.1) at 0.57 Å pixel size, 70 electrons. Å^{−2} total exposure at 7–8 electrons. pixel^{−1} sec^{−1} and in EER format, later fractionated into 70 frames during data processing. AFIS and FFI were employed to acquire 2–3 exposures per 1.2 μm ice hole and to accelerate data collection to approximately 600 movies/h by collecting data in holes within 12 μm of image-beam shift radius from the centered position. Approximately 5000 images were collected per grid for most grids (10,000 for THZ1 from two grids).

Pre-processing of high-resolution datasets in cryoSPARC

General pre-processing strategy. Datasets were acquired and processed in cryoSPARC live (cryoSPARC versions 3.3.1–4.1.1)³⁴ with the same settings as for the screening datasets (see above). In addition to blob picking during on-the-fly processing, two parallel template-based picking strategies were then carried out after the end of the data collection, using representative 2D classes from the corresponding live-processing session or 2D projections generated from a 3D

reconstruction as templates (Supplementary Fig. 14). Template-based picks were separately extracted from the micrographs marked as accepted during live processing, using an extraction box size of 160×160 pixels, and classified into 200 2D classes (100–140 iterations, circular mask diameter 104–110 Å, batch size per class 200). For most datasets, the best 2D classes from the live processing session (blob picking) and from the two post-acquisition template-based picking strategies were selected, combined and duplicate particles were removed. The combined particles were re-extracted to improve particle centering and any duplicate particles arising from the re-centering during re-extraction were removed again. This final particle set was exported and converted to a *.star file compatible with RELION using a conversion program available in the PYEM package⁴⁴ followed by adjustment of path names to adhere to RELION conventions. This strategy was applied to all datasets with occasional small variations, outlined in the paragraphs below.

CAK-THZ1. For each of the two CAK-THZ1 datasets, which were collected early and used for trialing of processing strategies, the particles selected from the three picking strategies (1× blob picking, 2× template picking) were separately re-extracted before combination into one dataset, duplicate particle removal, and export to RELION format (combined particles). Additionally, the particles selected from blob picking and on-the-fly 2D classification in cryoSPARC live were exported as well (blob particles). The combined particles and the blob particles were separately imported into RELION, giving a total of four imported particle sets (two from each CAK-THZ1 grid).

Apo-CAK. For the apo-CAK complex, particles were additionally classified into 50 2D classes after the first duplicates removal step to remove any classes with concentric ring patterns arising from sensor imperfections from the selected particle set.

CAK-ICEC0510-S. The final duplicate particle removal step occurred within RELION after a 3D refinement.

High-resolution data processing in RELION

General refinement strategy. All datasets were further processed using RELION 4.0 (Supplementary Fig. 14)³⁷. To ensure full RELION functionality, including Bayesian polishing³⁵, movies were motion corrected using the RELION CPU-based implementation of the motion correction algorithm⁴⁵, using a binning factor of 2.0 (giving a pixel size of 1.14 Å/pixel). Exported particles from cryoSPARC were re-extracted from the motion corrected micrographs using an extraction box size of 160×160 pixels. Extracted particles were subjected to masked 3D refinement using a previous CAK reconstruction as an initial reference¹⁵, with an initial angular sampling of 7.5 degrees. For ligand-bound datasets, the refined particles were classified into four 3D classes by alignment-free 3D classification ($\tau = 24$, mask diameter 104 Å, resolution E-step limited to 4 Å). The best class(es) were selected, refined, and subjected to Bayesian polishing³⁵, after first training the polishing on 10,000 particles, with re-windowing at a box size of 384 pixels and re-scaling to 256 pixels at 0.855 Å/pixel to account for CTF delocalization. Polished particles were refined and subjected to CTF refinement of per-particle defocus followed by refinement of beam tilt, trefoil, and fourth-order aberrations or vice versa (depending on which strategy provided the best result for each dataset)³⁶, followed by 3D refinement and a second round of polishing with re-windowing at a box size of 480 pixels and re-scaling to 384 pixels at 0.7125 Å/pixel. Re-polished particles were re-refined and the resulting map was post-processed in RELION. Deviations from this general strategy are outlined in the following paragraphs.

CAK-ATPyS. After completion of the processing pipeline outlined above, a final 3D classification step using 2 classes ($\tau = 36$) was inserted to identify the best-quality particle set used for the final refinement.

CAK-LDC4297. Due to sample heterogeneity, preferred orientation, and the presence of non-particle images, two additional 2D classification steps were inserted between the first 3D classification and the first Bayesian polishing step, selecting approximately 61,000 rare particle views to be added to the best 3D class, and eliminating non-particles from the resulting dataset. Subsequent polishing, refinements, and CTF refinements were performed according to the scheme outlined above. As a final step, a local refinement using a mask encompassing only CDK7 was performed to improve the appearance of the density of the inhibitor (Supplementary Fig. 2d, e).

CAK-BS-181, CAK-ICEC0942 (grid VC13-3), CAK-ICEC0943. The training step of Bayesian polishing failed, and particle polishing was instead run using the following ad hoc parameters, chosen based on the results of successful training runs from other samples, which used the same microscope and identical grid type: $\sigma_{\text{vel}} = 1.2$, $\sigma_{\text{div}} = 4500$, and $\sigma_{\text{acc}} = 1.5$.

Apo-CAK. Training for Bayesian polishing failed, and polishing was run with ad hoc parameters (as described above). Due to conformational heterogeneity of the β -sheet in the N-terminal kinase lobe in apo-CAK, particles were 3D classified while masking out the β -sheet after the initial motion correction, particle extraction, and initial refinement steps. This was done to focus the classification on the bulk of the particle density to select for particle quality, rather than letting the mobile β -sheet density dominate the classification. The selected particles were then refined using a full mask and polished only once (with re-windowing and re-scaling to a box size of 384 pixels at 0.855 Å/pixel) before a final refinement.

CAK-THZ1. Each of the two datasets (grids BG29-1, BG29-2) was motion corrected and each of the four exported particle sets from cryoSPARC (see above) were separately extracted from their corresponding micrographs. Extracted particles were separately refined and 3D classified, before selected particles were combined, duplicates were removed, and the resulting particle set refined before the initial polishing step. Training for Bayesian polishing failed, and polishing was run with ad hoc parameters (as described above). After the second polishing step, refined particles were additionally classified into 96 2D classes ($\tau = 2$, mask diameter 110 Å, E-step resolution limited to 6 Å) to remove non-particle classes before the final refinement.

CAK-ICEC0510-S, CAK-dinacilib. After the first 3D classification step, particles were selected from two different iterations (best class from iteration 15, two best classes from iteration 25), refined separately, joined, and subjected to duplicate removal. The resulting particle set was refined and polished (using ad hoc parameters because training failed, see above). Polishing and re-refinement steps were carried out according to the standard procedure above, but a 3D classification (3 classes) was inserted between CTF refinement and the second polishing run to improve the quality of the final particle set.

CAK-ICEC0574. CTF refinement consisted of aberrations followed by per-micrograph astigmatism.

CAK-ICEC0768. CTF refinement included only aberrations (beam tilt, trefoil, 4th order aberrations) and an additional 3D classification (3 classes, $\tau = 24$) was performed after the two polishing runs to identify the final particle set.

CAK-ICEC0880, CAK-ICEC0829. Additional 10-class 3D classifications (without resolution E-step limit) were carried out following the second round of polishing for ICEC0880 and ICEC0829. For ICEC0880, this revealed two distinct inhibitor conformations; classes corresponding to these two conformations were selected and refined. For ICEC0829, classes with the clearest inhibitor density were selected, combined, and refined.

CAK-ICEC0942 (grid VC14-1). An additional 3D classification step (4 classes, $\tau = 24$) after the first round of polishing and three rounds of CTF refinement (initially only refining beam tilt and third order aberrations, then defocus, then beam tilt and all aberrations) were performed.

CAK energy filter comparison. Only the first polishing step was performed for this dataset.

Model building and refinement

The CAK-THZ1 model was generated by rigid-body fitting of the previously published model¹⁵ (PDB ID 6XD3) into the post-processed map in UCSF ChimeraX⁴⁶. This was followed by several rounds of manual rebuilding in COOT (version 0.9.6)⁴⁷, during which water molecules were manually modeled based on inspection of the density and hydrogen bond distances, and real-space refinement in PHENIX (versions 1.20, 1.21)⁴⁸. To improve solvent modeling, we attempted to apply Segmentation-guided Water and Ion Modelling (SWIM)⁴⁹ to our model building procedure. This routine, as implemented in UCSF Chimera, added a large number of non-water atoms, such as Na⁺ ions, to our models. Considering that even very high resolution X-ray crystal structures of other CDKs (e.g. CDK2 at 1 Å resolution, PDB ID 6Q4G⁵⁰) crystallized in sodium-containing buffer do not contain modeled Na⁺ ions, we decided to adhere to a more conservative approach and modeled all water-like densities as waters, with the exception of the unambiguously identifiable hydrated Mg²⁺ ion coordinating ATPyS.

The refined CAK-THZ1 model was used as a starting point for building the remaining models, for which ligands and restraint files were generated using PHENIX eLBOW⁵¹. Ligands were manually modeled into the densities in COOT and models were adjusted and refined in the same way as for CAK-THZ1. For CAK-LDC4297, CAK-ICEC0942, and CAK-CT7030, the structure was additionally refined using the PHENIX-OPLS4 pipeline⁵² using the SCHRÖDINGER 2021-4 software package, followed by re-refinement in PHENIX while using reference restraints on the ligand, which resulted in improved overall MOLPROBITY score and model-to-map fit. The structure modeled into the 1.7 Å-resolution map of the combined dataset (Supplementary Fig. 6a, b) was refined using SERVALCAT (version 0.2.122)⁵³. For several models, the structures were refined using reference restraints on individual residues, typically in less-well resolved regions, resulting in improved geometry and model-to-map fit of these residues or nearby water molecules. These were: cyclin H E141 and CDK7 Y169 of CAK-BS-181; CDK7 Y169 of CAK-BS-194; CDK7 D92 of CAK-ICEC0574; CDK7 E62 and Y169 of CAK-ICEC0829; CDK7 E68 of CAK-ICEC0880 (ring-up conformer); cyclin H L248 and CDK7 Y169 of CAK-ICEC0942; and cyclin H M237 of CAK-CT7030. Water molecules with Q-score <0.70 were excluded from the models, except in the case of the water that is hydrogen bonded to N06 of the ligand in the CAK-LDC4297 structure, which has a Q-score of 0.66 but whose presence is clearly supported by the density and the presence of equivalent water molecules in several other structures. Refinement statistics obtained from the comprehensive validation tool in PHENIX are provided in Supplementary Tables 5–11.

Visualization of molecular models

Molecular models and cryo-EM maps were visualized in UCSF ChimeraX (versions 1.2.5)⁴⁶ and PyMOL (The PyMOL Molecular Graphics

System, versions 2.5.2–2.5.5 Schrödinger, LLC) for analysis, interpretation, and preparation of figures.

Further validation data

Local resolution maps, particle orientation distributions, and a second view of the ligands for all high-resolution CAK cryo-EM maps included in this manuscript are shown in Supplementary Figs. 15–17. Sphericity values to quantitatively assess the effects of preferred orientation on the cryo-EM reconstructions are provided in Supplementary Tables 5–11 and were computed using the 3D FSC validation server⁵⁴.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Cryo-EM maps generated from 1-hour Glacios screening in this study have been deposited to the Electron Microscopy Data Bank (EMDB) with accession codes [EMD-17470](#), [EMD-17471](#), [EMD-17472](#), [EMD-17473](#), [EMD-17474](#), [EMD-17475](#), [EMD-17476](#), [EMD-17477](#), [EMD-17478](#), [EMD-17479](#), [EMD-17480](#), [EMD-17481](#), [EMD-17482](#), [EMD-17483](#), [EMD-17484](#), [EMD-17485](#), [EMD-17486](#), [EMD-17487](#), [EMD-17488](#), [EMD-17489](#), [EMD-17490](#), [EMD-17491](#), [EMD-17492](#), [EMD-17493](#), [EMD-17494](#), and [EMD-17495](#). Cryo-EM maps resulting from 4-h Glacios screening have been deposited to the EMDB with accession codes [EMD-17496](#), [EMD-17497](#), [EMD-17498](#), [EMD-17499](#), [EMD-17500](#), [EMD-17501](#), [EMD-17502](#), [EMD-17503](#), [EMD-17504](#), [EMD-17505](#), [EMD-17506](#), and [EMD-17507](#). High-resolution cryo-EM maps generated in this study have been deposited to the EMDB using accession codes [EMD-17129](#), [EMD-17508](#), [EMD-17509](#), [EMD-17510](#), [EMD-17511](#), [EMD-17512](#), [EMD-17513](#), [EMD-17514](#), [EMD-17515](#), [EMD-17516](#), [EMD-17517](#), [EMD-17518](#), [EMD-17519](#), [EMD-17520](#), [EMD-17521](#), [EMD-17522](#), [EMD-17523](#), [EMD-17536](#), and [EMD-17754](#). Atomic coordinates of high-resolution ligand-bound complexes generated in this study have been deposited to the Protein Data Bank (PDB) using identifiers [8ORM](#), [8P6V](#), [8P6W](#), [8P6X](#), [8P6Y](#), [8P6Z](#), [8P70](#), [8P71](#), [8P72](#), [8P73](#), [8P74](#), [8P75](#), [8P76](#), [8P77](#), [8P78](#), [8P79](#), [8P7L](#), and [8PLZ](#) (as detailed in Supplementary Tables 5–11). Electron micrograph movies for selected datasets have been deposited to the Electron Microscopy Public Image Archive (EMPIAR) with accession codes [EMPIAR-11793](#), [EMPIAR-11799](#), [EMPIAR-11800](#), [EMPIAR-11807](#), [EMPIAR-11821](#), and [EMPIAR-11823](#). Atomic coordinate models used in this study are publicly available from the PDB under accession codes [1JKW](#), [3NS9](#), [4KD1](#), [5JQ5](#), [5JQ8](#), [6ATH](#), [6Q4G](#), [6XD3](#), [6Z4X](#). Source data are provided with this paper.

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Author contributions

B.J.G., A.K. and S.A. designed the cryo-EM-based project. A.G.M.B. and A.K.B. directed the inhibitor discovery program at Imperial College and Carrick Therapeutics, respectively. A.B., S.H.B.K., M.B. and B.S. synthesized CAK inhibitors and provided characterization data. K.J. characterized CAK inhibitors. B.J.G., V.I.C. and J.F. prepared the cryo-EM specimens and performed initial screening. A.F.K. and A.K. collected all cryo-EM data contained in the manuscript. V.I.C. and B.J.G. processed the cryo-EM data. V.I.C. interpreted the cryo-EM data under the guidance of B.J.G. B.J.G. and V.I.C. drafted the first version of the manuscript, and all authors contributed to its final form.

Competing interests

A.K. and A.F.K. are employees of Thermo Fisher Scientific, the manufacturer of the electron microscopes used in this study. S.A., A.G.M.B. and A.B. are named inventors on patents concerning CDK7 inhibitors, which have been licensed to Carrick Therapeutics (U.S. patents US 9932344 B2, US 8067424 B2, US11857552 B2, US8507673 B2). They have received milestone payments from the patent licensing, and own shares in Carrick Therapeutics. S.A. has received research funding and reagents from Carrick Therapeutics. S.A. has also received research funding from

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Additional information

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