Contribution of metabolic alterations to genome instability and its association with cancer development

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**CONTRIBUTION OF METABOLIC ALTERATIONS TO
GENOME INSTABILITY AND ITS ASSOCIATION WITH
CANCER DEVELOPMENT**

WOO REN HE

SCHOOL OF BIOLOGICAL SCIENCES

2021

**Contribution of metabolic alterations to genome instability and its
association with cancer development**

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SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Doctor of Philosophy

2021

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Summary

Metabolic reprogramming could promote genome instability, causing normal cells to acquire somatic mutations that favours tumourigenesis. To investigate this, ATP citrate lyase (ACLY) was overexpressed in normal epithelial cells, where it was found to cause the formation of micronuclei, nuclear budding and nucleoplasmic bridges in interphase cells, and chromatin bridges in mitotic cells. These nuclear abnormalities were found to be caused by elevated levels of DNA double stranded breaks (DSBs). It was discovered that phosphorylated ATR and Chk1 were deregulated through the proteosomal-dependent degradation pathway. This could have caused cells to become susceptible to DSBs via replication stress. Despite the increased presence of DSBs, no significant responses of the ATM pathway were detected. Furthermore, p53 phosphorylation was impaired in the presence of oxidative stress. These events could have enabled cells to sustain growth without undergoing apoptosis, causing DSBs to be left unrepaired or misrepaired and consequently giving rise to the formation of nuclear abnormalities. Hence, metabolic alterations induced by ACLY contributes to genome instability, which could have enabled cells to acquire an enhanced ability to grow under anchorage-independent conditions. Further works are required to determine the mechanisms of which ACLY exerts to disrupt ATR and ATM pathway, and p53 responses to DNA damage.

GLOSSARY

53BP1	p53 binding protein 1
Acetyl-CoA	Acetyl coenzyme A
ACLY	ATP citrate lyase
ACS	Acetyl-CoA synthetase
AMPK	Adenosine monophosphate-activated protein kinase
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia-telangiectasia and Rad3 related
Bax	Bcl-2-associated X protein
BRCA1	Breast cancer type 1 susceptibility protein
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CR	Caloric restriction
DDR	DNA damage response
Dox	Doxycycline
DSB	DNA double stranded breaks
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HME1	Human telomerase-immortalised normal breast epithelial cells
HME1-ACLY	HME1 with Tet-On inducible system for ACLY overexpression
HME1-Empty	HME1 with Tet-On inducible system without transgene
HR	Homologous recombination
KAT	Lysine acetyltransferase
MN	Micronuclei
NAD ⁺	Nicotinamide adenine dinucleotide
Nbs1	Nibrin
NBUD	Nuclear budding
NHEJ	Non-homologous end-joining
NPB	Nucleoplasmic bridges
PI3K-Akt	Phosphoinositide-3 kinase Akt
PUMA	p53 upregulated modulator of apoptosis
rDNA	ribosomal DNA
ROS	Reactive oxygen species
RPA32	Replication protein A 32kDa
RTK	Receptor tyrosine kinases
SSB	DNA single stranded breaks
tBHP	tert-butyl hydroperoxide
XPA	Xeroderma pigmentosum complementation group A
XPC	Xeroderma pigmentosum complementation group C

1. INTRODUCTION

1.1. Global disease burden of cancers and the need for continuous research efforts

Cancer is a disease where an individual's cells undergo a transformation away from what they ought to function in an organ. These cells are unable to maintain orderly tissue structures, and grow uncontrollably to form tumours, evading intrinsic cellular defence mechanisms that prevent them from doing so. These cells would continue to gradually transform themselves into a more malignant type by acquiring the ability to invade into the bloodstream, evade immune surveillance, and subsequently propagating themselves in other organs. The physiological consequence would usually be the experience of a gradual loss of vital bodily functions, resulting in poor quality of life.

Cancer is a complex disease that continues to affect many lives both young and old. In a recent annual health study, 24.5 million cancer incidences were reported worldwide in 2017 at a cost of 233.5 million disability-adjusted life years (DALYs), causing deaths of up to 9.6 million people (Fitzmaurice *et al.*, 2020). Among cancer incidences in men in 2017, 54% of the cases were attributed to skin, colorectal, tracheal, bronchus and lung (TBL) cancer. Cancers that caused more mortalities and DALYs in men were stomach, liver and TBL cancer. In women, common cancers reported in 2017 were colorectal, breast, and non-melanoma skin (NMSC) cancer. Cancers that inflicted more deaths and DALYs among affected women were colorectal, breast and TBL cancer.

The number of cancer incidences over a ten-year time period between 2007 and 2017 have increased by 33% (Fitzmaurice *et al.*, 2020). In 2017, cancers became

the top two leading causes of DALYs worldwide along with cardiovascular diseases. Study estimates revealed that 1 in every 3 men and 1 in every 4 women ages 0-79 are likely to develop cancers within their lifetime. These figures may continue to follow an upward trajectory in the future, affecting more lives globally. This presents a growing threat to healthcare systems, where resources are presently stretched to prepare and deal with present viral outbreaks and the inevitability of future ones on a global scale. Hence, research to find suitable and effective means to treat cancers are still relevant and necessary to reduce its global health burden.

1.2. Present knowledge on cancer development and progression

1.2.1. Conceptualisation of oncogenes and tumour suppressor genes

Discoveries made by Peyton Rous in the early 20th century pioneered research efforts that shaped our fundamental understanding about cancers. Curious to understand how cancers were formed, he transplanted tumour bits taken from an affected domesticated chicken into healthy ones of the same flock. He observed that these tumours were transplantable even after performing several serial transplantations (Rous, 1910). He subsequently collected tumour extracts and filtered them extensively to become cell-free before injecting them into closely related chickens. These cell-free tumour transplants were still able cause sarcoma with widespread metastases (Rous, 1911). Rous concluded that this phenomenon was caused by a transmissible virus, now known as the Rous sarcoma virus (RSV). In the later half of the 20th century, studies on the RNA genome uncovered the role of the v-Src gene (viral-Src) in causing sarcomas (Duesberg and Vogt, 1970; Wang *et al.*, 1976). Two notable scientists, Mike Bishop and

Harold Varmus, noticed the dispensability of the v-Src gene for the replication of RSV, and became intrigued on its origin. Investigations by these two scientists traced the v-*Src* gene to a variety of avian species, confirming that the gene was captured from a viral infected avian host (Stahelin *et al.*, 1976). This finding proved fundamentally that the cell, not the virus, has specific intrinsic genes associated with the ability to form cancers. Later works subsequently uncovered a human homologue of the v-*Src* gene, named c-*Src*, that encodes for Src proteins known to be overexpressed and active in diverse human cancers (Irby and Yeatman, 2000). Taken together, Rous' research and subsequent works by scientists including Bishop and Varmus helped to conceptualise that cancers are formed by the aberrant activation of specific genes in the cell known as proto-oncogenes.

Another early research that helped establish the principle of cancer development was by geneticist Alfred Knudson. To understand the genetic basis of retinoblastoma, a rare retinal tumour, Knudson studied a group of 48 patients who had developed tumours in one eye (unilateral) and both eyes (bilateral) (Knudson, 1971). He noticed that patients with family history of retinoblastoma developed bilateral tumours at a younger age. This was distinct from patients with no family history who developed unilateral retinoblastoma at a later age. These observations followed the trend of Knudson-proposed "two-hit" mathematical model. He postulated that cancers are caused by mutations on both alleles of a gene critical in preventing the formation of tumours. Patients carrying a mutated allele of a gene, otherwise known as the "first hit", are mathematically more probable and thereby more pre-disposed to acquire the "second hit",

resulting in the early onset of cancer. On the other hand, patients with two normal alleles would mathematically require more time to accumulate two consecutive mutations on both alleles. Research by Knudson led to the conceptualisation of tumour suppressor genes, where they serve as the cell's intrinsic defence system to suppress mechanisms that promote tumour formation. Research later identified that mutations on the retinoblastoma tumour suppressor gene (*Rb1*) disrupts the ability of the corresponding pRb protein in repressing E2F transcription factors, which are responsible for initiating DNA replication (Goodrich, 2006; Nevins, 2001).

Therefore, the initiation and progression of cancers as we understand today are caused by (1) the activation of proto-oncogenes and (2) the loss-of-function of tumour suppressor genes. Activation of proto-oncogenes transforms them to serve as oncogenes, where they can either overexpress proteins, or express proteins that remain constitutively active. On the other hand, the function of tumour suppressor genes can be lost through either reduced protein expression, or the expression of dysfunctional proteins. As a consequence, normal intracellular signalling pathways of the cell become perturbed, pushing its transformation to form neoplasms.

1.2.2. Role of somatic mutations in cancer development and progression

Rous achieved other notable discoveries when he used chemical carcinogens to transform benign viral papilloma tumours in rabbits into aggressive malignant tumours (Rous and Beard 1935; Rous and Kidd, 1940; Rous *et al.*, 1952). In other research, Ames developed a procedure to determine the mutagenicity of chemical

compounds, which is based on the principle that mutagens inflict DNA damage resulting in sequence alterations in genes (Ames *et al.*, 1973). Known as the Ames test, it utilises strains of *Salmonella* bacterium that are harbouring mutations in several genes necessary for histidine synthesis. The mutagenicity of the compound is measured by its ability to reverse those mutations thereby reverting the bacteria's wild-type prototrophic state (Ames *et al.*, 1973). These studies shaped our understanding that somatic mutations play a role in activating oncogenes and down-regulating tumour suppressor functions. Presently, we know somatic mutations include point mutations, chromosomal mutations and gene copy number variations (Clancy, 2008).

Point mutations involve either a nucleotide base substitution, deletion or insertion. Nucleotide base substitution is a single nucleotide change that results in an altered codon that codes for the same amino acid (silent mutation), codes for a different amino acid (missense mutation), or prematurely stops protein synthesis (nonsense mutation). Missense mutations can potentially alter the native conformational shape of the protein especially at its active site/s. Consequentially, the protein can become constitutively activated or become dysfunctional. Missense mutations have been seen on Ras oncogenes in many cancers, where they were discovered to disable the ability of Ras proteins to hydrolyse GTP (Hobbs and Der, 2019). This results them to be remain permanently locked in an active GTP-bound state, amplifying intracellular signalling pathways that promote aberrant cell proliferation (Hobbs and Der, 2019). Nonsense mutations lead to the synthesis of truncated proteins that are non-functional. Such mutations are common in BRCA1 and BRCA2 genes in

most breast cancers as reported (Hogervorst *et al.*, 1995; Spain *et al.*, 1999). Next, nucleotide deletions and insertions results in frameshift mutations, causing the nucleotide sequence of the gene to be read in the wrong frame during gene transcription and protein translation. Consequentially, protein products could either be larger or smaller than the normal protein, with its amino acid sequence altered starting from the point of nucleotide insertion or deletion.

As for chromosome mutations, they can come in various forms, where a chromosome region can be inverted or deleted, translocated from the original position to another, or simply duplicated (Clancy, 2008). Chromosomal translocations have been found to cause cancers in two ways. Firstly, the coding region of the proto-oncogene can be juxtaposed near an active promoter or enhancer site of another gene, enabling its overexpression as an oncogene. This is the case for non-Hodgkin's lymphoma, where the anti-apoptotic BCL-2 gene on chromosome 14 was found to be translocated near an enhancer on chromosome 18, enabling its overexpression to promote survival (Tsujimoto *et al.*, 1985). Similarly, Burkitt's lymphoma is caused by the translocation of c-Myc on chromosome 8 near an enhancer on chromosome 14, thereby allowing its overexpression to promote cell proliferation (Nambiar *et al.*, 2008). Next, another way chromosomal translocations can cause cancers is the formation of chimeric proteins with enhanced activity. Chronic myeloid leukemia (CML) is one such example, where translocations between chromosome 9 and 22 results in a fusion between the *BCR* and *ABL* gene. This *BCR-ABL* gene encodes for a large chimeric oncoprotein that has highly active tyrosine kinase activity to promote aberrant proliferation. Lastly, duplication of chromosome regions can potentially

generate extra copies of proto-oncogenes, resulting in increase dosage of the protein product that could drive tumourigenesis (Clancy, 2008).

Gene amplification can occur in cancers, where multiple copies of proto-oncogenes are generated to encourage overexpression of oncoproteins. The *MCYN* gene (N-Myc) has been observed to be amplified in some cases of neuroblastomas that corresponds with increased cancer malignancy. Another example is the *EGFR* gene (Epidermal growth factor receptor), where its amplification has been reported in some cases of gliomas. Also, amplification of the *ERBB2* gene is present in gastric, ovarian, breast cancers (Albertson, 2006).

The role of somatic mutations in causing cancers could lead one to postulate that the lack of response to DNA damage, and increased tolerance to faulty DNA replication processes could be causative agents.

1.2.3. Role of aberrant epigenetic modifications in cancer development and progression

Besides somatic mutations, aberrant epigenetic modifications have been found in cancers. These epigenetic modifications involve chemical modifications on nucleotide bases and histone proteins to activate or repress expression of oncogenes and tumour suppressor genes without altering DNA sequences. Firstly, DNA methylation occurs on cytosine residues in CpG islands to become 5-methylcytosine, and this chemical modification is mediated by DNA methyltransferase enzymes (Philips, 2008). Although it has been well established that DNA methylation represses gene expression, recent research has found the ability of methylated cytosine bases in interacting with transcriptional activators

to promote gene activation (Greenberg and Bourc'his, 2019). Next, histones can be methylated specifically at lysine and arginine residues at the N-terminal tail, and is mediated by a group of proteins known as histone methyltransferases (HMTs). Histone methylations on lysine residues has been characterised to either repress or activate gene expression, depending on the lysine position of which methylation has occurred (Jambhekar *et al.*, 2019). Histones can also be acetylated but only on lysine residues located at the N-terminal tail, and is facilitated by group of enzymes known as histone acetyltransferases (HATs). This type of modification neutralises the inherent positive charge on the lysine residue, which causes histones to lose their binding affinity with the negatively charged DNA, forming a relaxed chromatin structure that favours the recruitment of transcriptional factors and RNA polymerases to initiate transcription (Eberharter & Becker, 2002). Hence, histone acetylation has always been seen as an active mark for the initiation of gene expression (Eberharter & Becker, 2002).

Global DNA demethylation, also termed “hypomethylation”, have been found to cause chromosomal instability in mice tumours and human primary colorectal cancers (Howard *et al.*, 2008; Rodriguez *et al.*, 2006). One study determined that the activation of transposons, as a consequence of DNA hypomethylation, resulted in undesirable chromosomal translocations to favour tumour formation (Howard *et al.*, 2008). Not surprisingly, DNA hypomethylation has also been found to activate the transcription of oncogenes in gastric, melanoma, and colon cancers (Wilson *et al.*, 2007). Moreover, the loss of genomic imprinting due to aberrant DNA hypomethylation was found to be linked with an increased risk of colorectal cancer (Cui *et al.*, 2003). Interestingly, hypermethylation at specific

sites of the genome were found in several cancers, and has been associated with the silencing of tumour suppressor genes, leading to tumour initiation (Akiyama *et al.*, 2003; Baylin, 2005; Long *et al.*, 2007).

Cancers have also been revealed to display genome-wide changes in histone acetylation and methylation patterns. Global reduction of histone acetylation at lysine 16 of histone H4 (H4K16) was found in leukaemia (Fraga *et al.*, 2005), while global increase in histone acetylation at lysine 4 of histone H3 (H3K4) was seen in several breast cancers (Hałasa *et al.*, 2019). In addition, the overexpression of class I histone deacetylases (HDAC) has been observed in prostate and gastric cancers (Halkidou *et al.*, 2004; Song *et al.*, 2005). HDACs are a group of proteins that function to remove acetyl groups from acetylated histones to facilitate the formation of tight chromatin structures associated with gene repression. Hence, aberrant site-specific histone acetylations and deacetylations could activate oncogenes and silence tumour suppressor genes respectively to promote tumour initiation. On the other hand, changes in methylation patterns in cancers can have both repressive and activating effects on gene expression that altogether work in tandem to promote tumourigenesis (Sharma *et al.*, 2010). HMTs associated with the methylation of repressive marks, namely H3K9 and H3K27, are known to be overexpressed in breast, prostate, and liver cancers (Kondo *et al.*, 2007; Kondo *et al.*, 2008; Valk-Lingbeek *et al.*, 2004). In other studies, overexpression of lysine-specific histone demethylase 1 (LSD1) and the decrease in methylated H3K9 repressive mark has been linked to prostate cancer (Shi, 2007; Metzger *et al.*, 2005). Interestingly, a study found that carcinogenic treatment on normal human mammary epithelial

cells stimulated the upregulation of LDC1 and subsequent decrease in methylated H3K4 active mark, which could provide new insights on the early development of breast cancers (Bradley, 2007; Shi, 2007).

1.2.4. The acquisition of cancer hallmarks

Taken altogether, the (1) loss of genome integrity through somatic mutations, and (2) aberrant epigenetic modifications are drivers of tumourigenesis and cancer progression. Genes implicated by these two causative factors can be classified into two categories, namely oncogenes and tumour suppressor genes.

DNA mutations and epigenetic modifications can result in the gain-of-function of oncogenes, and the loss-of-function of tumour suppressor genes. These two outcomes enable cells to acquire and exhibit various distinct behaviours known as cancer hallmarks that enable them to thrive and exert detrimental effects on human health. The various cancer hallmarks have been well described by Hanahan and Weinberg (Hanahan and Weinberg, 2011) (Fig. 1).

Figure 1. The ten hallmarks of cancer. Cellular transformation can occur in diverse ways as they acquire distinct capabilities to form neoplasms. These neoplasms can progress further toward malignancy through the gradual acquisition of more hallmarks.

1.3. Importance of discovering novel approaches to study the complexities of cancer development and progression

1.3.1. Challenges in present cancer treatments

Our present understanding about cancers is a culmination of years of extensive research on many diverse disease models ranging from humans to avian birds. Studies on these disease models have enabled scientists to identify genes implicated in various cancer hallmarks (Fig. 1), which are important in (1) the determination of key therapeutic targets for drug development and (2) the development of tools and processes for accurate cancer diagnosis. However, though we have an in-depth knowledge about cancers and the technological

advancements to combat them, cancer treatments have been plagued with many obstacles.

The prescribed effective dose of a chemotherapeutic drug used for treatment has been known to non-specifically target healthy proliferating cells for destruction, causing patients to experience toxic side effects (Bayat Mokhtari *et al.*, 2017). Furthermore, cancer cells are able to utilise alternative salvage pathways for survival in response to conventionally used monotherapy techniques that usually target a specific pathway (Bayat Mokhtari *et al.*, 2017). Another key complication is that tumours between patients can be different from one another despite sharing the same tissue of origin, which research has coined this phenomenon as between-tumour heterogeneity (Palmer and Sorger, 2017; Dagogo-Jack and Shaw, 2017) (Fig. 2). As a result, the efficacy of a particular therapy can vary between patients (Palmer and Sorger, 2017). Clinicians have been circumventing these complex problems by pursuing personalised combinatorial drug therapy (Bayat Mokhtari *et al.*, 2017; Palmer and Sorger, 2017). This involves extensive molecular biomarker testing to identify key therapeutic targets, which helps to direct a customised combination of immunotherapy and chemotherapy (Bayat Mokhtari *et al.*, 2017; Jhan and Andrechek, 2017). As such, multiple pathways could be targeted simultaneously using different drugs at lower therapeutic doses, which are sufficient to work in synergy to exert the desired cytotoxic effects on cancer cells (Bayat Mokhtari *et al.*, 2017). This approach would attenuate the toxic side effects of treatment, and reduce the likelihood of drug resistance seen in cancer relapses that would otherwise narrow treatment options dramatically. Further developments are

required in defining molecular signatures in different types of cancer and to determine corresponding treatment regimes that produces the best therapeutic response.

Figure 2. Patient-to-patient variability to cancer treatment. For example, within a population of patients who are having the same cancer type through the exhibition of similar cancer biomarkers, a subgroup would respond well to both Drug A and B, some would respond to either Drug A or B only, and some would not respond to both (non-responders) (Palmer and Sorger, 2017).

Another obstacle that has gained recognition is within-tumour heterogeneity, where a population of cells within a tumour of a patient can display genetic and epigenetic divergence (Greaves and Maley, 2012; Palmer and Sorger, 2017). This makes cancer diagnosis challenging as biopsies may not provide comprehensive information on the biomarkers enriched in the tumour as a whole, which are vital for clinical decisions on personalised treatment (Almendro *et al.*, 2013; Bedard *et al.*, 2013). As a result, the effectiveness of a therapeutic regime employed can be affected. Furthermore, minor subpopulations of cells in the tumour that are

able to survive the initiate wave of treatment could re-emerge and evolve further with increased malignancy and cause relapses (Almendo *et al.*, 2013). Secondary tumours implicated in these relapses have been shown to display genomic divergence from the initial cancer diagnosis (Almendo *et al.*, 2013), and may show changes in epigenetic patterns. A study showed that patients encountering relapses often display cancer phenotypes that are more aggressive and usually exhibit multi-drug resistance (Mitra *et al.*, 2015). Such cancers are challenging to treat as therapeutic options available to clinicians are typically reduced severely.

Hence, the complex nature of cancers has brought challenges to determine effective therapies, since its formation and progression does not follow a fixed predictable course. Therefore, new approaches have to be adopted to accurately understand how neoplasms are formed and how it evolves to become malignant.

1.3.2. Complex nature of cancers: Cancer evolution

The complex nature of cancers can be explained by the classical model of clonal evolution first theorised by Nowell in 1976 (Greaves and Maley, 2012; Nowell, 1976). The model describes that the formation of cancers from a single cell of origin is driven by the progressive acquisition of somatic mutations, producing sub-clonal populations of cells of which some display dominance over others and are selected to clonally expand. Further acquisition of somatic mutations in these dominant sub-populations continue to occur, producing more sub-clonal population of cells with some displaying increased malignancy. Selection pressure placed on the tumour microenvironment, by means of medical

interventions, can enrich the selection of sub-clonal populations that exhibit increased malignancy and drug-resistance. Besides somatic mutations, cancers can arise through the sequential acquisition of epigenetic alterations (Greaves and Maley, 2012). Interestingly, this model fits the Darwinian model of natural selection.

Figure 3. Model of Clonal Evolution to explain the complex nature of cancers. Cancers evolve through the gradual acquisition of somatic mutations or epigenetic changes over time, leading to the emergence of sub-clonal populations with different genotypes and phenotypes. Some of these sub-clonal populations display dominance over others in response to limited space and nutrient availability and are allowed to clonally expand. These dominant sub-clonal populations would continue to acquire further somatic mutations and epigenetic changes to give rise to more sub-clonal populations with some displaying increased malignancy and forming metastases. These cells would clonally expand while acquiring more mutations and epigenetic modifications. Due to the heterogeneity of tumour microenvironment in these metastases, some sub-clonal populations are able to survive initial therapeutic treatment, and are responsible for cancer relapses commonly experienced (Greaves and Maley, 2012).

Another model that has gained support by several research studies is the cancer stem cell (CSC) hypothesis (Greaves and Maley, 2012; O’Flaherty *et al.*, 2012, Tan *et al.*, 2006). In this model, tumour initiation is caused by subset of cancer cells that have stem-cell like characteristics that include unlimited self-renewal, low replication rates, and the ability to generate differentiated progenies (O’Flaherty *et al.*, 2012; Tan *et al.*, 2006). Slow replication of CSCs causes them to be unresponsive to chemotherapy and radiotherapy, enabling them to give rise to cancer-like progenitors and consequentially new tumours seen in relapses. CSCs have been postulated by studies to originate from somatic stem cells, since both share almost the same tissue identity (Tan *et al.*, 2006). In addition, it is also possible for progenitors of these somatic stem cells to acquire self-renewal capabilities, thereby enabling them to give rise to aberrant progenies (Tan *et al.*, 2006).

The ability of cancers to evolve over time poses limitations on disease-model based approaches to study cancers and uncover therapeutic targets, as findings may not fully represent how neoplasms are formed from normal cells. Hence, novel approaches are needed to be adopted to better understand how genetic and possibly epigenetic divergences occur within tumours and between tumours of patients, which altogether gives a holistic picture of how cancers evolve and how to effectively prevent it.

1.4. Altering cellular metabolism as a novel approach to study tumourigenesis and cancer progression

1.4.1. Positive influence of caloric restriction on health

Studies have shown how diet could impact overall health in various animal models. Caloric restriction (CR), a diet that involves a reduction in food intake while retaining essential nutrients, was reported to increase average lifespan in yeast, worms, and flies (Fontana *et al.*, 2010). In various mammalian models, CR has been found to prevent metabolic diseases and delay the onset of age-associated diseases, thereby promoting healthy lifespan (Fontana *et al.*, 2010). In mice models, CR was found to help with obesity by encouraging the browning of adipose tissues to form beige adipocytes, which then leads to increased energy expenditure and consequently fat loss (Fabbiano *et al.*, 2016). Other works on mice and rodent models revealed the role of CR in improving cardiovascular health, namely by (1) inhibiting pathological remodelling of the cardiac tissue associated with left ventricular hypertrophy, (2) enabling speedy restoration of heart functions upon ischemic injury, and (3) inhibiting systematic inflammation to reduce likelihood of developing age-related cardiomyopathy (Dolinsky and Dyck, 2011). The health benefits of CR were also evident in monkeys, where it was found to reduce the incidence of age-associated mortalities (Colman *et al.*, 2014).

Whether CR extends human lifespan remains to be determined, however, studies have found its role in reducing the risk of developing age-associated diseases, which include diabetes, cardiovascular diseases and cancer (Fontana *et al.*, 2010). More recently, a human study has determined how a 30-day intermittent fasting was sufficient to induce an upregulation of proteins known to confer protection against metabolic diseases, cancer, Alzheimer's disease, and neuropsychiatric disorders (Mindikoglu *et al.*, 2020).

1.4.2. Molecular mechanisms caloric restriction exerts on the cell

Studies have been done to elucidate the molecular mechanisms CR exerts on the cell that confers its beneficial effects as aforementioned. CR reduces calorie intake, causing the cells respond by increasing cellular AMP-to-ATP ratio (Canto and Auwerx, 2011). This increased imbalance of metabolites is sensed by AMP-activated protein kinase (AMPK), causing it to trigger intracellular pathways to recover cellular ATP levels and also increase cytoplasmic NAD^+ levels (Canto and Auwerx, 2011).

Elevated levels of cytoplasmic NAD^+ has been found to found to modulate a group of enzymes known as sirtuins (Schemies *et al.*, 2010). Sirtuins are class III HDACs, that requires NAD^+ as a metabolic co-substrate for its catalytic activity to remove acetyl groups from histones (Schemies *et al.*, 2010), promoting gene silencing. Increased activity of specific sirtuins has been associated with the extension of healthy lifespan in both transgenic invertebrate and mammalian models (Watroba & Szukiewicz, 2016). This conserved effect of sirtuin activity in a wide diversity of organisms can provide insights on the aforementioned health benefits of CR (Watroba & Szukiewicz, 2016). Interestingly, an age-dependent decline of intracellular of NAD^+ levels has been observed in various mammalian models, which can be correlated with the onset of age-associated diseases (Imai and Guarente, 2014).

Studies have elucidated the role of sirtuins in maintaining genome integrity. In mice models, the knockdown of five sirtuins, specifically SIRT1-4 and SIRT6,

were discovered to cause defects in DNA repair, increase the presence of DNA damage, and inflict chromosomal mutations (Bosch-Presegué and Vaquero, 2014). Not surprisingly, knockdown studies on yeast Sir2, a mammalian ortholog of SIRT6, was found to cause genome instability and increase the cell's susceptibility to DNA damage (Imai and Guarente, 2014; Tasselli *et al.*, 2017). Studies on SIRT1 revealed its ability to suppress the transcription of ribosomal DNA (rDNA) genes, reducing the risk of unwanted homologous recombination events between the rDNA loci and other repeat elements found in the genome such as retroelements and transposons, which can ultimately lead to chromosomal translocations (Murayama *et al.*, 2008). Sirtuins are also known to maintain telomeres. This is the case of SIRT1, where it was found to promote telomerase activity, reducing the effects of aging associated with telomere shortening (Bosch-Presegué and Vaquero, 2014). SIRT6 has also been discovered to maintain telomere function, preventing any undesirable telomere end-fusions that would give rise to dicentric chromosomes (Bosch-Presegué and Vaquero, 2014). In the presence DNA damage, SIRT1 is able to (1) deacetylate XPA and facilitate the expression of XPC to promote nuclear excision repair (NER) at single stranded breaks, and (2) signal the formation of Nbs1, γ H2AX, Rad51 and BRCA1 foci at double stranded breaks (Wang *et al.*, 2008; Fan and Luo, 2010; Ming *et al.*, 2010). SIRT6 also participates in DNA damage responses by orchestrating multiple pathways to respond to DNA damage, which include (1) chromatin remodelling through histone H3K56 deacetylation and (2) facilitating the recruitment of 53BP1 and BRCA1 repair proteins at damaged foci (Tasselli *et al.*, 2017). Next, sirtuins can be active at specific cell cycle checkpoints. During mitosis, SIRT1 ensures the stability of chromosomes as they

condense by facilitating the incorporation of condensin I and histone H1 into chromatin. At the G2/M checkpoint, SIRT2 deacetylates H4K16 to initiate H4K20 monomethylation (Serrano *et al.*, 2013), where the latter has been found to be a key epigenetic marker for DNA repair (Jørgensen *et al.*, 2013). Altogether, sirtuins promote genome stability by maintaining the integrity of rDNA and telomeres, mediating DNA damage responses, and ensuring chromosome stability at specific cell cycle checkpoints. With this, genome instability as a result of sirtuin inactivity may contribute to age-associated diseases such as cancer, diabetes, and cardiovascular diseases.

1.4.3. Over-nutrition could have implications on health

Having known the beneficial effects of CR, it can be postulated that over-nutrition could detrimentally affect human health. High calorie intake is known to increase the activity of metabolic pathways associated with aerobic respiration, resulting in the elevation of nuclear-cytoplasmic Acetyl-CoA levels (Cai *et al.*, 2011; Lee *et al.*, 2014). The nuclear-cytoplasmic Acetyl-CoA pool is contributed by two metabolic enzymes, namely ATP-citrate lyase (ACLY) and Acetyl-CoA synthetase (ACS). Acetyl-CoA that is not utilised in the mitochondrial compartment are transported out into the cytosol by its conversion into citrate, where it can be catalysed by ACLY to release oxaloacetate and Acetyl-CoA. On the other hand, ACS contributes to the Acetyl-CoA pool by metabolising acetate. Increased levels of nuclear-cytoplasmic Acetyl-CoA is known to modulate lysine acetyltransferases (KATs) (Galdieri and Vancura, 2012; Lee *et al.*, 2013b).

A sub-group of KATs that respond to the elevation of Acetyl-CoA are HATs that promote histone acetylation (Galdieri and Vancura, 2012). Low histone H3 acetylation has been found to prolong lifespan in yeast and flies when ACS activity was reduced (Eisenberg *et al.*, 2016). This observed effect was attributed to the transcription of genes associated with autophagy (Eisenberg *et al.*, 2016). Consistently, the down-regulation of ACLY activity in midlife flies, where they typically express increased levels of ACLY, was able to extend their lifespan (Peleg *et al.*, 2016). This was due to the reduced levels of Acetyl-CoA and the consequential decrease in acetylation at specific epigenetic marks on histones H3 and H4 (Peleg *et al.*, 2016). In mice models, histone acetylations specifically at H4K12 have been associated with cancer and neurodegeneration (Peleg *et al.*, 2016). Furthermore, when a Kras mutant linked to human pancreatic adenocarcinoma (PDA) was overexpressed in mice, histone H3 and H4 acetylations were observed. This finding implicates the role of these epigenetic modifications in the initiation of pancreatic cancers (Lee *et al.*, 2014). Also in monkey cells, PCAF (CREB-binding protein associated factor) acetyltransferase was found to induce histone acetylations at promoters of NF- κ B target genes to promote obesity-associated inflammations (Sheppard *et al.*, 1999). Taken together, these invertebrate and mammalian models seem to suggest a correlation between elevated histone acetylations and reduced longevity or poor health.

In humans, elevated histone acetylations have been identified in cancers. Several aggressive breast cancer cell lines such as MCF-7 and MDA-MB-231 were found to harbour increased genome-wide histone acetylations at H3K4 (Hałasa *et al.*, 2019). In another study, global histone hyperacetylation patterns were detected

in oral cancers (Arif *et al.*, 2010). However, reduction in global histone acetylations have also been identified in human cancers. For example, H4K16 histone acetylations are decreased in leukemia (Fraga *et al.*, 2005). In breast cancers, low levels of H4K16, H3K9, H3K14, and H4K12 acetylations have been found (Elsheikh *et al.*, 2009). In non-small cell lung carcinoma (NSCLC), low of levels H3K9 acetylation is associated with poor prognosis, while low levels of H4K12 and H4K16 hypoacetylation are associated with the disease progression (Kaypee *et al.*, 2016). As previously mentioned, increased deacetylation activity of class I HDACs, namely HDAC1 and HDAC2, have been discovered to correspond with increased malignancy in both prostate and gastric cancers (Halkidou *et al.*, 2004; Song *et al.*, 2005). Similar histone hypoacetylations have been observed in malignant colorectal cancers at H4K12 and H3K18, which were found to be caused by HDAC2 overexpression (Ashktorab *et al.*, 2009). Although histone hypoacetylations have been seen in various forms of cancers described here, they are more closely linked to aggressive phenotypes than to tumour initiation. Moreover, these hypoacetylations are likely caused by class I HDACs, which unlike class III HDACs, do not require NAD^+ as a co-substrate for their activity and thereby do not respond to any changes in nutrient availability. This means that the increased activity of class I HDACs are not caused by low nutrient intake, but through other underlying mechanisms. Hence, the possible contribution of excess nutrition to tumour initiation by means of genome-wide histone acetylations should not be disregarded.

Besides HATs, the elevation of nuclear-cytoplasmic Acetyl-CoA can modulate other lysine acetyltransferases (KATs), of which several of them have been

identified to be associated with metabolic diseases. Protein acetylations on various subunits of NF- κ B by p300/CBP were observed to impair its interaction with inhibitor I κ B, enabling it to interact with DNA freely to promote expression of genes associated with obesity-linked inflammations (Lee *et al.*, 2013b). These obesity-linked inflammations have been characterised to have links with insulin resistance and carcinogenesis (Lee *et al.*, 2013b). In another study, PGC-1 α , a known regulator of fatty acid oxidation, was found to be negatively regulated by GCN5-mediated acetylation (Lee *et al.*, 2013b). The consequential reduction in PGC-1 α activity has been linked to lipid accumulation in obesity, and even type II diabetes (Cheng *et al.*, 2018; Łukaszuk *et al.*, 2015). Interestingly, CR has been found to trigger PGC-1 α expression (Larrouy *et al.*, 1999).

Although sirtuin activity has been described to maintain genome integrity through facilitating DNA repair, few research has determined if the increase activity of KATs could destabilise the genome. In one study, ACLY overexpression was found to modulate histone acetylations to promote recruitment of BRCA1 and impair recruitment of 53BP1 at double stranded DNA breaks, thereby encouraging DNA repair via error-free homologous recombination pathway (Sivanand *et al.*, 2017). Although the study has implied the correlation between elevated histone acetylations and genome stability, it seems to contradict other studies that are consistent on the opposing roles Acetyl-CoA and NAD⁺ metabolism has on the cell. If an increase in sirtuin activity in response to elevated NAD⁺ levels promotes genome stability, it seems unlikely that elevated Acetyl-CoA levels could achieve the same beneficial effect. Moreover, such a notion implies that there are little consequences to over-

nutrition. Hence, further works are needed to better characterise the role Acetyl-CoA metabolism has on genome stability. The present study aims to investigate this.

1.4.4. Possible role of metabolic reprogramming in cancer development

The deregulation of cellular energetics, otherwise known as metabolic reprogramming, has been characterised as a cancer hallmark (Hanahan and Weinberg, 2011). The conceptualisation of metabolic reprogramming first began with early studies by Otto Warburg. In his research, he noticed that tumour cells exhibited an increased rate of glucose consumption, and tendency to ferment glucose into lactic acid despite the presence of oxygen (Warburg *et al.*, 1927; Warburg, 1956). Later research subsequently termed this phenomenon as the *Warburg effect*, which is caused by an enhanced rate of glycolysis, consequently favouring lactate production even in the presence of a functioning mitochondria (Liberti and Locasale, 2016). Extensive studies on the *Warburg effect* revealed how the elevation of glycolytic intermediates facilitates an increase in carbon flow toward different metabolic pathways, which promotes the syntheses of macromolecules such as DNA, polypeptides, and phospholipids necessary for cell growth (Vander Heiden *et al.*, 2009).

The phosphoinositide-3 kinase-protein kinase Akt (PI3K-Akt) pathway has been well known to be hyperactivated in a wide spectrum of spontaneous cancers (Liu *et al.*, 2009). It has been found to reprogramme cellular metabolism by modulating several kinases and catalytic proteins (described in Fig. 4), thereby contributing to the *Warburg effect* (Liu *et al.*, 2009). The initiation of this

pathway starts through the mitotic stimulation of receptor tyrosine kinases (RTK) that triggers the activation of a class of PI3K kinases. These PI3K kinases act to convert phosphatidylinositol 4,5-bisphosphate (PIP₂) to phosphatidylinositol 3,4,5-trisphosphate (PIP₃) at the membrane. The formation of PIP₃ enables the docking of protein kinase Akt and phosphoinositide-dependent kinase 1 (PDK1) at the plasma membrane. PDK1 is then able to partially activate Akt through phosphorylation at Thr308. Subsequent phosphorylation at Ser473 by the mTORC2 complex enables the full activation of Akt (Liu *et al.*, 2009) (Fig. 4). The PI3K-Akt pathway is also activated by Ras family of GTPases that are known to be hyperactive in colorectal, lung and pancreatic cancers (Castellano and Downward, 2011; Hobbs and Der, 2019). As mentioned previously, mutations on Ras results in the loss of its ability to hydrolyse GTP, causing it to remain in an active GTP-bound state. In addition, other mutations have been reported to enhance the nucleotide exchange rate of Ras as a guanine exchange factor (GEF), allowing the generation of more GTP-bound Ras (Hobbs and Der, 2019).

Besides creating the *Warburg effect* in cells, the PI3K-Akt pathway has been characterised to induce epigenetic modifications to promote tumourigenesis. In one study, Akt overexpression was discovered to elevate nuclear-cytoplasmic Acetyl-CoA levels, driving genome-wide histone acetylations to enrich transcription of genes associated with cell growth and proliferation (Lee *et al.*, 2014).

Therefore, metabolic reprogramming is conserved and necessary in a wide variety of cancers for cell growth and proliferation as shown by the presence of hyperactive Ras signalling and PI3K-Akt pathway. As such, its role in tumourigenesis can be postulated.

In summary, it is now understood that metabolic reprogramming involves the increase of carbon flow into the cell through accelerating the production of glycolytic intermediates (Fig. 5). This raises the generation of macromolecular precursors necessary for cell growth. One of the macromolecular precursors is nuclear-cytoplasmic Acetyl-CoA that is able to modulate the activity of KATs (Galdieri and Vancura, 2012; Lee *et al.*, 2013b). The consequential increased activity of KATs not only promotes histone acetylations to enrich expression of genes for cell growth and proliferation, it encourages acetylations on non-histone proteins to contribute to obesity and obesity-linked inflammations (Fig. 5) (Lee *et al.*, 2013b; Lee *et al.*, 2014). However, the contribution of KATs-mediated protein acetylations to genome instability remains unclear (Fig. 5). On the other hand, metabolic reprogramming lowers levels of NAD⁺ metabolites, resulting in decreased activity of sirtuins (class III HDACs). As a consequence, sirtuins are unlikely to exert their various protective functions to maintain genome integrity (see Section 1.4.2.), which may then result in genome instability (Fig. 5). Having known that metabolic reprogramming is conserved and necessary in most cancer cells, its role in tumourigenesis can be postulated. Hence, the present study aims to investigate if metabolic alterations contribute to genome instability through histone acetylations. The study will also address if genome instability would eventually push cells to acquire cancer hallmarks.

Figure 4. Role of PI3K/Akt pathway in altering cellular metabolism to promote cell growth. Ras signalling and mitotic stimulation of receptor tyrosine kinases (RTKs) triggers the activation of PI3K, where it subsequently activates Akt. Akt stimulates activity of hexokinase and phosphofructokinase-1 in the glycolysis pathway, thereby causing increased rate of aerobic glycolysis described by the *Warburg effect* (Deprez *et al.*, 1997; Gottlob *et al.*, 2001; Brendan and Lewis, 2007). Elevated rate of aerobic glycolysis results in increased carbon flow of glycolytic intermediates into pentose phosphate pathway and citric acid cycle, consequently raising nucleic acid and amino acid synthesis respectively (Berg *et al.*, 2002a; Berg *et al.*, 2002b). Increased carbon flow through the citric acid cycle also leads to increased production of citrate that is transported out of the mitochondria through an inner membrane-bound citrate carrier (Gnoni *et al.*, 2009). There, citrate is catalysed by activated ATP citrate lyase (ACLY) to produce cytoplasmic Acetyl-CoA, which is then utilised as a metabolic precursor for fatty acid synthesis (Bauer *et al.*, 2005; Liu *et al.*, 2009). ACLY has been known to be activated by Akt through phosphorylation at Ser455 (Bauer *et al.*, 2005). Elevation of Acetyl-CoA levels has also been found to induce genome-wide histone acetylation, enriching expression of genes associated with cell growth and proliferation (Lee *et al.*, 2014). On the other hand, Akt promotes protein synthesis by inhibiting GTPase activating protein (GAP) TSC2, allowing Rheb to retain its active GTP-bound state to stimulate mTORC1 complex (Inoki *et al.*, 2002; Rosner and Hengstschläger, 2011).

Figure 5. The role of metabolic reprogramming in tumour initiation. The *Warburg effect* enables an increase in carbon flow into the cell by accelerating the production of glycolytic intermediates. This raises the production of macromolecular precursors for the synthesis of amino acids, nucleic acids and lipids. Among the macromolecular precursors, nuclear-cytoplasmic Acetyl-CoA has been found to modulate the activity of lysine acetyltransferases (KATs). Non-histone protein acetylations mediated by KATs have been observed in metabolic diseases, including obesity-linked inflammations that are associated with diabetes and cancer development. Increased levels of histone acetylations have been seen in some cancers, where research has postulated its involvement in tumour initiation. Furthermore, genome-wide histone acetylations have been reported to enrich the expression of genes associated with cell growth and proliferation. However, little is known if elevated histone acetylations in normal cells contributes to genome instability. On the other hand, NAD^+ levels decline, impairing the function of sirtuins to perform DNA repair, which could potentially lead to genome instability and consequentially reduce longevity.

1.5. Hypothesis

1.5.1. The proposed model of tumour initiation

It can be theorised that the early events leading to the initiation of tumours from normal cells parallel the model of the clonal evolution as described (Greaves and

Maley, 2012; Nowell, 1976) (Fig. 3, 6). As normal cells undergo multiple rounds of divisions, they acquire aberrant somatic mutations and epigenetic changes. This leads to the emergence of sub-clonal cells carrying distinct genotypes and epigenetic marks. In response to limited tissue space and nutrient availability, a subset of these sub-clonal cells are able exhibit dominance and expand more efficiently than the rest. The remaining sub-clonal cells would either become extinct, or form small progeny populations and maintain dormancy.

Over time, the dominant sub-clonal cells would accumulate even more somatic mutations and epigenetic changes as they expand, creating even more sub-clonal cells with increasing genetic and epigenetic diversity. Similarly, some of these sub-clonal cells exhibit better fitness than others, favouring their clonal expansion over others. These events would continue to repeat over an extended period of time, generating a large heterogeneous pool of somatic cells, with a few acquiring the right mutation and epigenetic “hits” to display a cancer phenotype. Cells exhibiting cancer phenotypes are likely to acquire the appropriate aberrations that lead to the loss-of-function of tumour suppressor genes, and the gain-of-function of oncogenes.

This proposed theory assumes that acquisition of aberrant somatic mutations and epigenetic changes occur in a random manner, with some beneficial for dominance, some rendering the cell unfit, and some with no effect. As such, individuals who have no inheritance of any germline mutations would mathematically require more time to acquire sufficient and accurate “hits” for tumour initiation compared to those who have an inheritance. This fits Knudson’s

two-hit mathematical model, and could provide an explanation on why most spontaneous cancers are usually age-associated. In addition, the theory also assumes that these changes, especially genetic changes, are irreversible and remain permanent in subsequent progenies.

Figure 6. Proposed Model of Tumour Initiation. Tumour initiation from normal cells may follow the postulated model of clonal evolution. Acquisition of aberrant somatic mutations and epigenetic changes lead to the generation of sub-clonal cells with distinct genotype and epigenetic marks (represented by different coloured circles). Some of these sub-clonal cells are able to display dominance in their expansion in response to limited space and nutrient availability. The remaining sub-clonal cells remain dormant, or become extinct. These events continue to repeat over an extended period of time, generating more dominant and dormant sub-clonal populations. This would ultimately give rise to a large heterogeneous pool of somatic cells with increased genetic and epigenetic diversity. An extremely small number of sub-clonal cells would acquire the sufficient and appropriate aberrations to express a cancer phenotype.

1.5.2. Hypothesis on the contribution of ACLY-induced metabolic reprogramming to cancer development

The present study hypothesizes that metabolic reprogramming contributes to genome instability (Fig. 7). To investigate this, ATP citrate lyase (ACLY) was selected as the mode to influence cellular metabolism, since it contributes to the nuclear-cytoplasmic Acetyl-CoA pool and is regulated by the PI3K-Akt pathway through Ser455 phosphorylation (Fig. 4) (Bauer *et al.*, 2005). Furthermore, ACLY has been found to be overexpressed in breast, colorectal, gastric, and non-small cell lung (NSCLC) cancers, which may suggest its role in tumourigenesis (Hanai *et al.*, 2012; Migita *et al.*, 2008; Qian *et al.*, 2015; Wang *et al.*, 2017; Zhou *et al.*, 2013).

ACLY could cause genome instability through elevating levels of DNA damage and promoting chromosomal mis-segregations. These two events favour the acquisition of somatic DNA mutations, which include point mutations, chromosomal mutations and gene amplification (Aguilera and Gómez-González, 2008; Clancy, 2008; Fenech *et al.*, 2011) (See section 1.2.1.). DNA damage could be caused by the deregulation of proper DNA damage responses, resulting in unrepaired and/or misrepaired DNA. Misrepaired DNA could potentially lead to chromosomal mis-segregations during cell division. Also, metabolic reprogramming could cause an increase in the production of reactive oxygen species (ROS), which has been known to inflict DNA damage (Cooke *et al.*, 2003). On the other hand, chromosomal mis-segregations could be caused by centrosome amplifications and errors in kinetochore-microtubule attachment.

Elevated ACLY activity could promote genome-wide histone acetylations to remodel chromatin. This may silence the expression of DNA damage response proteins involved in cell cycle checkpoint activation, DNA repair, and apoptosis. (Fig. 7).

The possible accumulation of aberrant mutations and epigenetic marks could drive cells to acquire various cancer phenotypes. One known phenotype associated with increased malignancy of cancer cells is anchorage-independent growth (Mori *et al.*, 2009), which can be used to positively identify if malignant transformation has occurred.

Figure 7. The proposed model on the contribution of ACLY-induced metabolic reprogramming to tumourigenesis.

1.6. Research Aims

The first part of the investigation focussed on determining whether ACLY overexpression resulted in the increased formation of nuclear and chromosomal abnormalities in interphase and mitotic cells respectively. Upon detecting the abnormalities, works were done to establish if they were caused by chromosomal mis-segregations or the increased presence of DNA damage, or both. With regard to the detection of DNA damage, selected assays were performed to detect specifically for the presence of DNA double stranded breaks (DSBs) since they are well known to be associated with nuclear and chromosomal abnormalities (Fenech *et al.*, 2011).

Subsequently, the study attempted to determine how these DSBs could have occurred. Three possibilities were explored. First, ACLY overexpression could increase the generation of reactive oxygen species, resulting in DNA oxidative damage. Second, the deregulation of ATR- and ATM-mediated responses could decrease the robustness DNA damage responses against DNA damage caused from endogenous sources. Lastly, the deregulation of proteins involved in cell cycle arrest, DNA repair, and apoptosis could have allowed DNA damage to persist and remain unrepaired, thereby contributing to elevated levels of DSBs.

Changes in genome-wide histone acetylations was also examined to determine if epigenetic modifications had occurred and remained permanent. The results could reveal if ACLY overexpression epigenetically down-regulates the expression of DNA damage response proteins.

Lastly, it was determined if the increased presence of DSBs would favour the acquisition of cancer hallmarks. One characteristic that was examined in ACLY-overexpressing cells is the ability to proliferate under anchorage-independent conditions, which demonstrates the cell's metastatic potential.

1.7. Background literature on experimental approaches

1.7.1. Detecting nuclear and chromosomal abnormalities

Genome instability can be observable through the formation of abnormal nuclear structures in interphase cells and abnormal chromosome structures in mitotic cells. These anomalies are a hallmark of cancers (Hanahan and Weinberg, 2011), and are closely associated with the initiation and progression of tumours (Fenech *et al.*, 2011; Ganem & Pellman, 2012).

Nuclear abnormalities include micronuclei (MN), nuclear bud (NBUD), and nucleoplasmic bridge (NPB) (Fenech *et al.*, 2011). Firstly, MN are extra-nuclear bodies that appear separately from the primary nucleus. They are formed when acentric chromosome fragments or whole chromosomes fail to be incorporated into the daughter nuclei during cell division (Fenech *et al.*, 2011). Next, NBUD are abnormal budding structures that appear at the nuclear periphery of an interphase cell. Lastly, NPB can be characterised by a chromatin strand that links two separated daughter nuclei. This structure is formed when a dicentric chromosome is pulled toward both polar ends of the cell during mitosis (Fenech *et al.*, 2011). Dicentric chromosomes are formed due to DNA misrepair by error-prone repair pathways (Fenech *et al.*, 2011).

Abnormal chromosome structures that appear in mitotic cells include small extrachromosomes, chromatin bridges, and lagging chromosomes. The presence of these abnormal structures implies that genomic aberrations could potentially be passed down from one generation of cells to another. Small extrachromosomes are small fragments of DNA consisting of amplified DNA, and are known to be responsible for gene amplification in tumours (Ott, 2020). These structures may have likely originated from NBUDs, which then bud completely to form MNs and later condensed into a small extrachromosomes during mitosis. Chromatin bridges, otherwise known as anaphase bridges, occurs in conjunction with NPB since they share the same cause (Fenech *et al.*, 2011). Lastly, lagging chromosomes are characterised as centromere positive chromosomes that do not seem to properly migrate toward a pole of the cell during anaphase (Fenech *et al.*, 2011). These lagging chromosomes can form MNs as cells leave the mitotic phase.

Hence, to determine if ACLY overexpression cells induces genome instability, the study aims to detect for (1) the presence of MNs, NBUDs, and NPBs in interphase cells, and (2) the presence of small extrachromosomes, chromatin bridges and lagging chromosomes in mitotic cells.

1.7.2. Determine if nuclear and chromosomal abnormalities were caused by increased levels of DNA damage or chromosomal mis-segregations or both

MN, NBUD, and NPB structures are considered as biomarkers of DNA damage (Fenech *et al.*, 2011). Increased levels of DNA double stranded breaks and the cell's limited repair capacity could cause unrepaired DNA to break off from the primary nuclei and form acentric chromosome fragments. Since acentric

chromosome fragments do not have a centromere, it is unable to interact with the mitotic apparatus during mitosis. This prevents these fragments from incorporating themselves into the primary nuclei, which can be visualised as MN structures (Fenech *et al.*, 2011). DNA breaks can also be misrepaired as cells are likely to rely on error-prone repair mechanisms to cope with increased levels of DNA damage. This could detrimentally lead to the rise of NPBs due to error prone ligations. The formation of NPBs can also coincide with MN formation since DNA misrepair also results in the generation of acentric chromosome fragments (Fenech *et al.*, 2011). Lastly, DNA damage caused by ionising radiation has been found to lead to the emergence of NBUDs, of which they bud from the parent nucleus to form MNs (Fenech *et al.*, 2011; Haaf *et al.*, 1999).

Whole chromosomes that fail to be properly attached to the mitotic spindles are unable to faithfully segregate into sister chromatids, causing them to lag behind as other chromosomes migrate toward opposite poles (Fenech *et al.*, 2011; Santaguida & Amon, 2015). These lagging chromosomes eventually form MN structures as the cell exits the mitotic phase. Kinetochore-microtubule attachment errors, and the failure to activate the spindle assembly checkpoint (SAC), can lead to the presence of centromere-positive lagging chromosomes and MNs (Santaguida & Amon, 2015). Another factor that contributes to chromosomal mis-segregations is centrosome amplification. Centrosome amplification was found to induce multipolar spindle formation, resulting in asymmetric cell divisions that can give rise to MN structures and aneuploidy (Fenech *et al.*, 2011; Fukasawa, 2005; Santaguida & Amon, 2015; Yun *et al.*, 2004).

As such, MN structures in ACLY-overexpressing cells were further examined if they were mostly centromere-positive or centromere-negative to determine whether they were caused by chromosomal mis-segregations or DSBs respectively. In addition, to ascertain any defects in mechanisms associated with faithful chromosome segregation, mitotic spindle formations and centrosome numbers were observed in mitotic cells, and the presence of aneuploidy was monitored in populations of asynchronous cells. Also, assays were performed to quantify levels of DSBs occurring in ACLY-overexpressing cells.

1.7.3. Determine if ACLY overexpression elevated ROS production to induce oxidative DNA damage

Reactive oxygen species (ROS) are produced as by-products of normal cellular metabolism in the mitochondria through active oxidative phosphorylation (Starkov, 2008). ROS includes hydrogen peroxides, superoxide anions, and hydroxyl radicals. The generation of these oxygen radicals has been proposed to be caused by the accidental diversion of electrons in the electron transport chain to reduce oxygen molecules (Starkov, 2008).

Elevated levels of ROS has been known to induce oxidative stress in cells as they damage cellular lipids, proteins and DNA, and are closely linked to many pathological diseases (Schieber and Chandel, 2014). Increased ROS can inflict lesions on both nuclear and mitochondrial DNA, which can be deleterious if they are left unrepaired (Cooke *et al.*, 2003). Oxidative damage of nuclear DNA has been linked to the development of cancers and cardiovascular diseases (Cooke *et al.*, 2003), while mitochondrial DNA damage has been found in kidney, urinary and skin cancers (Lee *et al.*, 2013a; Wada *et al.*, 2006). Next, ROS can react with

polyunsaturated fats (PUFAs) at the plasma membrane, leading to the formation of lipid peroxides. One of the lipid peroxides formed is 4-hydroxy-2-nonenals (4-HNE), which has also been shown to damage DNA, proteins and lipids (Zhong and Yin, 2015). 4-HNE-associated protein damage has implicated in the development of liver and prostate cancers (Shoeb *et al.*, 2014), and the increased malignancy in both colon and kidney cancers (Chang *et al.*, 2005). In addition, 4-HNE was found to be responsible for the progression of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Chen *et al.*, 2012).

Metabolic reprogramming could possibly raise ROS levels. In one study, low NAD⁺ levels in cells were discovered to encourage the production of superoxides by α -ketoglutarate dehydrogenase (α -KGDH) and complex I of the electron transport chain (Tretter and Adam-Vizi, 2004; Adam-Vizi and Chinopoulos, 2006). This study suggests the possible role of metabolic reprogramming in inducing DNA damage.

With this, intracellular levels of ROS in ACLY-overexpressing cells were measured to determine if they were elevated. Findings would suggest the presence of oxidative DNA damage actively occurring.

1.7.4. Determine if ACLY overexpression led to the deregulation of ATR and ATM-mediated DNA damage responses

Eukaryotic cells are susceptible to DNA insults from endogenous sources and exogenous sources, especially during DNA replication. Endogenous sources include DNA lesions (DNA hydrolysis and oxidation) caused by metabolic by-products such as ROS and lipid peroxides (Hakem, 2008). These DNA lesions,

along with the presence of repetitive sequences and common fragile sites, can contribute to replication fork stalling (Hakem, 2008; Mazouzi, 2014). Another endogenous source is replication errors that can cause DNA mismatches or the misincorporation of ribonucleotides (Pray, 2008; Zeman & Cimprich, 2014). Exogenous sources of DNA damage can include UV radiation, ionising radiation, and cytotoxic chemicals (Hakem, 2008). The activation of DNA damage responses (DDR) is necessary to ensure proper and timely repair of any presence of DNA damage prior to cell division. In the event where DNA becomes irreparable, DDR can initiate apoptosis. All of these cellular decisions help to maintain genome integrity and thereby within-tissue homogeneity. Ataxia telangiectasia and rad3 related (ATR) and Ataxia telangiectasia-mutated (ATM) are important kinases in initiating the DDR (Awasthi *et al.*, 2015).

Replication stress or replication fork stalling typically generates single stranded DNA (ssDNA) as helicase activity continues to unwind the parent DNA (Paulsen and Cimprich, 2007). Also, the resection of DNA double stranded breaks necessary for homologous recombination (HR) repair generates ssDNA (Ferretti, 2013). These ssDNA can be stabilised through direct interaction with replication protein A heterotrimers (RPA), enabling the recruitment of ATR-ATRIP (ATR interacting protein) heterodimers (Awasthi *et al.*, 2015) and the subsequent autophosphorylation of ATR at Thr1989 (Liu *et al.*, 2011). ATR-ATRIP then facilitates the recruitment of Rad9-Rad1-Hus1 (9-1-1) complex, where Rad17 complex facilitates its loading onto the chromatin (Awasthi *et al.*, 2015; Zou *et al.*, 2002). These events are necessary for Rad9 to be phosphorylated at Ser387, promoting the association of DNA topoisomerase 2-binding protein 1 (TopBP1)

to the 9-1-1 complex (Liu *et al.*, 2011). TopBP1 subsequently interacts with ATR, enabling its full activation to initiate downstream DDR responses (Liu *et al.*, 2011). Several downstream targets of ATR includes Chk1, p53, and RPA32 subunit of the RPA complex. ATR phosphorylates Chk1 at Ser317 and Ser345 to trigger its full activation, where it can initiate cell cycle arrest through suppressing activity of CDC25 phosphatases (Patil *et al.*, 2013). ATR phosphorylates p53 at Ser15, releasing its inhibition from MDM2 to induce the expression of proteins to promote cell cycle arrest and apoptosis (Meek, 2009). Lastly, ATR phosphorylates RPA32 at Ser33, priming it to undergo further phosphorylation events that induces checkpoint activation (Liu *et al.*, 2012).

Prolonged stalling of replication forks can eventually lead to their collapse to form DNA double stranded breaks (DSBs) (Ong *et al.*, 2013). Endogenous and exogenous sources of DNA damage as described can also incur the formation of DNA DSBs. The Mre11-Rad50-Nbs1 (MRN) complex are able to detect these breaks, localising themselves at the vicinity of DNA breaks (Kozlov *et al.*, 2011). This enables the recruitment of inactive ATM dimers, where they undergo autophosphorylation at four sites, namely Ser367, Ser1893, Ser1981, and Ser2996 (Kozlov *et al.*, 2011). Autophosphorylation at Ser1981 is vital for ATM monomerisation and activation at DNA breakage sites (Kozlov *et al.*, 2011), where it is able to act on multiple substrates. Firstly, it suppresses inhibitory effects of HUWE1 on H2AX, promoting H2AX chromatin incorporation at sites of DNA DSBs (Atsumi *et al.*, 2015). This then allows ATM to phosphorylate H2AX at Ser139, which is necessary for the recruitment of BRCA1 or 53BP1 to trigger HR repair or non-homologous end-joining (NHEJ) repair respectively

(Daley and Sung, 2014; Kozlov et al., 2011). Secondly, ATM is able to phosphorylate Nbs1 of the MRN complex at Ser343, Ser397, and Ser615 (Wu *et al.*, 2000), where they play a role in facilitating DDR. Thirdly, ATM has overlapping functions with ATR shown by its ability to phosphorylate p53 at Ser15 to induce the same effects aforementioned (Meek, 2009). In addition, ATM can activate Chk1 through Nbs1-dependent phosphorylation at Ser317, suppressing cell cycle progression (Gatei et al., 2003). On the other hand, ATM uniquely phosphorylates checkpoint kinase 2 (Chk2) at Thr68, triggering its full activation to facilitate cell cycle arrest by inhibiting CDC25 phosphatases, and DNA repair via HR by activating BRCA1 (Cai *et al.*, 2009; Zhang *et al.*, 2004). Also, ATM can phosphorylate 53BP1 at multiple sites, activating its role in initiating NHEJ repair (Daley and Sung, 2014).

Taken altogether, ATR and ATM are important kinases in orchestrating DDRs through multiple and overlapping pathways to activate cell cycle arrest to enable time for DNA damage repair, and trigger apoptosis if DNA damage becomes irreparable (Fig. 8). Hence, the study investigated if ACLY-induced metabolic reprogramming would deregulate the activity of these kinases and their direct downstream substrates.

Figure 8. ATR and ATM kinases are important in the initiation of DNA damage responses. In the presence of replication stress, RPA heterotrimeric complexes are recruited to stabilise the presence of single stranded DNA. This allows the sequential recruitment of ATR-ATRIP heterodimers, Rad17 complexes, 9-1-1 complexes, and TopBP1, triggering a series of post-translational modifications that altogether promote the full activation of ATR with a distinct phosphorylation at Thr1989. This fully activated form of ATR then initiates the DNA damage response by phosphorylating downstream proteins such as RPA32 of the RPA complex, Chk1, and p53. When replication forks collapse or when endogenous and exogenous sources of DNA damage cause DNA double stranded breaks, it triggers the recruitment of DNA damage sensor MRN complex (Mre11-Rad50-Nbs1). This allows the recruitment of inactive ATM dimers, where undergoes autophosphorylation to become fully activated with distinct phosphorylation at Ser1981. ATM shares overlapping pathways by its ability to phosphorylate Chk1 and p53. ATM also acts to release suppressive effects of HUWE1 on H2AX, allowing H2AX to incorporate at DNA damage sites and be phosphorylated at Ser139 by the same kinase (Atsumi *et al.*, 2015). Distinct to ATM, it is able to phosphorylate Chk2 at Thr68, and Nbs1 at Ser343. All these substrates of ATR and ATM kinases are able to initiate mechanisms to induce cell cycle arrest, facilitate DNA damage repair, and trigger apoptosis if DNA damage becomes too extensive. These DDRs are important to maintain genome integrity within a tissue.

1.7.5. Determine if ACLY overexpression deregulated proteins involved in cell cycle checkpoint arrest, DNA damage repair, and apoptosis

DNA damage responses in response to replication stress or DSBs result in the activation of cell cycle checkpoints (cell cycle arrest), enabling time for DNA damage repair processes to occur. When DNA damage levels are extensive and beyond what the cell can cope, the apoptosis pathway is initiated to prevent the transfer of any somatic mutations into the next progeny after cell division.

These mechanisms are essential to maintain genome integrity. Hence, the study examined whether ACLY overexpression led to the deregulation of several key proteins important in (1) cell cycle arrest, (2) DNA repair, and (3) apoptosis.

Cell cycle arrest is facilitated by Cip and Kip family of cyclin-dependent kinase (CDK) inhibitors, where they function to inhibit various CDK-cyclin complexes. One of these inhibitors is p21^{Cip1}, which has been found by studies to inhibit kinase activity of CDK1 and CDK2 (Abbas and Dutta, 2009). As a result, (1) G₁-S phase transition and S phase progression cannot occur due to CDK2-Cyclin E inactivation, (2) S phase progression and S-G₂ phase transition cannot occur due to inactivation of CDK2-Cyclin A and CDK1-Cyclin A respectively, and (3) G₂ phase progression and G₂-M phase transition cannot occur due to CDK1-Cyclin B1 inactivation (Fig. 9) (Abbas and Dutta, 2009). In addition, p21^{Cip1} has been shown to also inhibit the activity of proliferating cell nuclear antigen (PCNA), halting DNA synthesis and thereby S phase progression (Abbas and Dutta, 2009). In the presence of DNA damage, phosphorylated p53 can induce transcriptional expression of p21^{Cip1}. Research has also shown that p21^{Cip1} can be expressed in a p53-independent manner (Abbas and Dutta, 2009).

Figure 9. The role of p21^{Cip1} in modulating CDK-Cyclin complexes to promote cycle cycle arrest. In the presence of DNA double stranded breaks, p21^{Cip1} can be transcriptionally activated in a p53-dependent or -independent manner. The up-regulation of p21^{Cip1} has been found to inhibit activity of CDK1 and CDK2 kinases, and PCNA.

The non-homologous end-joining (NHEJ) DNA repair pathway is predominantly utilised to resolve DNA double stranded breaks throughout the cell cycle, especially at the G₁/G₀ phase (Chang *et al.*, 2017; Charkirabarty *et al.*, 2016). In the presence of DSBs, phosphorylated H2AX (Ser139) (γH2AX) facilitates the recruitment of 53BP1 at damage sites, where it is subsequently phosphorylated by ATM at several sites (Jowsey *et al.*, 2007; Wu *et al.*, 2011) (Fig. 10).

Figure 10. Activation of 53BP1 in response to DNA double stranded breaks. In the presence of DNA double stranded breaks, H2AX is recruited at damage sites where it is phosphorylated by activated ATM kinase at Ser139. This is essential in facilitating the recruitment of 53BP1, where it is phosphorylated by ATM at multiple sites. There, 53BP1 exerts its role in initiating DNA repair via NHEJ pathway.

Lastly, in the event of irreparable DSBs, activated p53 can facilitate the induction of apoptosis by transcriptionally activating the expression of two pro-apoptotic proteins, namely Bax and PUMA (p53 up-regulated modulator of apoptosis) (Miyashita and Reed, 1995; Nakano and Vousden, 2001). Upon receiving apoptotic stimulus, Bax undergoes conformation changes to enable their localisation at the outer mitochondrial membrane (Stephen, 2010). There, they oligomerize to form a pore complex, inducing mitochondrial outer membrane permeabilisation (MOMP) and the subsequent release of cytochrome c (Stephen, 2010). Cytochrome c that is freed into the cytoplasm could then interact with Aparf-1 and caspase-9 to form the apoptosome complex, which acts to initiate the apoptotic pathway via the intrinsic pathway (Loreto, 2014). On the other hand, PUMA interacts with pro-survival proteins Bcl-xL, Bcl-2, Bcl-w, and Mcl-1, sequestering their inhibitory effects on pro-apoptotic Bax and Bak proteins. This consequently frees up Bax or Bak to oligomerize and initiate MOMP (Yu

and Zhang, 2008). Hence, both Bax and PUMA function in a concerted manner to promote apoptosis (Fig. 11).

Figure 11. Apoptosis via the intrinsic pathway is facilitated by PUMA and Bax. Phosphorylated p53 can promote apoptosis through transcriptional activation of PUMA and Bax. Apoptotic stimulus promotes Bax recruitment at the outer mitochondrial membrane, where they oligomerize to form a pore complex, resulting in mitochondrial outer membrane permeabilisation (MOMP). PUMA on the other hand interacts with pro-survival proteins (e.g. Bcl-2, Bcl-X_L, and Bcl-w) outer membrane of the mitochondria, releasing Bax from inhibition. The activities of PUMA and Bax ultimately leads to the release of cytochrome c into the cytosol, triggering apoptosis via the intrinsic pathway.

1.7.6. Determining presence of global histone acetylations in ACLY-overexpressing cells

As previously mentioned, histone acetylations are closely associated with changes in cellular metabolism. Since site-specific histone acetylations have been found in several cancers (Hałasa *et al.*, 2019; Peleg *et al.*, 2019; Shen *et al.*, 2019), and Akt-induced genome-wide histone acetylations have been found to

enrich genes associated with cell growth and proliferation (Lee *et al.*, 2014), experiments were performed to determine if ACLY overexpression would result in an overall increase in histone acetylations.

The presence of genome-wide histone acetylations may indicate the presence of chromatin remodelling that would affect the expression of genes associated with cell cycle arrest, DNA repair, and apoptosis.

1.7.7. Determine if the effects of ACLY overexpression in the cell enabled the acquisition of cancer phenotypes

The gradual acquisition of somatic mutations and epigenetic changes could drive some cells to exhibit cancer phenotypes as hypothesized. One such phenotype is the ability to grow under anchorage-independent conditions, which is seen in aggressive cancers.

Proper control of cell proliferation is necessary to maintain tissue homeostasis. Two rules have to be fulfilled for cell proliferation to occur (Guadamillas *et al.*, 2011). First, cells have to be properly attached to the extracellular matrix (ECM) of the tissue. This is mediated by a group of surface receptors known as integrins that interacts with ECM to trigger signalling cascades through the cytoskeleton, collaborating with other pathways to promote cell growth (Lee *et al.*, 2004). Second, cells require mitogenic stimulation of receptor tyrosine receptors induced by growth factors (Guadamillas *et al.*, 2011). At times, aberrant cell proliferation can occur to push cells into an inappropriate tissue environment, where proper contact to the ECM becomes absent. The resultant lack of intracellular signalling due to the loss of ECM-integrin contact causes normal

cells to induce cell cycle arrest, and trigger caspase-mediated cell death known as anoikis (Guadamillas *et al.*, 2011). Anoikis is important to eliminate cells that are misplaced within the tissue, preventing the formation of neoplasms. Anoikis can involve both intrinsic and extrinsic apoptotic pathways, which ultimately result in DNA fragmentation and subsequently cell death (Guadamillas *et al.*, 2011). Tumour cells have been shown to overcome anoikis by omitting the requirement to anchor themselves onto the ECM (Guadamillas *et al.*, 2011). Furthermore, they can acquire mutations on receptor tyrosine kinases to eliminate the requirement of growth factors necessary for cell proliferation (Hanahan and Weinberg, 2011). As such, the acquisition of these two capabilities allow these tumour cells to invade into other tissues of the body and grow in an anchorage-independent manner, forming metastases (Guadamillas *et al.*, 2011).

As such, the study investigated whether ACLY-induced metabolic reprogramming enhanced the proliferative fitness of cells when grown under anchorage-independent conditions. Findings would be indicative that metabolic alterations can result in cancer development.

1.8. Significance of study

Extensive studies on many disease models have built our current understanding about cancers. Such approaches have enabled us to identify therapeutic targets to drive drug discovery and developments. However, they have not been successful as cancer incidences continue to follow an upward trend worldwide (Fitzmaurice *et al.*, 2020). This reveals the complexity of cancers. The way cancers develop differs from one person to another (between-tumour heterogeneity), which can

account the variations in drug responses (Palmer and Sorger, 2017; Shaw and Dagogo-Jack, 2017). Furthermore, not all cancer cells appear the same within the tumour of a patient (within-tumour heterogeneity), placing limits on biopsies to provide wholesome diagnosis and risking unsuccessful therapies (Greaves and Maley, 2012; Palmer and Sorger, 2017). Cancer development involves a series of random evolutionary events that confers them dominance over normal cells. As quoted by Nowell in his paper in 1976, “research should be directed toward understanding and controlling the evolutionary process in tumours before it reaches the late stage usually seen in clinical cancer” (Nowell, 1976).

Therefore, the present study proposes the role of metabolic reprogramming in driving evolutionary processes that ultimately favours cancer development and progression. Findings will motivate research to identify novel metabolic targets, thereby driving drug developments that hopefully limits cells from evolving over time. In addition, it is also hoped that this research would motivate future works to establish new models to better characterise how cancers emerge from a normal phenotype. With this, there is a possibility to build novel prediction tools to determine how the tumours would evolve over time in each patient based on their genomic and epigenomic profile. This would then enable clinicians to better direct customised therapy while minimising risk of any side effects.

2. MATERIALS AND METHODS

2.1. Cell lines and maintenance

2.1.1. The consideration on the use of cell lines that best represents normal cell phenotype

The use of cancer cell lines in the study may introduce variances in the experimental data, and that the results may not accurately depict the early events of tumourigenesis. Firstly, cancer cell lines derived from different individuals exhibit between-tumour heterogeneity, where genetic and epigenetic divergences can cause variances in responses upon ACLY knockdown or overexpression. This is because the way individuals acquire genetic and epigenetic aberrations are unique (Fig. 12). Secondly, these cancer cells lines can harbour other unidentified irreversible mutations and epigenetic modifications that can potentially contaminate any effects caused by ACLY knockdown.

To minimise the possibility of variances in the data, cell lines that closely represents a normal phenotype were used. Doing so will also allow future works to accurately characterise the events required to transform cells into cancers. In this study, immortalised human mammary epithelial cells (hTERT-HME1) (ATCC), and immortalised retinal epithelial cells (hTERT-RPE1) (ATCC) were used. The hTERT-HME1 cell line was generated by infecting normal mammary epithelial cells with retroviruses to induce the expression of telomerase reverse transcriptase (hTERT), a catalytic subunit of the human telomerase. These normal mammary epithelial cells were taken from a patient with no history of breast cancers. On the other hand, the hTERT-RPE1 cell line was obtained by transfecting the hTERT vector into RPE-340 primary cells.

Figure 12. Limitations imposed through the use of cancer cell lines in the study. Based on the model of clonal evolution, the way individuals acquire mutations and epigenetic modifications are unique (as annotated by differences in coloured circles between patient 1 and 2). Hence, cancer cell lines taken from different individuals would display genetic and epigenetic divergence. This could potentially cause variations in the experimental data. In addition, genetic and epigenetic aberrations in these cancer cell lines can remain permanent and thereby irreversible. Consequentially, these aberrations may contaminate cellular events caused by ACLY knockdown. As such, the results obtained through works on cancer cell lines may not be representative on the role of ACLY-induced metabolic reprogramming on tumourigenesis.

2.1.2. Culturing conditions of cell lines

For hTERT-HME1 cells, they were cultured in DMEM/F12 media (Gibco, 12500) supplemented with 10% fetal bovine serum (FBS) (Hyclone, SV30160), 1% penicillin/streptomycin (pen/strep) (Nacalai Tesque, 09367-34), and 1% mammary epithelial growth supplement (MEGS) (containing 0.4% v/v bovine pituitary extract, 1.0 µg/mL recombinant human insulin-like growth factor-I, 0.5 µg/mL hydrocortisone, 3 ng/mL human epidermal growth factor) (Gibco,

S0155). This was the complete media used on hTERT-HME1 cells. For regular maintenance, cells were washed with 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) before treatment with trypsin (0.25% w/v Trypsin, 1mM EDTA) (Gibco, 25200) for 5 min. Trypsin was subsequently inactivated with complete medium. A subcultivation ratio of 1:5 to 1:6 was used during each passage.

As for hTERT-RPE1 cells, they were cultured in DMEM/F12 media (Gibco, 12500) supplemented with 10% FBS (Hyclone, SV30160) and 1% pen/strep (Nacalai Tesque, 09367-34). This is the complete medium for hTERT-RPE1 cells. For regular maintenance, hTERT-RPE1 cells were washed with 1x PBS, before treating with trypsin for 3 min. After which trypsin was inactivated with complete medium. A subcultivation ratio of 1:8 was used during each passage.

Highly transfectable 293FT cells (Invitrogen, R70007) were used to generate lentiviruses for the establishment of doxycycline-inducible cell lines. 293FT cells were cultured in DMEM media (Gibco, 12100) supplemented with 10% FBS (Hyclone, SV30160), 0.1 mM minimal essential medium non-essential amino acids (MEM NEAA) (Gibco, 11140), 1 mM sodium pyruvate (Gibco, 11360), 6 mM glutamine (Gibco, 25030), and 1% pen/strep (Nacalai Tesque, 09367-34). This was the complete medium used in all subsequent works. To maintain 293FT cells, they were washed with 1x PBS and subsequently treated with trypsin for 3 min. Trypsin was then inactivated with complete medium. A subcultivation ratio of 1:8 to 1:10 was used for every passage, with Geneticin™ antibiotic (G418) (Gibco, 10131) added to final concentration of 500 µg/mL.

One aggressive breast cancer cell line, namely double positive MCF-7 cell line (estrogen receptor and progesterone receptor positive) (ATCC), was selected as model of breast cancer with increased malignancy. In addition, U-2OS cell line was used to establish human cDNA library. These cell lines were cultured in DMEM media (Gibco, 12100) supplemented with 10% FBS (Hyclone, SV30160) and 1% pen/strep (Nacalai Tesque, 09367-34). Both of these cell lines were passaged by washing the cells with 1x PBS before treatment trypsin for 3 min. Trypsin was then inactivated with the same complete medium. For these two cell lines, a subcultivation ratio of 1:8 was used for every passage.

The cell lines used throughout this study, namely hTERT-HME1, hTERT-RPE1, 293FT, MCF-7, and U-2OS cells, were incubated in a humidified 37°C incubator with 5% CO₂.

2.2. Generating pXJ40-FLAG-ACLY vector and transfection

2.2.1. Generating pXJ40-FLAG-ACLY vector

Ectopic expression of ACLY in hTERT-HME1 and hTERT-RPE1 cells was accomplished through transfection with the pXJ40-FLAG-ACLY vector. To generate this vector, total RNA was first obtained from U-2OS cells using the PureLink® RNA mini kit (Ambion, 12182018). Stable human cDNA libraries were then established through reverse transcription-polymerase chain reaction (RT-PCR) from the total RNA extract using the RevertAid reverse transcriptase (Thermo Scientific™, EP0441). From the cDNA library, the ACLY cDNA was amplified through polymerase chain reaction (PCR) using a high fidelity DNA

polymerase from the KAPA HiFi PCR kit (KAPA Biosystems, KK2102A). A forward primer (5' – ATAAAGCTTATGTCGGCCAAGGC – 3') (Sigma Aldrich) and reverse primer (5' – ATGCGGCCGCTTACATGCTCAT – 3') (Sigma Aldrich) were designed to introduce a HindIII restriction enzyme site (5' – AAGCTT – 3') and NotI restriction enzyme site (5' – GCGGCCGC – 3') at the N and C terminal of ACLY respectively. The PCR cycling protocol is summarised in Table 1. Successful amplification of ACLY cDNA product was determined through gel electrophoresis using 0.75% agarose gel (Vivantis, PC0701) in 1 x Tris-acetate EDTA (TAE) buffer (40 mM Tris-base, 20 mM acetic acid, 1 mM EDTA), where an expected amplified ~3.3 kilo base pair (kbp) DNA fragment was detected that corresponds to the coding DNA sequence (CDS) of human ACLY. Once verified, the amplified ACLY cDNA product was extracted using the NucleoSpin™Gel and PCR Clean-up kit (Macherey-Nagel™, 740609.250).

Table 1. PCR cycling protocol for the amplification of ACLY cDNA for insertion into pXJ40-FLAG vector

Step	Process	Temperature	Time	Repeat
1	Initial denaturation	95°C	2min	30 cycles
2	Denaturation	98°C	20sec	
3	Annealing	67°C to 69°C (gradient)	40sec	
4	Extension	72°C	3min	
5	Final Extension	72°C	3min	
6	Cooling	4°C	Minimum 15min	

Sequential digestion of ACLY cDNA and pXJ40-FLAG vector was then performed with HindIII (NEB, R0104) and NotI (NEB, R0189) restriction enzymes, where digested products were isolated through gel electrophoresis and DNA gel extractions (using the same kit). The final digested products were then

ligated using T4 DNA ligase (NEB, M0202). Cloning of ACLY cDNA at HindIII and NotI restriction enzyme sites of the PXJ40-FLAG vector allows ACLY to be FLAG-tagged at the N-terminal.

After which, DH5 α competent cells (Invitrogen, 18265017) were transformed with the ligation mix and grown on selective Luria-Bertani (LB) agar (BD Difco, 240110) containing 100 μ g/mL of ampicillin (Gold Biotechnology, A-301-25). Several colonies were isolated, grown in selective LB broth (BD Difco, 240230) containing 100 μ g/mL of ampicillin, and lysed using the QIAprep Spin Miniprep kit (QIAGEN, 27106) to extract plasmids. To determine successful insertion of ACLY cDNA in the PXJ40-FLAG vector, plasmids from each of the selected colonies were digested with NotI, which should yield $\sim$ 7.6 kbp DNA fragments (ACLY cDNA has $\sim$ 3.3 kbp; PXJ40-FLAG vector has $\sim$ 4.3 kbp). In addition, DNA sequencing was also done from the T7 promoter. The colony exhibiting the appropriate insertion was grown at a larger quantity of selective LB broth, where the pXJ40-FLAG-ACLY vector was extracted using the HiSpeed Plasmid Midi Kit (QIAGEN, 12643).

2.2.2. LipofectamineTM2000-based transfection of pXJ40-FLAG-ACLY and pXJ40-FLAG vectors

Ectopic expression of ACLY was achieved through transfecting pXJ40-FLAG-ACLY vector into hTERT-HME1 and hTERT-RPE1 cells. As a negative control, the empty pXJ40-FLAG vector was transfected into these cells also. hTERT-HME1 and hTERT-RPE1 cells were first seeded into six-well plates at cell density of 3.0×10^5 and 2.6×10^5 cells per well respectively. After an overnight incubation, the media of both cells were removed and replaced with 1 mL of

serum free F12-DMEM media. In each well, the cells were transfected with either 1.25 µg of pXJ40-FLAG-ACLY or 1.25 µg of PXJ40-FLAG using the Lipofectamine™2000 reagent (Invitrogen™, 11668019) at a DNA:Lipofectamine™2000 ratio of 1:2.5. These conditions were applied on both hTERT-HME1 and hTERT-RPE1 cells.

As per manufacturer's instructions, 1.25 µg of plasmid DNA and 3.125 µL of Lipofectamine™2000 were diluted in two separate tubes containing 150 µL of OptiMEM reduced serum media (Gibco, 31985070) each. The diluted Lipofectamine™2000 reagent was left to incubate at room temperature for 5 min before the addition of diluted DNA. The resultant mixture was then left to incubate for another 20min at room temperature for DNA-Lipofectamine™2000 complexes to form. Finally, the DNA-Lipofectamine™2000 were added onto the cells, where transfection was allowed to take place for 5hrs before replacing the transfection mixture with fresh complete media. Transfected hTERT-HME1 and hTERT-RPE1 cells were then left to incubate for 24hrs to allow ectopic expression of ACLY. These cells were then fixed and stained to observe for nuclear abnormalities. Also, they were harvested and lysed to determine levels of phosphorylated ACLY (Ser455) and total ACLY.

2.3. Generation and maintenance of stable cell clones for doxycycline-inducible expression of ACLY

2.3.1. Generating pSLIK-Neo-TRE-FLAG-ACLY expression vector

Stable doxycycline-inducible hTERT-HME1 cell line was produced using a third generation lentiviral system. The pSLIK-Neo vector (Addgene, #25735), a third generation lentiviral vector, was selected to integrate the FLAG-ACLY transgene

into the genome. The pSLIK-Neo vector encodes for a reverse-tTA (rtTA) protein that responds to the presence of doxycycline by binding to the Tet-response element (TRE) promoter, triggering the expression of the transgene. As the pSLIK-Neo vector does not have the TRE promoter, the FLAG-ACLY coding sequence was first spliced into entry vector pEN_Tmcs (Addgene, #25751) that has the TRE promoter. Through Gateway™ cloning, the gene cassette containing both TRE-promoter and FLAG-ACLY coding sequence was transferred onto the pSLIK-Neo vector. The final product pSLIK-Neo-TRE-FLAG-ACLY vector was then used to generate lentiviruses.

Using pXJ40-FLAG-ACLY as a template, PCR was performed with a forward primer (5' – TCCGCGGTTCACCATGGACTAC – 3') (Sigma Aldrich) and reverse primer (5' – ATGCGGCCGCTTACATGCTCAT – 3') (Sigma Aldrich). These two primers were designed to generate a FLAG-ACLY cDNA amplified product with a SacII restriction enzyme site (5' – CCGCGG – 3') at the N terminal, and NotI restriction enzyme site (5' – GCGGCCGC – 3') at the C terminal. The same high fidelity DNA polymerase was used, with slight alterations in the the PCR cycling protocol shown in Table 2. Similarly, successful FLAG-ACLY cDNA amplification was verified through gel electrophoresis using 0.75% agarose gel in 1xTAE buffer, and isolated using the same NucleoSpin™Gel and PCR Clean-up kit.

Table 2. PCR cycling protocol for the amplification of ACLY cDNA for insertion into pEN_Tmcs vector

Step	Process	Temperature	Time	Repeat
1	Initial denaturation	95°C	2min	30 cycles
2	Denaturation	98°C	20sec	
3	Annealing	68°C	40sec	

4	Extension	72°C	3min	
5	Final Extension	72°C	3min	
6	Cooling	4°C	Minimum 15min	

The amplified FLAG-ACLY cDNA and pEN_Tmcs vector were sequentially digested with SacII (NEB, R0157) and NotI restriction enzymes. After a series of gel electrophoresis and DNA gel extractions to isolate the desired digested products, they were ligated using the same T4 DNA ligase. The resultant ligation mixes were then used to transform JM109 competent cells (L2005), where they were grown on selective LB agar containing 10 µg/mL of gentamycin. The pEN_Tmcs vector contains a ccdB gene that is flanked by SacII and NotI restriction enzymes sites at the 5'-end and 3'-end respectively. The ccdB gene encodes for a toxic peptide that interferes with DNA gyrase activity in *Escherichia coli* bacteria, resulting in chromosomal and plasmid DNA damage and consequentially cell death. SacII and NotI digestion on pEN_Tmcs vector facilitates the replacement of the ccdB gene with the FLAG-ACLY coding sequence. Hence, successful ligation of FLAG-ACLY cDNA to the pEN_Tmcs vector should yield colony formation. Next, successful colonies were propagated in selective LB broth containing 10 µg/mL of gentamycin, and plasmids were extracted using the QIAprep Spin Miniprep kit. To confirm successful integration of the FLAG-ACLY cDNA into the pEN_Tmcs vector, DNA sequencing was done with the pCEP-forward primer (5' – AGAGCTCGTTTAGTGAACCG – 3'). The colony that has the correct pEN_Tmcs-FLAG-ACLY product, was used as an entry vector for Gateway™ cloning.

Gateway™ cloning is an efficient method to clone a gene-of-interest into an expression vector through the DNA recombination reactions. For this study, the LR reaction was used to clone the TRE-FLAG-ACLY gene cassette into the pSLIK-Neo vector. The LR reaction involves the use of an enzyme mix that recognises *attL* sites that are flanking the gene-of-interest in the entry vector, and *attR* sites that flanking the *ccdB* gene in the destination vector. When the enzymes become active, DNA recombination occurs between the *attL* and *attR* sites to generate *attP* and *attB* sites. As a result, the gene-of-interest is transferred from the entry vector to the destination vector to generate a new expression vector. In this expression vector, the gene of interest is flanked by *attB* sites. On the other hand, the *ccdB* gene is transferred from the destination vector to the entry vector, where it is flanked by newly formed *attP* sites. This toxic vector induces cell death when transformed into competent cells.

Gateway™ cloning was performed through the initiation of an LR reaction between entry vector pEN_Tmcs-FLAG-ACLY and destination vector pSLIK-Neo using the LR Clonase™ II enzyme mix (Invitrogen™, 11791019) (Fig. 13). The clonase reaction mix consisted of 150 ng of entry vector, 150 ng of destination vector, and Tris-EDTA (TE) buffer (10 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0), making a total volume of 8 µL. As per manufacturer's instructions, 2 µL of LR Clonase™ II enzyme mix were added, where the LR reaction was allowed to occur for 1hr at 25°C. To terminate the reaction 1 µL of Proteinase K solution (Invitrogen™, 11791019) was added to the sample and allowed to incubate for 10min at 37°C. The final LR reaction mix was used to transform JM109 competent cells, which were then grown on selective LB agar

containing 100 µg/mL of ampicillin and 25 µg/mL of chloramphenicol (Sigma, C0378). Any colonies formed should only harbour the pSLIK-Neo-TRE-FLAG-ACLY expression vector, since the *ccdB* gene in the other by-product of the LR reaction will not lead to any viable bacteria cells. Successful colonies were propagated in selective LB broth containing 100 µg/mL of ampicillin and 25 µg/mL of chloramphenicol, and plasmids were extracted using the QIAprep Spin Miniprep kit.

Figure 13. Generation of the pSLIK-Neo-TRE-FLAG-ACLY expression vector using Gateway™ cloning. The LR reaction of the Gateway™ cloning system involves DNA recombination between *attL* sites of the entry vector and *attR* sites of the destination vector. This results in the integration of the gene-of-interest into the destination vector, forming a new expression vector containing *attB* sites. The LR reaction also generates a by-product vector containing a *ccdB* gene that is flanked by newly formed *attP* sites. This toxic vector encodes for a toxic peptide that promotes cell death in transformed competent cells, thereby allowing the selective amplification of the expression vector.

2.3.2. Lentiviral production

Lentiviral production was achieved using a 3rd generation lentiviral packaging system (Invitrogen, K4975-00), which consists of (1) packaging plasmids pLP1 and pLP2, (2) envelop plasmid pLP/VSVG, (3) transfer plasmid, and (4) 293FT cells (Dull *et al.*, 1998). Packaging plasmid pLP1 expresses Gag and Pol proteins, while packaging plasmid pLP2 expresses Rev proteins (Dull *et al.*, 1998). On the other hand, pLP/VSVG plasmid expresses a viral envelop protein that has been known to interact with a broad range of mammalian cells (Burns *et al.*, 1993; Emi *et al.*, 1991). Lastly, the transfer plasmid not only contains the expression cassette, but also elements that facilitates its packaging into virions. To generate the lentivirus, all four plasmids are transfected into 293FT cells. There, the expression of Gag, Pol, Rev, and envelop proteins work in a concerted manner to facilitate the packaging of the expression cassette into the lentivirus. In this study, the pSLIK-Neo-TRE-FLAG-ACLY construct was used as the transfer plasmid to express FLAG-tagged ACLY in hTERT-HME1 cells. As a negative control to determine off-target effects of viral transduction and doxycycline treatment, an empty pSLIK-Neo construct was used.

293FT cells were seeded in a 10 cm culture dish at cell density of 5×10^6 cells. After the cells were left to recover for 24hrs, conditioned medium was removed and replaced with fresh 5 mL complete medium without G418. In a sterile tube, 3 µg of pLP1, 3 µg of pLP2, 3 µg of pLP/VSVG, and 3 µg of pSLIK-TRE-FLAG-ACLY or pSLIK-Neo was diluted in 1.5 mL of OptiMEM. In another sterile tube, 36 µL of Lipofectamine™2000 was diluted into 1.5 mL of OptiMEM, where it was allowed to incubate at room temperature for 5min. The diluted DNA was

then mixed with the diluted Lipofectamine™2000, and were left to incubate at room temperature for another 20min to allow DNA-Lipofectamine™2000 complexes to form. After incubation, the DNA-Lipofectamine™2000 complexes were added to the cells, and they were left to incubate for about 16hrs. Following 16hrs of incubation, the medium containing the DNA-Lipofectamine™2000 complexes was removed and replaced with fresh 10 mL complete medium without G418. The transfected 293FT cells were left to incubate for a further 24hrs for the lentivirus to be generated. The virus was harvested by transferring the medium-containing virus into a sterile tube. This medium was then purified from any cellular debris by centrifuging them at 3000 rpm for 15min at 4°C. The resultant virus-containing supernatant was transferred into cryovials. The virus stocks were stored at -80°C. In addition, the same transfected 293FT cells could be incubated further with 10 mL of fresh complete medium without G418 for another 24hrs to yield another batch for lentivirus.

2.3.3. Determining titer of lentiviral stocks

The titer of SLIK-Neo-TRE-FLAG-ACLY and SLIK-Neo lentiviral stocks were determined using hTERT-HME1 cells. The pSLIK-Neo construct contains a Neomycin resistance gene that confers G418 antibiotic resistance to cells (Shin *et al.*, 2006). To determine antibiotic sensitivity of hTERT-HME1 cells to G418, cells were first seeded into six-well plates at a density of 1.0×10^5 cells per well, achieving approximately 30% confluence. After an overnight incubation, the cells were treated at various working concentrations of G418, namely, 400 µg/mL, 500 µg/mL, 600 µg/mL, 700 µg/mL, 800 µg/mL, 900 µg/mL, 1000 µg/mL, and 1100 µg/mL. Cells were then allowed to incubate for a total of 6 days

with replenishment of the selective medium on the third day. It was determined that a working concentration of 700 µg/mL was sufficient to eliminate most of the untransduced hTERT-HME1 cells.

Next, hTERT-HME1 cells were seeded in six-well plates at a cell density of 8.0×10^4 cells per well, after which the cells were incubated overnight. The following day, lentiviral stocks were thawed and serially diluted by ten-folds in complete medium from 10^2 to 10^6 (a total of 5 dilutions). Each dilution was prepared to a final volume of 1mL per well. Prior to transduction, the medium was removed from hTERT-HME1 cells. There, each dilute was added into one well of cells. In the mock well representing untransduced cells, 1 mL of complete medium was only added. To facilitate efficient transduction, hexadimethrine bromide (Polybrene®) (Sigma, 107689) was added into each well at a final working concentration of 6 µg/mL. Viral transduction was allowed to take place for 24hrs in the incubator. After incubation, the virus-containing medium was removed from each well and replaced with 2 mL of complete medium without G418. The cells were then left to incubate for another 24hrs before replacing with 2 mL of selective complete medium containing 700 µg/mL of G418. The cells were allowed to incubate in selective medium for the next 12 days with routine replenishment every 3 days.

After 12 days, untransduced cells in the mock well was found to be mostly eliminated. At this time, cells were washed with 1x PBS twice before fixing them in 100% ice-cold methanol (Sigma, 106009) for 10min. Subsequently, the cells were stained with crystal violet solution (0.5% w/v crystal violet, 25% v/v

methanol) (Sigma, C6158) for 5min at room temperature before rinsing it off with double distilled H₂O. Stained colonies in each well were counted, where the titer was calculated in transducing units per mL (TU/mL) by multiplying the number of colonies with the dilution factor. The titer of these wells were averaged to derive the actual titer of SLIK-Neo-TRE-FLAG-ACLY and SLIK-Neo lentiviral stocks.

2.3.4. Lentiviral transduction to generate stable HME1-ACLY and HME1-Empty cells

hTERT-HME1 cells were seeded in six-well plates at a cell density of 2.5×10^5 cells per well, where they were left to incubate overnight. The following day, SLIK-Neo-TRE-FLAG-ACLY and SLIK-Neo lentiviral stocks were thawed and diluted with the appropriate amount of virus in complete medium to achieve a multiplicity of infection (MOI) of 1.8. The MOI was calculated based on the formula below. To initiate viral transduction, the medium was removed from the cells, where diluted SLIK-Neo-TRE-FLAG-ACLY and SLIK-Neo lentiviruses were added into separate wells.

$$MOI = \frac{\text{Viral Titer} \left(\frac{TU}{mL} \right) \times \text{Volume of lentiviral stock (mL)}}{\text{Number of cells}}$$

To each well, Polybrene® was added at working concentration of 10 µg/mL. The cells were then incubated for 24hrs. After incubation, the virus-containing medium was removed and replaced with fresh complete medium without G418, and was allowed to incubate for a further 24hrs. Subsequently, the transduced cells were grown in selective complete medium containing 700 µg/mL of G418 for a total of 10 days, with routine replenishment every 3 days. To verify

successful transduction, the cells were harvested, lysed and checked via Western Blotting for the expression of the rtTA protein.

hTERT-HME1 cells that were transduced with the SLIK-Neo-TRE-FLAG-ACLY lentivirus were labelled as “HME1-ACLY”, while cells that were transduced with the SLIK-Neo lentivirus were labelled as “HME1-Empty”. These stable cell lines were maintained in selective complete medium containing 700 µg/mL of G418, with similar passaging and culturing conditions as the hTERT-HME1 cell line. In addition, a Tet-system approved fetal bovine serum (Tet-free FBS) (TakaraBio, 631106) was used instead of the commonly-used FBS (Hyclone, SV30160) in the complete media, since the latter may contain small traces of tetracycline that can inadvertently trigger the Tet-On system for transgene expression. ACLY overexpression was achieved by treating HME1-ACLY cells with 40 ng/mL of doxycycline (Sigma, D9891) without G418 in complete media containing Tet-free FBS. To detect any off-target effects of doxycycline treatment, HME1-Empty cells were treated with the similar amount of doxycycline under similar media conditions. HME1-Empty cells were used to determine if the interaction between doxycycline and rtTA would cause any off target effects on gene expression.

2.4. Immunofluorescent staining

2.4.1. Cell fixation, permeabilisation, and immunostaining conditions

All specimens were washed twice with 1x PBS and fixed with 4% paraformaldehyde solution (4% w/v paraformaldehyde in 1x PBS) for 10min. The fixative solution was removed subsequently and the cells were washed three

times with ice-cold 1x PBS. The cells were then permeabilised with 0.25% Triton X-100 (0.25% v/v Triton X-100 in 1x PBS) for 10min at room temperature before washing them three times with 1x PBS, 5min each. After which, the specimens were incubated with antibodies overnight at 4°C in 1% BSA (1% w/v bovine serum albumin, 0.1% v/v Tween-20, in 1x PBS) (Capricorn, BSA-1S). The following day, the cells were washed three times with 1x Tris-buffered saline and Tween 20 (TBST) (20 mM Tris-HCl, 136 mM NaCl, 3 mM KCl, 0.1% v/v Tween-20, adjusted to pH 7.4), for 5min each. The cells were then incubated with the appropriate secondary Alexa-Fluor® antibody in 1% BSA for 1hr at room temperature. In the last step, the cells were washed three times with 1x TBST for 5min each, before mounting the coverslip onto DAPI-containing mounting medium (Life Technologies, P36931). All the cells were observed under fluorescence microscope using 63x objectives.

2.4.2. Studies on transfected hTERT-HME1 and hTERT-RPE1 cells

To determine transfection efficiency, hTERT-HME1 and hTERT-RPE1 cells that were transfected with pXJ40-FLAG-ACLY were probed with mouse anti-FLAG antibodies (Cell signalling, #8146) at a dilution of 1:1000. The cells were then incubated with goat anti-mouse Alexa-Fluor®594 secondary antibodies (Invitrogen A-11005) at a dilution of 1:1000.

To observe micronuclei structures, transfected hTERT-HME1 and hTERT-RPE1 cells were mounted directly onto DAPI-containing mounting medium after permeabilisation, thereby staining only the nuclei.

2.4.3. Studies on stable HME1-ACLY and HME1-Empty cell lines

HME1-ACLY cells were induced to overexpress ACLY through the addition of doxycycline in complete media without G418. There, the presence of genome instability was determined through observing a range of nuclear abnormalities and chromosomal aberrations in the cells. Also, levels of γ H2AX and 53BP1 foci in each nuclei were quantified. As a positive control, etoposide (VP16) was used to induce DNA damage. To prepare positive control, HME1-ACLY cells were treated with 5 μ M of VP16 (Sigma, E1383) for 2hrs in complete media.

First, the presence of nuclear abnormalities and chromosomal aberrations were observed and quantified. HME1-ACLY and HME1-Empty cells were first seeded in separate 35 mm dishes at cell density of 0.7×10^5 cells per dish. After an overnight incubation, media from both cell lines were replaced with fresh complete medium containing doxycycline. The cells were then left to incubate for 72hrs. For the positive control, 2.5×10^5 of HME1-ACLY cells were seeded in a 35mm dish. The following day, these cells were treated with VP16 and later incubated for another 24hrs in fresh complete media. All specimens were fixed and permeabilised as aforementioned (refer to Section 2.4.1.). Micronuclei (MN) and nuclear budding (NBUD) structures were visualised through DAPI staining. To determine if the MN structures were derived from lagging chromosomes, acentric chromosomes, or fragmented DNA, the specimens were stained against centromere marker CENPA (centromere protein A). Furthermore, to identify anaphase and telophase cells for the detection of small extrachromosomal structures, chromatin bridges, and lagging chromosomes, the specimens were stained against DAPI and α -tubulin. Lastly, to identify the presence of multipolar

spindles in mitotic cells, the specimens were stained against centrosome marker pericentrin and α -tubulin.

Next, the cytokinesis-block micronucleus cytome (CBMN) assay was performed to observe nuclear abnormalities, namely MN, NBUD and nucleoplasmic bridges (NPB), in once-divided cells. HME1-ACLY and HME1-Empty cells were seeded in separate 35 mm dishes at cell density of 0.6×10^5 cells per dish, where they were left to incubate overnight. The following day, the media was removed and replaced with fresh complete medium containing doxycycline. The cells were then left to incubate for 72hrs. After which, the conditioned medium was removed and replaced with fresh complete media containing doxycycline and $1.0 \mu\text{g/mL}$ of Cytochalasin B (CytB) (Sigma, C6762). On the other hand, the positive control was prepared by first seeding 2.5×10^5 of HME1-ACLY cells in a 35mm dish. These cells were treated with VP16 the following day before replacing with fresh complete media containing same concentration of CytB. All specimens were incubated with CytB for 16hrs, allowing cells to arrest at the cytokinesis stage to yield binuclear cells. In a similar fashion, the cells were fixed and permeabilised accordingly (refer to Section 2.4.1.). The specimens were stained against CENPA to determine if the MN structures are derived from lagging chromosomes or acentric chromosomes. Also, to distinguish binuclear cells from others, the specimens were stained against α -tubulin to mark cellular boundaries.

Lastly, changes in levels of γH2AX and 53BP1 foci in response to ACLY overexpression were observed and quantified at 48hr and 72hr time-points. First, HME1-ACLY and HME1-Empty cells were seeded in separate 35 mm dishes at

a cell density of 1.5×10^5 cells (48hr time-point), and 0.7×10^5 cells (72hr time-point) per dish. After an overnight incubation, the media was replaced with fresh complete media containing doxycycline. The cells were then incubated for 48hrs and 72hrs. On the other hand, the positive control was prepared by seeding 3.0×10^5 of HME1-ACLY cells in a 35 mm dish. The following day, these cells were then treated with VP16 and immediately processed. All specimens were fixed and permeabilised accordingly (refer to Section 2.4.1). Following that, the specimens were stained against γ H2AX or 53BP1.

The study was also interested in observing the levels of γ H2AX foci in cells that overexpressed ACLY for 5 weeks. To do so, HME1-ACLY and HME1-Empty cells were first seeded in 60 mm dishes at cell density of either 0.5×10^6 or 0.3×10^6 cells per dish, where they were allowed to incubate in doxycycline for 3 or 4 days respectively. These doxycycline-treated HME1-ACLY and HME1-Empty cells were subsequently passaged twice every week following the same seeding and incubation procedures for up to 4 weeks and 3 days. At this point, the cells were transferred into 35 mm dishes at a cell density of 0.7×10^5 cells per dish, and were incubated for an additional 4 days in doxycycline, thereby achieving 5 weeks of doxycycline treatment. The specimens were fixed and permeabilised accordingly (refer to Section 2.4.1), and stained against γ H2AX.

Primary antibodies and fluorophore-conjugated secondary antibodies that were used are summarised in Table 3 along with their respective dilutions.

Table 3. Primary antibodies and fluorophore-conjugated secondary antibodies used for immunostaining.

Detection	Primary antibodies		Fluorophore-conjugated Secondary antibodies	
	Antibody	Dilution	Antibody	Dilution
CENPA (Centromere marker)	Mouse anti-CENPA (Stressgen, KAMCC006)	1:2000	Goat anti-mouse Alexa-Fluor®594 (Invitrogen, A11005)	1:1000
Pericentrin (Centrosome marker)	Rabbit anti-Pericentrin (Abcam, ab4448)	1:2000	Goat anti-rabbit Alexa-Fluor®594 (Invitrogen, A11012)	1:1000
α -tubulin (Mitotic spindles)	Mouse anti- α -tubulin-FITC (Sigma, F2168)	1:1000	-	-
γ H2AX	Rabbit anti-gamma H2A.X (phospho S139) (Abcam, ab11174)	1:1000	Goat anti-rabbit Alexa-Fluor®488 (Invitrogen, A11008)	1:1000
53BP1	Mouse anti-53BP1 (BD Transduction, 612522)	1:1000	Goat anti-mouse Alexa-Fluor®488 (Invitrogen, A11001)	1:1000

2.5. Acid histone extraction

Changes in histone acetylations levels were determined in response to ACLY overexpression over a period of 48hrs and 72hrs. To determine baseline levels of histone acetylations, HME1-ACLY cells were grown in complete media without doxycycline treatment. As a positive control, HME1-ACLY cells were treated with sodium butyrate (NaB), which has shown to inhibit the activity of histone deacetylases (HDACs), thereby promoting an increase in genome wide histone acetylation levels (Steliou *et al.*, 2012).

First, HME1-ACLY cells were seeded in 10 cm dishes at a cell density of 4.0×10^6 cells (0hr time-point), 2.0×10^6 cells (positive control), 1.0×10^6 cells (48hr time-point) and 0.5×10^6 cells (72hr time-point) per dish. After allowing the cells to incubate overnight, the 0hr time-point specimen was harvested first. As for the remaining cells, the media was removed and replaced with fresh complete media. These cells were then allowed to incubate for 48hrs and 72hrs with or without

doxycycline. On the other hand, the positive control cells were treated with 2 mM of NaB (Sigma, 07596) in fresh complete media for 24hrs.

All specimens were harvested by washing cells with 1x PBS before treating them with trypsin at 37°C for 5 min to attain single cell suspensions. After deactivating trypsin with complete media, cell suspensions were transferred into sterile microcentrifuge tubes. There, the cells were spun down by centrifugation at 200 g for 5 min at 4°C into pellets. After the supernatants were removed, the remaining cell pellets were washed twice by resuspending them with 1x PBS with 5 mM of NaB and spinning them down again under the same centrifugation conditions. The resultant cell pellets were frozen quickly in liquid nitrogen before storage in a -80°C freezer. As a note, NaB was added into 1x PBS at a concentration of 5 mM during the harvesting procedures to retain histone acetylations in the cells.

Next, the cells in each specimen were lysed in 400 µL of Triton extraction buffer (TEB) (0.5% v/v Triton X-100, 2 mM phenylmethanesulfonyl fluoride, 0.02% w/v sodium azide, 5 mM NaB) for 10min at 4°C under constant gentle agitation. The lysed cells were then centrifuged at 6,500 g for 10 min at 4°C to spin down the nuclei. After removing the unwanted supernatant, the remaining nuclei pellet was washed with 200 µL of TEB without agitation and subsequently spun down with the same centrifugation conditions. The nuclei pellet was then suspended in 200 µL of 0.4N H₂SO₄ (Merck, 112080) overnight at 4°C with constant gentle agitation. The following day, the nuclear debris was spun down by centrifugation at 16,000 g for 10min at 4°C. The supernatant containing solubilised histones was

isolated and precipitated with 64 μ L of 100% trichloroacetic acid (TCA) (Sigma, T6399) for 30min at 4°C. Precipitated histones were pelleted down by centrifugation at 16,000 g for 10min at 4°C. After removing the supernatant, the histone pellet was washed twice by adding ice-cold acetone (Merck, 100014) without agitating it and centrifuging it at 16,000 g for 5min at 4°C. The histone pellet was allowed to be air-dried for 20min before dissolving it in double distilled H₂O. These histone extracts were subsequently analysed through western blotting and Coomassie blue staining.

2.6. Western blotting and Coomassie blue staining

2.6.1. Protein extraction, sample preparation and SDS-PAGE

To attain single cell suspensions, cells were washed with 1x PBS before treatment with trypsin for 5min at 37°C. After deactivating trypsin with complete media, the resultant cell suspensions were centrifuged at 200 g for 5min at 4°C. The cells were then washed twice by re-suspending the cell pellets with 1x PBS and spinning the cells down under the same centrifugation conditions. After aspirating the spent 1x PBS to leave only the cell pellets, they were quickly frozen in liquid nitrogen and stored at -80°C. The cell pellets were then subsequently lysed to extract proteins.

To extract whole cell lysates, the cell pellets were re-suspended in radio-immunoprecipitation assay buffer (RIPA) (50 mM Tris-HCl, 1% v/v NP40, 0.5% w/v sodium deoxycholate, 0.1% w/v SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF) supplemented with a cocktail of protease inhibitors (cOmplete™ protease inhibitor cocktail tablets) (Roche, 11836145001) and phosphatase inhibitors

(PhosSTOP™) (Roche, 04906837001). The cells were then incubated in lysis buffer for 30min on ice, with agitation every 10min through high-speed vortexing. The cell lysates were then centrifuged at 16,000 g for 15min at 4°C. The supernatants containing the whole protein extracts were then isolated in fresh microcentrifuge tubes.

Concentration of extracted proteins (i.e. RIPA-based whole cell lysates or histone extracts) were determined through the Bradford assay. First, protein standards were prepared with BSA at concentrations of 10 µg/µL, 8 µg/µL, 6 µg/µL, 4 µg/µL, 2 µg/µL, 1 µg/µL and 0 µg/µL. Protein extracts and protein standards were then plated in a 96-well plate in duplicates at aliquots of 4 µL per well. Following this, the protein assay dye reagent (BioRad, #5000006) was added at a volume of 200 µL per well. There, the absorbance in each well at the wavelength of 595 nm was read using a spectrophotometer.

Next, the specimens were prepared for sodium-dodecyl sulphate-based polyacrylamide gel electrophoresis (SDS-PAGE). This was done by first denaturing an appropriate amount of proteins from each specimen in 2x sample buffer (125 mM Tris-HCl pH 6.8, 4% w/v SDS, 20% v/v glycerol, 10% β-mercaptoethanol, 0.004% bromophenol blue) on a heat block at 95°C for 10min. On the other hand, the SDS-PAGE gel was prepared by first making the resolving gel followed by the stacking gel. The resolving gel consists of the appropriate percentage of acrylamide/bis (37.5:1) (BioRad, #1610158), resolving buffer (375 mM Tris-HCl, 0.1% w/v SDS, adjusted to pH 8.8), 1% w/v ammonium persulfate (Promega, V3131), and 0.1% v/v TEMED (BioRad, 161-0800). As for the

stacking gel, it consists of 5% acrylamide/bis (37.5:1), stacking buffer (125 mM Tris-HCl, 0.1% w/v SDS, adjusted to pH 6.8), 1% w/v ammonium persulfate, and 0.1% v/v TEMED. Lastly, the proteins were loaded into the SDS-polyacrylamide gels before running gel electrophoresis. Gel electrophoresis in 15% polyacrylamide gels were run at 150V for 90min, while other polyacrylamide gels (i.e. 6%, 8%, and 10%) were run at 120V for 90-100min.

2.6.2. Western blotting

After SDS-PAGE, proteins were transferred onto 0.2 μ m polyvinylidene fluoride (PVDF) membranes (BioRad, 1620177) using the wet transfer method. For most wet transfers, they were run in Towbin buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol) at 100V for 60-100min at 4°C. However, for high molecular weight proteins (i.e. > 150 kDa), the transfers were performed in a modified Towbin buffer containing smaller percentage of methanol (25 mM Tris, 192 mM glycine, 5% v/v methanol) at 30V for approximately 20hrs at 4°C.

Subsequently, the PVDF membranes were blocked with blocking buffer containing 5% BSA or 5% fat-free milk in 1x TBST (19 mM Tris-HCl, 135 mM NaCl, 2.7 mM KCl, 0.1% v/v Tween-20, adjusted to pH 7.4) for 1hr at room temperature. Subsequently, the membranes were incubated with primary antibodies overnight at 4°C in the appropriate blocking buffer. The following day, the membranes were washed three times with 1x TBST for 5min each to remove unbound primary antibodies. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for approximately 1hr at room temperature in blocking buffer. After which the

membranes were washed three times with 1x TBST for 5min each. The protein bands were visualised through enhanced chemiluminescence (ECL) by coating the membranes with entry-level HRP substrates (Thermo Scientific™, 32106), or highly sensitive HRP substrates (Amersham, RPN2236).

2.6.3. Coomassie blue staining

The SDS-polyacrylamide gels were stained in Coomassie® blue solution (0.125% Coomassie® brilliant blue R250, 50% v/v methanol, 10% v/v acetic acid) (Merck, 112553) for 10min at room temperature under gentle shaking. The stain was then removed by washing the gels with de-staining solution (40% v/v methanol, 10% v/v acetic acid in double distilled H₂O) multiple times until the protein bands became visible. The gels were then submerged in 5% acetic acid and stored at 4°C.

2.6.4. Studies on transfected hTERT-HME1 and hTERT-RPE1 cells

Western blot was performed to confirm the expression of FLAG-tagged ACLY in hTERT-HME1 and hTERT-RPE1 cells transfected with the pXJ40-FLAG-ACLY vector. To determine possible off-target effects of transfection on ACLY expression, both cell lines were transfected with the pXJ40-FLAG vector. Transfection procedures were as follows in Section 2.2.2. Cells were harvested and lysed in RIPA buffer, where levels of phosphorylated ACLY (Ser455), total ACLY, and FLAG-tagged ACLY were analysed through the following SDS-PAGE and Western Blot procedures and conditions (Refer to Section 2.6.1 and 2.6.2.) (Table 4 and 5).

2.6.5. Studies on stable HME1-ACLY and HME1-Empty cell lines

To confirm successful viral transduction, about 1.0×10^6 HME1-ACLY and HME1-Empty cells were harvested and lysed in RIPA buffer, where levels of rtTA proteins were analysed according to the SDS-PAGE and western blotting procedures (Refer to Section 2.6.1 and 2.6.2.) (Table 4 and 5).

Stable overexpression of ACLY in HME1-ACLY cells over 24hrs, 48hrs, and 72hrs was verified in response to doxycycline treatment. HME1-ACLY cells were first seeded in 60 mm dishes at a cell density of 2.0×10^6 (0hr time-point), 0.7×10^6 (24hr time-point), 0.5×10^6 (48hr time-point) and 0.3×10^6 (72hr time-point) cells per dish. After an overnight incubation, the 0hr time-point was harvested. The media of the remaining cells was removed and replaced with fresh complete media. These cells were then left to incubate with or without doxycycline for 24hrs, 48hrs and 72hrs, where they were harvested and lysed in RIPA buffer (Refer to Section 2.6.1). Levels of phosphorylated ACLY, total ACLY, and FLAG-tagged ACLY were analysed according to the SDS-PAGE and western blotting procedures (Refer to Section 2.6.1 and 2.6.2.) (Table 4 and 5).

To observe possible changes in genome-wide histone acetylations, an appropriate amount of histone extracts (attained according to Section 2.5.) were used for SDS-PAGE and western blotting (See Table 4). To detect for the presence of acetylations, a broad-detecting primary antibody that specifically interacts with acetylated-lysine residues was used (Table 5).

The levels of selected DNA damage response proteins and their activities were analysed in HME1-ACLY cells that were induced to overexpress ACLY for 24hrs, 48hrs, and 72hrs. With the consideration of any off-target effects of doxycycline on DNA damage responses (DDRs), HME1-Empty cells were treated with doxycycline for the aforementioned time-points. From the start, HME1-ACLY and HME1-Empty cells were seeded in 60 mm dishes at a cell density of 0.7×10^6 (24hr time-point, baseline controls), 0.5×10^6 (48hr time-point) and 0.3×10^6 (72hr time-point) cells per dish. After an overnight incubation, the media was removed from the cells and replaced with fresh complete media. Baseline controls (-) were allowed to incubate for another 24hrs without doxycycline before they were collected. As for the remaining cells, they were treated with doxycycline and left to incubate for 24hrs, 48hrs, and 72hrs. As positive controls for DNA damage and replication stress, HME1-ACLY cells were treated with VP16 and hydroxyurea (HU) respectively. HME1-ACLY cells were seeded in 60 mm dishes at a cell density of 2.0×10^6 cells. After overnight incubation, the cells were treated with 5 μ M of VP16 or 4 mM of HU (Sigma, H8627) for 2hrs, where they were immediately harvested. All specimens were harvested and lysed with RIPA buffer (Refer to Section 2.6.1). Total protein levels and phosphorylation status of ATR, ATM, Chk1, Chk2, and Nbs1 were analysed. Furthermore, protein levels of RPA32, 53BP1, Bax, PUMA, and p21^{Cip1} were observed. Specimens were prepared in accordance to the SDS-PAGE and western blotting specifications for the analysis of the above mentioned proteins (Refer to Section 2.6.1 and 2.6.2.) (Table 4 and 5).

To determine if ATR and Chk1 were down-regulated by the proteosomal degradation pathway or autophagy pathway, ACLY-overexpressing cells were treated with MG132 and ammonium chloride (NH₄Cl). MG132 inhibits proteolytic activity of proteasomes (Han *et al.*, 2009), while NH₄Cl is a known inhibitor of autophagy (Sun *et al.*, 2015). HME1-ACLY cells were first seeded in 60 mm dishes at cell density of 0.7×10^6 (24hr time-point, baseline control) and 0.5×10^6 cells (48hr time-point) per dish. After an overnight incubation, media was removed replaced with fresh complete media. Baseline controls were allowed to incubate overnight without doxycycline for 24hrs before being harvested. The remaining cells were incubated with doxycycline for 24hrs. After incubation, some of the cells were harvested (+Dox 24hr time-point), some were treated with a working concentration of 5 μ M of MG132 (Sigma, M8699) or 10 mM of NH₄Cl (Riedel-de Haen, 11209) for 24hrs, and the rest were treated with equal volumes of MG132 or NH₄Cl diluents for 24hrs. All specimens were harvested and lysed with RIPA buffer (Refer to Section 2.6.1). There, specimens were prepared accordingly to detect total protein and phosphorylation levels of ATR and Chk1 (Refer to Section 2.6.1 and 2.6.2.) (Table 4 and 5).

Lastly, DDRs in response to oxidative stress were observed in ACLY-overexpressing cells. HME1-ACLY and HME1-Empty cells were seeded in 60 mm dishes at a cell density of 0.5×10^6 cells per dish, where they were incubated overnight. The following day, media from both cell lines were removed and replace with fresh complete media containing doxycycline, where they were allowed to incubate for 24hrs. After 24hrs of incubation, some of the cells were harvested (-), while the rest of the cells were treated with 200 μ M tert-Butyl

hydroperoxide (tBHP) (Sigma, 416665) for 2hrs. Following treatment, some of the cells were immediately harvested (0hr post-treatment), and the remaining cells were washed twice with 1x PBS before adding fresh complete media containing doxycycline. These cells were then allowed to incubate for 4hrs, 8hrs, 12hrs, and 24hrs before being harvested. All specimens were lysed with RIPA buffer (Refer to Section 2.6.1). There, specimens were prepared accordingly to detect total protein and phosphorylation levels of Chk1, Chk2, Nbs1 and p53 (Refer to Section 2.6.1 and 2.6.2.) (Table 4 and 5). Specimens were also prepared to analyse total protein levels of p21^{Cip1}.

Table 4. SDS-PAGE conditions for each protein-of-interest

Protein-of-interest	Approximate molecular weight	Amount of protein load per well	Percentage SDS-polyacrylamide gel
Phospho-ACLY (Ser455) and ACLY	125 kDa	20 µg	8%
rtTA	27.8 kDa	20 µg	15%
Histone extracts (containing H2A, H2B, H3, and H4)	12-15 kDa	2 µg	15%
FLAG-tagged ACLY	125 kDa	20 µg	8%
Phospho-ATR (Thr1989) and ATR	301 kDa	30 µg	6%
Phospho-ATM (Ser1981) and ATM	350 kDa	30 µg	6%
Phospho-Chk1 (Ser345) and Chk1	56 kDa	20 µg	10%
Phospho-Chk2 (Thr68) and Chk2	62 kDa	20 µg	10%
RPA32	32 kDa	20 µg	15%
Phospho-Nbs1 (Ser343) and Nbs1	95 kDa	20 µg	8%
Phospho-p53 (Ser15) and p53	53 kDa	20 µg	10%
53BP1	450 kDa	20 µg	6%
Bax	20 kDa	20 µg	15%
PUMA	22 kDa	20 µg	15%
p21 ^{Cip1}	21 kDa	20 µg	15%

Table 5. Primary antibodies used in Western Blotting.

Protein-of-interest	Primary antibodies		HRP-conjugated Secondary antibodies		Blocking Buffer
	Antibody	Dilution	Antibody	Dilution	
Phospho-ACLY (Ser455)	Mouse anti-phospho ACLY (Ser455) (Cell Signalling, #4331)	1:2,000	Goat anti-rabbit (Invitrogen, G-21234)	1:10,000	5% BSA
ACLY	Rabbit anti-ACLY (D1X6P)	1:2,000	Goat anti-rabbit (Invitrogen, G-21234)	1:10,000	5% Milk

	(Cell Signalling, #13390)				
rtTA	Mouse anti-TetR (Clontech, 631132)	1:500	Goat anti-mouse (Invitrogen, G-21040)	1:10,000	5% Milk
FLAG-tag	Mouse anti-DYKDDDDK Tag (Cell Signalling, #8146)	1:1,000	Goat anti-mouse (Invitrogen, G-21040)	1:10,000	5% Milk
Acetylated-lysine	Rabbit anti-acetyl lysine (Abcam, ab80178)	1:1,000	Goat anti-rabbit (Invitrogen, G-21234)	1:10,000	5% BSA
Phospho-ATR (Thr1989)	Rabbit anti-phospho-ATR (Thr1989) (Cell Signalling, #58014)	1:1,000	Goat anti-rabbit (Invitrogen, G-21234)	1:5,000	5% Milk
ATR	Rabbit anti-ATR (E13S3S) (Cell Signalling, #13934)	1:1,000	Goat anti-rabbit (Invitrogen, G-21234)	1:5,000	5% Milk
Phospho-ATM (Ser1981)	Rabbit anti-phospho ATM (Ser1981) (D25E5) (Cell Signalling, #13050)	1:1,000	Goat anti-rabbit (Invitrogen, G-21234)	1:5,000	5% BSA
ATM	Mouse anti-ATM (11G12) (Cell Signalling, #92356)	1:1,000	Goat anti-mouse (Invitrogen, G-21040)	1:5,000	5% Milk
Phospho-Chk1 (Ser345)	Rabbit anti-phospho Chk1 (113D3) (Cell Signalling, #2348)	1:500	Goat anti-rabbit (Invitrogen, G-21234)	1:10,000	5% BSA
Chk1	Mouse anti-Chk1 (G-4) (Santa cruz, sc-8408)	1:500	Goat anti-mouse (Invitrogen, G-21040)	1:10,000	5% Milk
RPA32	Rabbit anti-RPA32 (E8X5P) (Cell Signalling, #35869)	1:2,000	Goat anti-rabbit (Invitrogen, G-21234)	1:10,000	5% BSA
Phospho-Chk2 (Thr68)	Rabbit anti-phospho Chk2 (Thr68) (Cell Signalling, #2197)	1:1,000	Goat anti-rabbit (Invitrogen, G-21234)	1:10,000	5% BSA
Chk2	Mouse anti-Chk2 (A-11) (Santa cruz, sc17747)	1:500	Goat anti-mouse (Invitrogen, G-21040)	1:10,000	5% Milk
Phospho-Nbs1 (Ser343)	Rabbit anti-phospho-Nbs1 (Novus, AF4994)	1:1,000	Goat anti-rabbit (Invitrogen, G-21234)	1:10,000	5% BSA
Nbs1	Rabbit anti-p95/Nbs1 (D6J4I) (Cell Signalling, #14956)	1:1,000	Goat anti-rabbit (Invitrogen, G-21234)	1:10,000	5% Milk

Phospho-p53 (Ser15)	Rabbit anti-phospho-p53 (Cell Signalling, #9284)	1:1,000	Goat anti-rabbit (Invitrogen, G-21234)	1:10,000	5% BSA
p53	Mouse anti-p53 (DO-1) (Santa cruz, sc-126)	1:500	Goat anti-mouse (Invitrogen, G-21040)	1:10,000	5% Milk
53BP1	Mouse anti-53BP1 (19/53BP1) (BD Transduction, 612522)	1:1,000	Goat anti-mouse (Invitrogen, G-21040)	1:10,000	5% BSA
Bax	Rabbit anti-Bax (Cell Signalling, #2772)	1:1,000	Goat anti-rabbit (Invitrogen, G-21234)	1:10,000	5% BSA
PUMA	Rabbit anti-PUMA (Cell Signalling, #4976)	1:5,000	Goat anti-rabbit (Invitrogen, G-21234)	1:10,000	5% BSA
p21 ^{Cip1}	Rabbit anti-p21 Waf1/Cip1 (12D1) (Cell Signalling, #2947)	1:1,000	Goat anti-rabbit (Invitrogen, G-21234)	1:10,000	5% BSA
Vinculin (Loading control for high molecular weight proteins)	Mouse anti-Vinculin (Abcam, ab130007)	1:10,000	Goat anti-mouse (Invitrogen, G-21040)	1:10,000	10% Milk
Actin (Loading control)	Mouse anti-Actin (Millipore, MAB1501)	1:20,000	Goat anti-mouse (Invitrogen, G-21040)	1:10,000	10% Milk

2.7. Flow cytometry analysis

2.7.1. DNA staining

To analyse DNA content in cells, specimens were first washed with 1x PBS before trypsinizing them for 5min at 37°C to attain single cell suspensions. After deactivating trypsin with complete media, cell suspensions were transferred into fresh sterile tubes. There, the cells were spun down by centrifugation at 200 g for 5min at room temperature. The supernatants were removed and the cell pellets were washed twice by re-resuspending the cells in 1x PBS and spinning them under the same centrifugation conditions. The cell pellets were then re-suspended in 500 µL of 1x PBS, and then transferred drop-wise into ice-cold 70% ethanol under gentle vortexing. The fixed specimens were then kept in a 4°C fridge

overnight. The following day, the fixed cells were spun down by centrifugation at 200 g for 5min at room temperature to remove the ethanol. Cell pellets were then re-suspended in 1x PBS for 1min before spinning the cells down under the same centrifugation conditions. After removing the supernatants, the cell pellets were re-suspended in propidium iodide (PI) staining solution (20 µg/mL propidium iodide, 10 µg/mL RNaseA, 0.1% v/v Triton X-100, in 1x PBS) and incubated for 15min at 37°C. The specimens were then analysed under flow cytometry to determine DNA content in each cell.

2.7.2. Intracellular staining

To probe against intracellular proteins in the cell, specimens were first harvested by washing the cells with 1x PBS and trypsinizing them for 5min at 37°C to attain single cell suspensions. Trypsin was then deactivated by the addition of complete media. The resultant cell suspensions were transferred into fresh sterile tubes, where they were spun down by centrifugation at 200 g for 5min at room temperature. The cells were then washed twice by resuspending them in 1x PBS and spinning them down under the same centrifugation conditions. After removing the spent 1x PBS, the cell pellets were suspended in ice-cold methanol, where fixation was allowed to take place for 10min at -20°C. Following fixation, the cells were spun down by centrifugation at 300 g for 5min at 4°C to remove methanol. The remaining cell pellets were then washed twice by re-suspending them with 1% BSA (1% w/v BSA in 1x PBS) and spinning the cells down by centrifugation at 300 g for 5min at 4°C. Following the removal of supernatants, the cells were permeabilised with 0.25% Triton X-100 (0.25% v/v Triton X-100 in 1x PBS) at room temperature in the dark for 15min. After that, the cells were

centrifuged at 300 g for 5min at 4°C to remove the permeabilisation buffer. There, the cell pellets were washed with 0.1% Triton X-100 (0.1% v/v Triton X-100 in 1x PBS) and centrifuged under the same conditions.

Next, the cells were incubated with 5 µg/mL of primary antibodies in 3% BSA (3% w/v BSA in 1x PBS) for 1hr at 4°C under constant gentle agitation. After incubation, the cells were washed three times by centrifugation at 400 g for 5min at 4°C and re-suspending them with ice-cold 1x PBS. Following that, the cells were incubated with 5 µg/mL of fluorophore-conjugated secondary antibodies in 3% BSA in the dark for 1hr at 4°C under constant gentle agitation. Similarly, the cells were washed three times by centrifugation using the same conditions and re-resuspending them with ice cold 1x PBS. After removing the spent 1x PBS, the cell pellets were re-suspended in PI staining solution (3% w/v BSA, 1% w/v sodium azide, 20 µg/mL propidium iodide, 10 µg/mL RNaseA, in 1x PBS). The specimens were then analysed under flow cytometry to determine expression levels of the protein-of-interest and DNA content in each cell.

2.7.3. Studies on stable HME1-ACLY and HME1-Empty cell lines

To identify if ACLY overexpression leads to an increased population of sub-G₁ cells (DNA content < 2N), and polyploid cells (DNA content ≥ 4N), HME1-ACLY and HME1-Empty cells were first seeded in 10 cm dishes at a cell density of 0.5×10^6 cells per dish. After an overnight incubation, the media was replaced with fresh complete media. The cells were then allowed to incubate for 72hrs with or without doxycycline before they were harvested. To detect the presence of sub-G₁ and polyploid cells, the cells were fixed with 70% ethanol and stained

with PI (Refer to Section 2.7.1). To detect the presence of tetraploid cells (DNA content = 4N), each specimen was prepared in triplicates containing 2.0×10^6 cells each. The cells were then fixed with methanol, permeabilised, and stained against cyclin B1 using mouse anti-cyclin B1 (D-11) primary antibodies (Santa cruz, sc-7393) and goat anti-mouse Alexa-Fluor®488 (Invitrogen, A11001) (Refer to Section 2.7.2). The cells were also stained with a non-specific mouse IgG primary antibody (eBioscience, 14-4714-82) as an isotype control to detect background signals.

Next, the influence of ACLY overexpression on cell cycle progression from S phase to mitotic (M) phase was determined by performing a double thymidine block. HME1-ACLY and HME1-Empty cells were first seeded in 10 cm dishes at a cell density of 0.5×10^6 cells, where they were incubated overnight. The following day, the media was removed and replaced with fresh complete media. The cells were then allowed to incubate for 72hrs with or without doxycycline. After 72hrs of incubation, the cells were trypsinized and seeded into 60 mm dishes at a cell density of 0.5×10^6 cells per dish, where 4 mM of thymidine (Sigma, T0895) was added into complete media with or without doxycycline. The cells were then allowed to incubate for 18hrs to initiate the first thymidine block. Following that, the cells were washed twice with 1x PBS, and released from thymidine block by adding fresh complete media with or without doxycycline. The cells were then left to incubate for 10hrs before adding thymidine at working concentration of 2 mM. There, the cells were incubated for another 18hrs to initiate the second round of thymidine block. After 18hrs of thymidine treatment, the cells were released by washing them twice with 1x PBS,

and adding fresh complete media with or without doxycycline. At the same time, some of the cells were harvested (0hr double thymidine block release). The remaining cells were allowed to incubate for 2hrs, 4hrs, 6hrs, 8hrs, and 10hrs before they were harvested. All specimens were fixed and stained to analyse DNA content in each cell (Refer to Section 2.7.1).

2.8. Neutral comet assay

The neutral comet assay is designed to detect the presence of DNA double stranded breaks in cells (Olive and Banáth, 2006). HME1-ACLY cells were induced to overexpress ACLY for 48hrs, 72hrs and 5 weeks, where its effect on DNA damage was analysed. As a positive control, HME1-ACLY cells were treated with 5 μ M VP16 to induce DNA double stranded breaks. First, HME1-ACLY cells were seeded into 60 mm dishes at a cell density of 0.5×10^6 (48hr time-point) and 0.3×10^6 (72hr time-point) cells per dish. After an overnight incubation, the media was removed and replaced with fresh complete media. The cells were then incubated for 48hrs and 72hrs with or without doxycycline before they were collected for the assay. For the positive control, 2.0×10^6 of HME1-ACLY cells were seeded in a 60 mm dish. The following day, these cells were treated with VP16 for 2hrs before being harvested for the assay. To overexpress ACLY for 5 weeks, HME1-ACLY cells were seeded at a cell density of either 0.5×10^6 or 0.3×10^6 cells per 60 mm dish, where they were allowed to incubate for 3 days or 4 days respectively in the presence or absence of doxycycline. Subsequently, these cells were passaged twice every week under similar cell seeding and incubation procedures until the fifth week. At the end point, all specimens at various time-points were prepared by trypsinizing the cells to attain

single-cell suspensions, where they were washed twice in 1x PBS by centrifugation at 200 g for 5min at 4°C. There, the cells were re-suspended in 1x PBS to a final concentration of 2×10^4 cells/mL.

Before the commencement of the neutral comet assay, the microscope slides were pre-coated with 1% low-gelling temperature agarose (Sigma, A9414) (1% w/v low-gelling temperature agarose in double distilled H₂O) and allowed to air-dry. To start, 0.4 mL of cells of each specimen were transferred into a sterile tube. Then, 1.2 mL of 1% low-gelling temperature agarose (melted and preheated at 40°C) was added and mixed rapidly. Immediately, 1.2 mL of cell suspension was transferred on the pre-coated microscope slide, where the gel was allowed to solidify for approximately 2min. The slide was then submerged in neutral lysis solution (2% N-lauroylsarcosine sodium salt, 0.5 M Na₂EDTA, 0.5 mg/mL proteinase K, adjusted to pH 8.0) (Promega, V3021) at 4°C in a covered dish. Following that, the slide was allowed to incubate in neutral lysis solution for 18hrs at 37°C in the dark. After 18hrs of incubation, the microscope slide was rinse three times in electrophoresis buffer (90 mM Tris-HCl, 90 mM boric acid, 2 mM Na₂EDTA, adjusted to pH 8.5) for 30min each. The slide was then transferred into an electrophoresis chamber, which was filled with electrophoresis buffer at a level that was approximately 1-2 mm above the top of the gel. Electrophoresis was performed at 0.6 V/cm for 25min. After electrophoresis, the slide was rinsed in 400 mL of distilled water and submerged in PI staining solution (2.5 µg/mL propidium iodide in distilled water) for 20min. After staining, the slide was rinsed in 400 mL of distilled water before it was briefly submerged in 70% ethanol and air-dried. Prior to image acquisition, the

slides were coated with a thin layer of 1% low-gelling temperature agarose and re-stained with PI staining solution.

Comet images were captured under 20x objectives, avoiding pixel saturation. About 300 comet images were taken for each specimen, and were analysed using an automated OpenComet software that was developed by a research group (Gyori *et al.*, 2014). Levels of DNA damage was calculated based on the olive tail moment (Olive and Banáth, 2006; Gyori *et al.*, 2014).

Olive tail moment (Arbitrary Units)
 $= (\text{Distance between the centers of mass of head and tail}) \times \% \text{ DNA in tail}$

2.9. Detecting levels of reactive oxygen species in live cells

The study determined if ACLY overexpression causes an increase in reactive oxygen species (ROS) in the cell. To achieve this, a commercially available CellRox™Green reagent (Invitrogen, C10444) was used to detect levels of ROS. This reagent becomes oxidised in the presence of ROS, where it then binds to DNA to display green fluorescence with an absorption and emission maxima of 485 nm and 520 nm respectively.

HME1-ACLY cells were treated with doxycycline to induce ACLY overexpression for 48hrs and 72hrs, where ROS levels were observed using the CellRox™Green reagent. As a positive control, HME1-ACLY cells were treated with tBHP to induce oxidative stress. To start, HME1-ACLY and HME1-Empty cells were seeded in 35 mm glass-bottom dishes (Ibidi, 81218-200) at a cell density of 1.0×10^5 (48hr time-point) and 0.7×10^5 cells (72hr time-point) per

dish. After an overnight incubation, the media was replaced with fresh complete media containing doxycycline. The cells were then incubated for 48hrs and 72hrs. As for the positive control, HME1-ACLY cells were seeded in a 35 mm glass-bottom dish at a cell density of 3.0×10^5 cells per dish. The following day, the cells were treated with 200 μ M of tBHP for 2hrs. All specimens were immediately stained with CellRox™Green reagent and observed live under fluorescence microscopy.

As per manufacturer's instructions, staining was performed by first adding the CellRox™Green reagent in the media at a working concentration of 5 μ M. The cells were then incubated with the reagent for 30min in a humidified incubator at 37°C with 5% CO₂. After incubation, the media was removed and the cells were washed three times with 1x PBS. Prior to fluorescence-based imaging, Leibovitz's L-15 media (Gibco, 11415064) supplemented with 1% MEGS was added to the cells. Images were acquired under 10x objectives.

2.10. MTT cell proliferation assay

The MTT cell proliferation assay was performed to determine if ACLY overexpression alters the growth rate of cells over a time period of 24hrs, 48hrs, and 72hrs. NAD(P)H-dependent oxidoreductase enzymes active in viable cells reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to form purple formazan salts (Vistica *et al.*, 1991), which can be dissolved in an appropriate solubilisation buffer to form a homogenized coloured solution. Hence, the MTT assay is a colorimetric assay to indicate cell viability.

First, HME1-ACLY and HME1-Empty cells were seeded into 96-well plates in triplicates at a cell density of 4.0×10^3 cells per well. After an overnight incubation, the media was removed and replaced with fresh complete media. The cells were then allowed to incubate with or without doxycycline for 24hrs, 48hr, and 72hrs. Prior to the commencement of the MTT assay, the conditioned media was removed and replaced with 100 μ L of fresh complete media. Using either HME1-ACLY or HME1-Empty cell lines, standards were prepared at a range of cell concentrations via two-fold serial dilutions, namely 1×10^6 , 0.5×10^6 , 0.25×10^6 , 0.125×10^6 , 0.0625×10^6 , 0.03125×10^6 cells/mL. Standards were then seeded in triplicates into 96-well plates at volumes of 100 μ L per well.

In each well, 10 μ L of MTT stock (5 mg/mL MTT in 1x PBS) (ThermoScientific, M6494) was added into the media. The cells were allowed to incubate for 5hrs before allowing the formazan salts to be dissolved in MTT solubilisation solution (20% w/v SDS, 50% v/v dimethyl formamide, 0.6% v/v acetic acid, adjusted to pH 4.8) overnight (approximately 16hrs) in the dark. The following day, the absorbance values in each well was determined at the wavelength of 570 nm, which was subtracted by the values at the reference wavelength of 650 nm.

2.11. TUNEL assay

The TUNEL assay was performed to determine if ACLY-overexpressing cells were undergoing apoptosis. The TUNEL assay typically detects cells that are at the late stages of the apoptotic pathway, where the DNA is highly fragmented by caspase-activated DNase (ICAD). The assay exploits the presence of these DNA fragments by using an enzyme, namely terminal deoxynucleotidyl transferase, to

attach modified fluorescein-dUTPs (fluorescein incorporated with dUTP) by their 3'-OH end. Hence, the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) reaction allows apoptotic cells to be visualized through fluorescence microscopy.

HME1-ACLY cells were treated with doxycycline to induce ACLY overexpression for 48hrs and 72hrs, where the TUNEL assay was performed to determine if there was an increase presence of apoptotic cells. First, HME1-ACLY and HME1-Empty cells were seeded into separate 35 mm dishes at a cell density of 1.0×10^5 (48hr time-point), and 0.7×10^5 (72hr time-point) cells per dish. After an overnight incubation, the media was replaced with fresh media containing doxycycline, where the cells were incubated for 48hrs and 72hrs before being fixed. Negative and positive experimental controls were also prepared by seeding 3.0×10^5 HME1-ACLY cells in 35 mm dishes, incubated overnight, and fixed subsequently.

All specimens were fixed in 4% paraformaldehyde for 15min at room temperature. The cells were then washed twice with ice-cold 1x PBS for 5min each. Following that, the cells were permeabilised with 0.25% Triton X-100 for 3min at room temperature. The cells were then washed three times with 1x PBS for 5min each. Next, positive control cells were treated with 20 U/mL of DNaseI (ThermoScientific, 89836) in DNAaseI buffer (1 mg/mL BSA, 10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM EDTA, adjusted to pH 7.5) for 10min at room temperature. These cells were then washed four times with distilled water.

TUNEL assay was then performed on all specimens using the in-situ cell death detection kit (Roche, 11684795910). As per manufacturer's instructions, the TUNEL mixture was prepared by adding the Enzyme Solution into the Label Solution. With the exception of the negative experiment control, 50 μ L of TUNEL mixture was added on each coverslip. As for the negative experiment control, 50 μ L of Label Solution was added instead. After coverslips were covered with a thin layer of parafilm, the specimens were allowed to incubate at 37°C for 1hr. After incubation, the specimens were washed three times with 1x PBS, and mounted onto a mounting medium containing DAPI. The cells were then examined under fluorescence microscopy using an excitation wavelength of 450-500 nm and a detection range of 515-565 nm.

2.12. Soft agar colony formation assay

The soft agar colony formation assay was performed to determine if ACLY overexpression enables cells to grow under anchorage-independent conditions. This assay involves suspending the cells in a layer of soft agar gel that is mixed with complete media. Cells in this layer are placed over a higher percentage soft agar layer that is also mixed with complete media. By doing so, the cells are prevented from adhering to the bottom of the culture dish, where it is typically surface-treated to encourage optimal cell adhesion and growth. HME1-ACLY cells were treated with doxycycline to induce overexpression of ACLY for one week before the assay was performed. As a negative control, HME1-Empty cells were treated with the similar amount of doxycycline. Also, MCF-7 cells were used as a positive indicator for anchorage-independent growth.

HME1-ACLY and HME1-Empty cells were seeded in separate 60 mm dishes at a cell density of 0.3×10^5 cells per dish. After an overnight incubation, the media was removed and replaced with fresh complete media. The cells were then left to incubate for 7 days with or without doxycycline. During this incubation period, the media was replenished every 3 days. After 7 days of incubation, the cells were harvested for the assay.

To set up for the assay, the bottom layer and feeder layer were first prepared. To prepare the bottom layer (0.6% agarose gel), 3% agarose gel (melted and pre-warmed in 45°C water bath) (3% w/v 2-Hydroxyethyl agarose in double distilled H₂O) (Sigma, A9045) was diluted in fresh complete media (pre-warmed at 37°C) at a dilution factor of 1:5. Immediately, 2 mL of freshly constituted molten 0.6% agarose gel was added into each well of 6-well plates. The culture plates were then incubated at 4°C for 1hr to allow the gels to solidify. Subsequently, the plates were placed in a humidified incubator at 37°C with 5% CO₂ for 30min. Next, the feeder layer (0.6% agarose gel) was prepared by diluting 3% agarose gel in fresh complete media, with or without doxycycline, at a dilution factor of 1:10. The freshly constituted molten 0.3% agarose gel was quickly added onto the bottom agarose layer at a volume of 1mL per well. The culture plates were then incubated at 4°C for 15 minutes before warming them in the incubator.

Next, single cell suspensions were prepared from each specimen, which includes HME1-ACLY (+Dox), HME1-ACLY (-Dox), HME1-Empty (+Dox), and MCF-7 cells. This involves trypsinizing the cells and diluting them in fresh complete media, with or without doxycycline, at a concentration of 2×10^4 cells/mL. On

the other hand, an appropriate amount of 0.6% agarose gel was prepared by diluting 3% agarose gel (melted and pre-warmed at 45°C) in fresh complete media (pre-warmed at 37°C) at a dilution factor of 1:5. The cell suspensions were then mixed with molten 0.6% agarose gels at a 1:1 ratio. Immediately, the cell-containing 0.3% agarose gels were plated in triplicates onto pre-layered culture plates at volumes of 1mL per well. Subsequently, they were incubated at 4°C for 15 minutes for the gels to solidify before placing them in a humidified incubator at 37°C with 5% CO₂. On a weekly basis, feeder layers were prepared and added over the existing layer to replenish cells with fresh media.

After 4 weeks of incubation, 350 µL of nitroblue tetrazolium chloride (NBT) solution (1 mg/mL NBT in 1x PBS) was added into each well. The culture plates were then incubated overnight at 37°C to stain viable cells. In actively respiring cells, soluble NBT is reduced by naturally occurring superoxides into blue precipitates (Davis Volk and Moreland, 2014). After the colonies were stained, they were imaged under brightfield microscopy. Multiple images were taken and superimposed to image whole wells using the AxioVision software, where all the colonies in each well were subsequently analysed using the Fiji software.

2.13. Biosafety, chemical safety and workplace safety

2.13.1. Safe handling of biological agents

In general, proper personal protective equipment (PPE), which includes lab coat and nitrile gloves, was used during the handling of all biological agents. The safety data sheets of all mammalian cells required for this research project were examined. U2-OS osteosarcoma and MCF-7 breast cancer cell lines were found

to be biosafety level 1 (BSL1) biological agents. On the other hand, hTERT-HME1 breast epithelial cells, hTERT-RPE1 retinal epithelial cells, 293FT human embryonic kidney cells, and 3rd generation lentiviruses were found to be biological safety level 2 (BSL2) biological agents. As such, all the biological agents were managed in a BSL2 laboratory, where live cells and lentiviruses were handled in a class II biological safety cabinet (BSC). Regarding the competent bacteria cells used in this project, namely JM109 and DH5 α , they were also managed in the same BSL2 facility. All used and unused biological agents were treated with bleach for up to 24hrs before being discarded in biological waste carboys or bio-hazardous bags.

The laboratory standard operating procedures classify biological spills into three categories, namely (1) spillages occurring within BSC, (2) spillages occurring outside of BSC, and (3) spillages resulting in accidental personnel exposure. Procedures to manage each spill were understood and adhered to with the appropriate PPE worn. For spillages within the BSC, the blower should remain functional with the sash lowered. Spilled biological agents, including any vessels and broken contaminated sharps, should be covered with paper towels. Once the spillage is contained without any immediate threat of spreading within the BSC, pour the disinfectant (either 70% ethanol or bleach) on the paper towels starting from the outer edges of the spill towards the inside, and allow the disinfection to occur for 30min. After which, all paper towels are to be discarded into a bio-hazardous bag, and any broken sharps discarded into the bio-hazardous sharps bin. Broken sharps should always be handled with forceps or a brush and dustpan. The spillage area is then cleaned with 70% ethanol to remove any residual

disinfectant. Next, for spillages occurring outside of BSC, the spill should be contained immediately by placing absorbent material (e.g. paper towels) at the outer edges of the spill. Then, pour disinfectant starting from the outside of the absorbent material towards the inside of the spill. Similarly, allow for disinfection to occur for 30min before discarding the absorbent material and any contaminated objects into a bio-hazardous bag. Any broken sharps involved in the spill should be picked up with forceps or a brush and dust-pan, and later discarded into a bio-hazardous sharps bin. The spillage area is then cleaned with bleach to eliminate any possible residual biological agents. Lastly, for a biological spill resulting in accidental personnel exposure, which includes direct contact of biological agents with compromised (cuts or open wound) or intact skin, and with mucous membranes of the mouth, eye and nose, the personnel should immediately evacuate to the closest sink, eyewash, or safety shower to flush out the biological agents. If the PPE has been impacted, it should be removed immediately. Any impacted skin should be washed thoroughly with 4% chlorohexidine and water.

2.13.2. Safe handling of hazardous and flammable chemicals

Hazardous chemicals that were handled in this project included concentrated 37% hydrochloric acid (Merck, 100317), 100% trichloroacetic acid (Sigma, T6399), glacial acetic acid (Merck, 100063), sulphuric acid (Merck, 112080), paraformaldehyde (Alfa Aesar, A11313), sodium hydroxide (Alfa Aesar, B24414), sodium azide (Sigma, S8032) and boric acid (Merck, A577065442). On the other hand, flammable chemicals used included ethanol (Merck, 100983), methanol (Merck, 106009), dimethylformamide (Fluka, 40255) and acetone

(Merck, 100014). The appropriate PPE, which includes lab coat, nitrile gloves and safety goggles, were worn during the handling of these hazardous and flammable chemicals. These chemicals were handled in a fume hood that operated at a face velocity of 100 fpm (feet per minute) to minimise the spread of any toxic fumes generated. To maximise protection, the sash was lowered and chemicals were handled at least 15cm into the hood from the plane of the sash.

An operating procedure was understood and adhered to in response to a chemical spill. In the event of a chemical spill, all personnel should be evacuated from the laboratory immediately, and the fume hood promptly activated to exhaust any toxic fumes generated. Before managing the spill, the appropriate PPE should be worn, which includes lab coat, chemical resistant boots, chemical resistant gloves, safety goggles, N95 or respirator (for toxic fumes). First, contain the spill by placing absorbent material (e.g. paper towels) around the edges of the spill. Based on the safety data sheet of the chemical, follow the appropriate spill management procedures to neutralise the hazardous chemical. Once the spill is no longer a threat, absorbent material should be used to absorb the spillage before being discarded it into a chemical waste bag. The waste bag should be labelled on the type of chemical involved in the spillage.

2.13.3. Workplace safety

Prolonged usage of the microscope could lead to eye fatigue and dry eye syndrome (Lin *et al.*, 2019). Furthermore, one case reported that a patient developed retinopathy as a result of constant exposure to fluorescent light over

the microscope (Quarato *et al.*, 2017). To limit exposure to fluorescent light, breaks were scheduled at every 4hrs.

Long working hours are prevalent in most research environments. In addition, extensive studies have shown that this negatively impacts the occupational health of workers, increasing their risk of developing cardiovascular diseases, chronic fatigue, and diverse mental health issues (Wong *et al.*, 2019). Hence, it seems imperative to reduce the frequency of working overtime. This was done by scheduling two 15-30min breaks a day, where up to 10-14hrs were spent performing laboratory work.

3. RESULTS

3.1. Nuclear abnormalities in a form of micronuclei structures were observed in cells that were ectopically expressing ACLY

hTERT-HME1 (HME1) and hTERT-RPE1 (RPE1) cells were transfected with pXJ40-FLAG-ACLY to induce ectopic expression of ACLY. To observe off-targets of transfection, these cells were transfected with an empty vector pXJ40-FLAG.

To determine transfection efficiency, three biological repeats of immunostaining were performed (n=3), where approximately 500 cells were analysed from each specimen. It was determined that the transfection efficiency of the pXJ40-FLAG-ACLY vector was 33.57% and 35.05% in HME1 and RPE1 cells respectively (Fig. 14).

Figure 14. Transfection efficiency of pXJ40-FLAG-ACLY vector. HME1 and RPE1 cells were transfected with pXJ40-FLAG-ACLY. Transfection efficiency in HME1 and RPE1 cells was found to be 33.57% and 35.04% respectively.

On the other hand, three biological repeats of western blots were performed to analyse levels of phosphorylated ACLY (Ser455) and total ACLY levels (n=3). Transfection with the pXJ40-FLAG-ACLY vector led to a 16-fold and 11-fold increase in total ACLY levels in HME1 and RPE1 cells respectively (Fig. 15A-B). It also led to 6-fold and 7-fold increase in phosphorylated ACLY in HME1 and RPE1 cells respectively (Fig. 15A-B). These changes were significant with *p values* < 0.05 according to two-tailed student *t*-tests (Fig. 15B).

Figure 15. Ectopic expression of FLAG-ACLY after transfection with pXJ40-FLAG-ACLY. (A and B) Transfection with pXJ40-FLAG-ACLY induced ectopic expression of FLAG-tagged ACLY in HME1 and RPE1 cells, achieving a 16- and 11-fold increase in total ACLY respectively compared to control cells. This resulted in a 6- and 7-fold increase in phosphorylated ACLY in HME1 and RPE1 cells respectively. Normalised signal intensities are represented in arbitrary units (A.U.).

Next, to observe the presence of nuclear abnormalities in response to ACLY overexpression, three biological repeats of immunostaining were performed (n=3), where approximately 500 cells were analysed from each specimen. Although micronuclei structures were found in both cell lines transfected with the empty vector, there was a statistically significant increase in such structures when ACLY was overexpressed (Fig. 16A). For HME1 cells, there was a 19.52% increase in cells harbouring micronuclei structures, with *p value* = 0.0022 (Fig. 16B). Whereas for RPE1 cells, a 32.09% increase in cells with such structures were found, with *p value* = 1.81×10^{-4} (Fig. 16B). The *p values* were calculated from two-tailed student *t*-tests.

Figure 16A. Micronuclei structures observed in HME1 and RPE1 cells upon ectopic expression of ACLY. The presence of micronuclei structures (marked “▶”) were examined in DAPI-stained cells transfected with pXJ40-FLAG and pXJ40-FLAG-ACLY (Scale bar = 10 μ m). Specimens were prepared in three biological repeats (n=3), where 500 cells were analysed each.

Figure 16B. Both HME1 and RPE1 cells displayed a statistically significant increase in micronuclei structures upon ectopic ACLY overexpression. Transfection with pXJ40-FLAG empty vector in both HME1 and RPE1 cells resulted in the formation of background micronuclei structures. However, pXJ40-FLAG-ACLY transfection in HME1 and RPE1 cells generated more cells harbouring micronuclei structures, as shown by *p* values of < 0.01 based on two-tailed student *t*-tests. (Statistical significance is represented by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$)

3.2. HME1-ACLY stable cells were verified to overexpress ACLY upon doxycycline induction

HME1-ACLY inducible cell line was generated to stably overexpress ACLY for extended periods of time in the presence of doxycycline. To detect off-target effects of doxycycline treatment on cellular proteins, HME1-Empty cells were generated as a control cell line. HME1-Empty cells were also used with the consideration that doxycycline-rtTA interactions could lead to off-target gene expression. A third generation lentiviral system was utilised to transduce the lentiviral vector pSLIK-Neo-TRE-FLAG-ACLY into HME1 cells, thereby producing HME1-ACLY cell line. The same system was also used to transduce the empty lentiviral vector pSLIK-Neo into HME1 cells, producing HME1-Empty cell line.

The pSLIK-Neo vector contains the gene that encodes for rtTA proteins. These rtTA proteins interact with doxycycline to enable its binding at the TRE promoter, initiating gene expression of ACLY. Western blot analysis confirmed that both stable cell lines were expressing similar amounts of rtTA proteins relative to HME1 cells (Fig. 17).

Figure 17. HME1-ACLY and HME1-Empty cells both expressed reverse tetracycline-controlled transactivator (rtTA). Stable cell lines were established through lentiviral transduction using a third generation lentiviral vector pSLIK-Neo. The FLAG-tagged ACLY cDNA was inserted into the pSLIK-Neo vector and subsequently introduced into HME1 cells, yielding an inducible stable cell line (HME1-ACLY) capable of expressing FLAG-tagged ACLY upon doxycycline treatment. A control cell line was also generated by transducing the empty pSLIK-Neo vector into HME1 cells (HME1-Empty). The pSLIK-Neo vector expresses rtTA that interacts with doxycycline to initiate gene expression (Tet-On system). Both HME1-ACLY and HME1-Empty cell lines express similar levels of rtTA. Normalised signal intensities relative to HME1 are represented in arbitrary units (A.U.).

Next, HME1-ACLY cells were treated with a low dose of 40 ng/mL doxycycline (+Dox) to induce overexpression of FLAG-tagged ACLY over time periods of 24hrs, 48hrs, and 72hrs. As a negative control, HME1-ACLY cells were allowed to incubate without doxycycline (-Dox). Western blot was performed over three biological repeats (n=3) to examine levels of phosphorylated ACLY (Ser455) and total ACLY. HME1-ACLY (-Dox) cells displayed little changes in phosphorylated ACLY and total ACLY at all time-points compared to 0hr time-point (Fig. 18A-B). Doxycycline treatment (+Dox) led to a significant increase in levels of phosphorylated ACLY and total ACLY at all time-points compared to HME1-ACLY (-Dox) cells. Levels of phosphorylated ACLY increased by folds of 2.13 at 24hr time-point, 3.60 at 48hr time-point, and 5.26 at 72hr time-point, displaying *p values* < 0.01 according to two-tailed student *t*-tests (Fig. 18A-B). On the other hand, levels of total ACLY increased by folds of 4.76 at 24hr time-point, 7.55 at 48hr time-point, and 7.76 at 72hr time-point, displaying *p values* < 0.05 according to two-tailed student *t*-tests (Fig. 18A-B). In addition, it was noted that levels of ACLY overexpression stabilised at 48hr and 72hrs in HME1-ACLY (+Dox) cells (Fig. 18A-B).

A)

B)

Figure 18. HME1-ACLY cell line was able to respond to low doses of doxycycline as shown by the expression FLAG-tagged ACLY. (A) Doxycycline treatment at 40 ng/mL was performed at 24hr, 48hr, and 72hr time-points. **(B)** In all three time-points, there was a significant increase in both phosphorylated ACLY (Ser455) and total ACLY levels in doxycycline-treated HME1-ACLY cells. (Statistical significance is represented by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$)

3.3. Nuclear abnormalities associated with genome instability were caused by DNA damage

3.3.1. Increased population of cells with micronuclei and nuclear budding structures were observed

HME1-ACLY and HME1-Empty cells were treated with doxycycline (+Dox) for 72hrs to determine the possible impact of extended ACLY activity on genome stability. As a positive control, the cells were exposed to VP16 to induce DNA damage. HME1-ACLY (-Dox), HME1-ACLY (+Dox), and HME1-Empty (+Dox) cells were immunostained to detect the presence of micronuclei (MN) and nuclear budding structures (NBUD). Specimens were prepared in six biological repeats (n=6), where approximately 500 cells were sampled each. Two-tailed student *t*-tests were performed to determine significant changes in the percentage of cells harbouring MN and NBUD structures between specimens.

VP16 treatment was able to successfully induce the formation MN and NBUD structures, accounting for 27.57% and 13.33% respectively of the cells sampled. These changes were significant compared to HME1-ACLY (-Dox) and HME1-Empty (+Dox) cells, generating *p values* < 0.001 (Fig. 19A-B). ACLY overexpression in HME1-ACLY (+Dox) cells resulted in an increased population of cells harbouring MN, accounting for 15.06% of the cells sampled. This increase occurred by a factor of 3.08 and 2.55 when compared to HME1-ACLY (-Dox) and HME1-Empty (+Dox) specimens respectively, displaying *p values* of < 0.001 (Fig. 19A-B). Also, an increased population of HME1-ACLY (+Dox) cells harbouring NBUD structures was observed, accounting for 9.82% of the cells sampled. This increased occurred by a factor of 5.01 and 3.32 when compared to HME1-ACLY (-Dox) and HME1-Empty (+Dox) specimens

respectively, displaying *p* values of < 0.001 (Fig. 19A-B). Although there was a percentage increase of HME1-ACLY (+Dox) cells with MN and NBUD structures, it did not reach levels similar to VP16-treated cells.

Figure 19A. Increased presence of centromere-negative micronuclei and nuclear budding structures were detected in ACLY-overexpressing cells. HME1-ACLY and HME1-Empty cells were treated with doxycycline (+Dox) for 72hrs. For positive control, HME1-ACLY cells were treated with VP16 to induce DNA double stranded breaks. A total of six biological repeats were prepared ($n=6$), where ~ 500 cells per specimen were analysed in each repeat. MN structures are marked in red boxes, where they were further examined if they were CENPA negative (-ve) or positive (+ve). NBUD cells are marked “►” (Scale bar = 10 μ m).

Two-tailed Student <i>t</i> -test (<i>p</i> values)				
		VP16	HME1- ACLY (-Dox)	HME1- ACLY (+Dox)
MN	HME1- ACLY (-Dox)	4.61E-08	-	-
	HME1- ACLY (+Dox)	7.81E-07	4.52E-07	-
	HME1- Empty (+Dox)	2.00E-08	1.28E-01	3.71E-07
NBUD	HME1- ACLY (-Dox)	3.10E-05	-	-
	HME1- ACLY (+Dox)	2.53E-02	1.65E-04	-
	HME1- Empty (+Dox)	4.88E-05	1.79E-02	3.22E-04

Figure 19B. ACLY-overexpressing cells displayed a statistically significant increase in micronuclei and nuclear budding structures. HME1-ACLY (+Dox) cells displayed a significant increase in both MN and NBUD structures compared to HME1-ACLY (-Dox) and HME1-Empty (+Dox) cells. This was confirmed with two-tailed student *t*-tests where *p* values were < 0.001. However, the increase of these abnormalities did not reach levels similar to VP16-treated cells, where *p* values were < 0.05. (Statistical significance is represented by **p* < 0.05, ***p* < 0.01, and ****p* < 0.001)

MN structures were further stained for the presence of centromere marker, CENPA, to determine if they were derived from lagging chromosomes (CENPA +ve), acentric chromosomes (CENPA -ve), or fragmented DNA (CENPA -ve) (Fig. 19A). It was determined that majority of the micronuclei structures in HME1-ACLY (-Dox), HME1-ACLY (+Dox), and HME1-Empty (+Dox) cells were CENPA (-ve), displaying percentages of 81.07%, 76.30%, and 79.20% respectively (Fig. 19C). Between these three specimens, no statistically significant changes were found. Although VP16-treated cells displayed a significant increase in MN structures with no CENPA staining compared to the other specimens, it demonstrates that DNA damage can cause DNA fragmentation and thereby MN formation.

Figure 19C. Micronuclei structures in ACLY-overexpressing cells were mostly centromere-negative. Percentages of CENPA positive (+ve) and negative (-ve) micronuclei structures were analysed in all specimens. Micronuclei structures in HME1-ACLY (-Dox), HME1-ACLY (+Dox) and HME1-Empty (+Dox) were mostly CENPA (-ve). VP16-treated cells displayed a significantly higher percentage of CENPA (-ve) micronuclei structures. (Statistical significance is represented by * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$)

3.3.2. The CBMN assay revealed an increased population of binuclear cells harbouring micronuclei structures, nuclear budding structures, and nucleoplasmic bridges

Next, the cytokinesis block micronucleus cytome (CBMN) assay was performed to observe MN, NBUD, and nucleoplasmic bridges (NPB) in once-divided binuclear cells. This assay is mostly used in genotoxicity studies to measure DNA damage (Fenech *et al.*, 2011). HME1-ACLY and HME1-Empty cells were treated with doxycycline (+Dox) for 72hrs before treatment with cytochalasin B (CytB) for a further 16hrs to induce the formation of binuclear cells. As a positive control, the cells were exposed to VP16 to induce DNA damage. Specimens were prepared in six biological repeats (n=6), where approximately 200 cells were sampled each. Two-tailed student *t*-tests were performed to determine any significant changes in the percentage of cells harbouring these nuclear abnormalities.

VP16 treatment was able to induce the formation of MN, NBUD, and NPB structures, accounting for 37.18%, 24.08% and 7.38% respectively of the binuclear cells sampled. These changes were significant compared to HME1-ACLY (-Dox) and HME1-Empty (+Dox) specimens, with *p values* < 0.001 (Fig. 20A-B). ACLY overexpression led to an increased percentage of binuclear cells carrying MN structures, accounting for 20.66% of binuclear cells sampled. This increase was significant compared to HME1-ACLY (-Dox) and HME1-Empty (+Dox) specimens, with *p values* < 0.001 (Fig. 20A-B). However, the percentage of HME1-ACLY (+Dox) binuclear cells with MN structures was comparatively lower than VP16-treated cells. Next, there was an increased population of HME1-ACLY (+Dox) binuclear cells carrying NBUD structures, accounting for

24.95% of binuclear cells sampled. This increase was significant when compared to HME1-ACLY (-Dox) and HME1-Empty (+Dox) specimens, displaying *p values* < 0.001 (Fig. 20A-B). Interestingly, the percentage of HME1-ACLY (+Dox) binuclear cells carrying NBUD structures was statistically similar to VP16-treated specimens, where *p value* was found to be > 0.05 (Fig. 20B). Lastly, HME1-ACLY (+Dox) specimens displayed more NPB-harboured binuclear cells than those in HME1-ACLY (-Dox) and HME1-Empty (+Dox) specimens, with *p values* < 0.01 (Fig. 20A-B). However, this increase was comparatively lower than VP16-treated cells.

Figure 20A. The cytokinesis block micronucleus cytochrome assay revealed an increased presence of centromere-negative micronuclei structures, nuclear budding structures, and nucleoplasmic bridges in ACLY-overexpressing cells. HME1-ACLY and HME1-Empty cells were treated with doxycycline for 72hrs. For positive control, HME1-ACLY cells were treated with VP16. Binuclear cells were generated by treating all specimens with Cytochalasin B before fixation and staining. Six biological repeats (n=6) were prepared, and ~200 binuclear cells per specimen were analysed in each repeat. MN structures are marked in red boxes, where they were further examined if they were CENPA positive or negative. NBUD structures are marked “▶”, while NPB structures are marked in yellow arrow “→” (Scale bar = 5 μ m).

Figure 20B. ACLY-overexpressing binuclear cells displayed a statistically significant increase in micronuclei structures, nuclear budding structures, and nucleoplasmic bridges. HME1-ACLY (+Dox) cells displayed about a twofold increase in both MN and NBUD structures compared to HME1-ACLY (-Dox) and HME1-Empty (+Dox) cells, showing p values < 0.001 based on two-tailed student t -tests. On the other hand, HME1-ACLY (+Dox) specimens have more binuclear cells with NPB than HME1-ACLY (-Dox) and HME1-Empty (+Dox) specimens, where p values were < 0.01 . In addition, the percentage frequency of NBUD structures in HME1-ACLY (+Dox) cells were statistically similar to VP16-treated cells, where p value was 0.697. (Statistical significance is represented by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$)

Next, the MN structures were stained for CENPA. Similarly, majority of the micronuclei structures in HME1-ACLY (-Dox), HME1-ACLY (+Dox), and HME1-Empty (+Dox) binuclear cells were CENPA (-ve), displaying percentages

of 87.98%, 85.59%, and 86.17% respectively (Fig. 20C). Between these three specimens, no statistically significant changes were found.

Figure 20C. Micronuclei structures in ACLY-overexpressing binuclear cells were mostly centromere-negative. There were no significant changes in the percentage frequency of CENPA (+ve) and (-ve) micronuclei structures between HME1-ACLY (-Dox), HME1-ACLY (+Dox), and HME1-Empty (+Dox). Although VP16-treated cells displayed a statistically significant increase in CENPA (-ve) micronuclei structures, majority of the micronuclei structures in all four specimens were CENPA (-ve). (Statistical significance is represented by * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$)

3.3.3. Summary of observations

Taken together, ACLY overexpression was found to promote the formation of MN structures, NBUD structures, and NPBs. Majority of the MN structures formed were CENPA (-ve), indicating that they were derived from either acentric chromosomes or DNA fragments. Also, NPBs in ACLY-overexpressing cells were likely dicentric chromosomes, which are formed by the misrepair of DNA damage. Hence, the increased presence of these abnormal nuclear structures were due to elevated levels DNA damage, which was demonstrated in VP16-treated cells. Nevertheless, ACLY overexpression resulted in an unstable genome.

3.4. Chromatin bridges observed in ACLY-overexpressing cells indicate the increased presence of DNA damage

Three different chromosomal abnormalities were examined in ACLY overexpressing cells, they were namely small extrachromosomes (SES), chromatin bridges (CB) and lagging chromosomes (LC). HME1-ACLY and HME1-Empty cells were treated with doxycycline for 72hrs. HME1-ACLY (-Dox), HME1-ACLY (+Dox), and HME1-Empty (+Dox) cells were then immunostained to observe the presence of these abnormal structures in mitotic cells, particularly anaphase and telophase cells. Specimens were prepared in six biological repeats (n=6), where a mixture of approximately 50-100 anaphase and telophase cells were sampled each (Fig. 21A). Two-tailed student *t*-tests were performed to detect any significant presence of these abnormal structures.

Figure 21A. Increased presence of chromatin bridges were found in ACLY-overexpressing mitotic cells. HME1-ACLY and HME1-Empty cells were treated with doxycycline for 72hrs. These cells were then subjected to fixation and immunostaining. Specimens were prepared over six biological repeats (n=6), where a mixture of ~50-100 anaphase and telophase cells per specimen were sampled. The presence of small extrachromosomes, chromatin bridges (marked “►”) and lagging chromosomes were analysed in all specimens (Scale bar = 5 μ m).

Although HME1-ACLY (+Dox) specimens showed an increased percentage of mitotic cells having SES compared to HME1-ACLY (-Dox) specimens, it was not the case when compared to HME1-Empty (+Dox) specimens (Fig. 21B), where p value = 0.06 was reported. Furthermore, the percentage frequency of mitotic cells with SES in all three specimens were considerably low. Hence, it can be concluded that there was little or no change in the formation of SES structures in HME1-ACLY (+Dox) specimens. Next, an increased percentage of anaphase and telophase cells bearing chromatin bridges was observed in HME1-ACLY (+Dox) specimens (Fig. 21A). This increase was significant compared to HME1-ACLY (-Dox) and HME1-Empty (+Dox) cells, showing p values < 0.05 (Fig. 21B). Lastly, HME1-ACLY (+Dox) specimens did not reveal any significant increase in mitotic cells harbouring lagging chromosomes, showing p values > 0.05 (Fig. 21B).

Hence, ACLY overexpression led to an increase in mitotic cells bearing chromatin bridges, which is consistent with data showing an increased percentage frequency of cells harbouring nucleoplasmic bridges (Fig. 20B). This affirms the previous conclusion that there was an increased presence of DNA damage in ACLY-overexpressing cells. Moreover, DNA misrepair could have led to the formation of these chromatin bridges and nucleoplasmic bridges.

		Small Extrachromosomes			Chromatin Bridges			Lagging Chromosomes		
		HME1-ACLY (-Dox)	HME1-ACLY (+Dox)	HME1-Empty (+Dox)	HME1-ACLY (-Dox)	HME1-ACLY (+Dox)	HME1-Empty (+Dox)	HME1-ACLY (-Dox)	HME1-ACLY (+Dox)	HME1-Empty (+Dox)
Two-tailed Student t-test (p values)	HME1-ACLY (-Dox)	-	3.77E-3	7.67E-1	-	1.60E-2	8.77E-1	-	3.02E-1	5.90E-1
	HME1-ACLY (+Dox)	-	-	6.65E-2	-	-	4.84E-2	-	-	9.29E-1

Figure 21B. The increased percentage of ACLY-overexpressing mitotic cells bearing chromatin bridges were statistically significant. HME1-ACLY (+Dox) specimens showed an increase in anaphase and telophase cells with chromatin bridges compared to HME1-ACLY (-Dox) and HME1-Empty (+Dox) specimens. This was confirmed by two-tailed student *t*-tests showing *p* values of < 0.05. Although HME1-ACLY (+Dox) specimens showed an increase in small extrachromosomes compared to HME1-ACLY (-Dox) specimens, it was not as dramatic when compared with HME1-Empty (+Dox) specimens where *p* > 0.05. Lastly, HME1-ACLY (+Dox) specimens did not show a significant increase in anaphase and telophase cells bearing lagging chromosomes. (Statistical significance is represented by **p* < 0.05, ***p* < 0.01, and ****p* < 0.001)

3.5. Centrosome amplifications detected in ACLY-overexpressing cells were caused by elevated levels of DNA damage, and did not result in chromosomal mis-segregations

Chromosomal mis-segregation could contribute to the formation of micronuclei structures as observed in the previous data. To investigate this, HME1-ACLY and HME1-Empty cells were incubated with doxycycline for 72hrs. HME1-ACLY (-Dox), HME1-ACLY (+Dox), and HME1-Empty (+Dox) cells were subsequently stained against pericentrin, a centrosome marker, and α -tubulin. Specimens were prepared in six biological repeats (n=6), where approximately 100 mitotic cells were analysed each to detect for the presence of amplified centrosomes. Two-tailed student *t*-tests were performed to determine significant changes between the three specimens. An increased percentage of mitotic cells with amplified centrosomes (> 2 centrosomes) were found in HME1-ACLY (+Dox) specimens, accounting for 27.37% of mitotic cells sampled. This increase was significant compared to HME1-ACLY (-Dox) and HME1-Empty (+Dox) specimens, displaying *p values* < 0.001 (Fig. 22).

Figure 22. Increased presence of centrosome amplifications was found in ACLY-overexpressing mitotic cells. HME1-ACLY and HME1-Empty cells were treated with doxycycline for 72hrs. HME1-ACLY (-Dox), HME1-ACLY (+Dox) and HME1-Empty (+Dox) mitotic cells were then examined for the presence amplified centrosomes, which is associated with the formation of multi-polar spindles. Cells were fixed and stained for α -tubulin and pericentrin, a centrosome marker (Scale bar = 5 μ m). A total of six biological repeats were prepared (n=6), where ~100 mitotic cells per specimen were sampled for analysis. Cells displaying more than two pericentrin signals (marked “►”) were deemed to have amplified centrosomes. There was a statistically significant increase in the population of HME1-ACLY (+Dox) mitotic cells displaying amplified centrosomes when compared to HME1-ACLY (-Dox) and HME1-Empty (+Dox) mitotic cells, showing *p* values of < 0.001 according to two-tailed student *t*-tests. (Statistical significance is represented by **p* < 0.05, ***p* < 0.01, and ****p* < 0.001)

Since ACLY overexpression was reported to induce centrosome amplification, further works were done to determine if it contributes to aneuploidy. In a similar way, HME1-ACLY and HME1-Empty cells were incubated with doxycycline for 72hrs. After which, HME1-ACLY (-Dox), HME1-ACLY (+Dox), and HME1-Empty (+Dox) cells were harvested and stained with propidium iodide (PI) and analysed through flow cytometry to detect the presence of sub-G₁ (DNA content < 2N) and polyploid cells (DNA content > 4N). Three biological repeats were performed (n=3), where in each specimen, 10,000 cells were randomly sampled. There, the percentage frequency of sub-G₁ and polyploid cells between the three specimens were subjected to two-tailed student *t*-tests. HME1-ACLY (+Dox) specimens revealed a low percentage of sub-G₁ and polyploid cells, accounting for 0.28% and 0.89% of the cells sampled respectively. These figures were similar to both HME1-ACLY (-Dox) and HME1-Empty (+Dox) cells, showing *p values* > 0.05. The data indicates that ACLY overexpression did not promote the formation of aneuploid cells (Fig. 23).

		Average % cells	Std. Dev. ±	Two-tailed Student <i>t</i> - test (<i>p</i> values)	
				HME1- ACLY (-Dox)	HME1- ACLY (+Dox)
Sub-G1 Cells	HME1-ACLY (-Dox)	0.33%	0.02%	-	0.39
	HME1-ACLY (+Dox)	0.28%	0.08%	-	-
	HME1-Empty (+Dox)	0.33%	0.10%	0.98	0.52
Polyploid Cells	HME1-ACLY (-Dox)	1.19%	0.56%	-	0.51
	HME1-ACLY (+Dox)	0.89%	0.50%	-	-
	HME1-Empty (+Dox)	0.35%	0.17%	0.13	0.22

Figure 23. Percentage of sub-G₁ and polyploid cells remained low and unaltered in ACLY-overexpressing specimens. HME1-ACLY and HME1-

Empty cells were treated with doxycycline for 72hrs. Since amplified centrosomes are known to cause chromosomal mis-segregations, HME1-ACLY (-Dox), HME1-ACLY (+Dox), and HME1-Empty (+Dox) cells were subjected to flow cytometry analysis to detect the presence of aneuploidy, which includes sub-G₁ and polyploid cells. Sub-G₁ cells typically have DNA content < 2N, while polyploid cells have DNA content ≥ 4N. Three biological repeats were performed (n=3), each consisting of three replicates. HME1-ACLY (+Dox) cells did not show a statistical increase in sub-G₁ and polyploid cells when compared to HME1-ACLY (-Dox) and HME1-Empty (+Dox) cells, showing *p* values > 0.05. (Statistical significance is represented by **p* < 0.05, ***p* < 0.01, and ****p* < 0.001)

The aforementioned experiment was insufficient to detect tetraploid cells (DNA content = 4N), which are considered as polyploid cells. To do so, HME1-ACLY (-Dox), HME1-ACLY (+Dox), and HME1-Empty (+Dox) cells were further stained with cyclin B1 to distinguish G₂ and mitotic cells from tetraploid cells. G₂ phase and mitotic cells typically have elevated levels of cyclin B1, while tetraploid cells have low cyclin B1 levels similar to G₀ and G₁ phase cells. Three biological repeats were performed (n=3), each in triplicates where 10,000 cells were randomly sampled per replicate through flow cytometry. The percentage frequencies of these cells in the three specimens were subjected to two-tailed student *t*-tests. There was no discernable increase in polyploid cells detected in HME1-ACLY (+Dox) specimens when compared to HME1-ACLY (-Dox) and HME1-Empty (+Dox) specimens, where *p values* were > 0.05 (Fig 24). Similarly, levels of polyploid cells remained low at 0.97% in HME1-ACLY (+Dox) specimens (Fig 24).

Taken together, ACLY overexpression resulted in an increased presence of cells with amplified centrosomes, but they did not lead to the aneuploidy. Studies have determined the role of centrosome amplifications in promoting chromosomal instability, which is a phenomenon seen in tumours (Löffler *et al.*, 2013). However, other studies have shown how centrosome amplification is essential to initiate mitotic catastrophe in cells carrying DNA damage that cannot be repaired, thereby maintaining genome integrity (Löffler *et al.*, 2013). Hence, the presence of more mitotic cells with amplified centrosomes indicates the increased presence of DNA damage. The results also suggest that chromosomal mis-segregations

were not responsible for the formation of nuclear abnormalities as reported (Fig. 19A-C, 20A-C).

	Average % Polyploid cells	Std. Dev. ±	Two-tailed Student <i>t</i> -test (<i>p</i> values)	
			HME1-ACLY (-Dox)	HME1-ACLY (+Dox)
HME1-ACLY (-Dox)	1.33%	0.51%	-	0.42
HME1-ACLY (+Dox)	0.97%	0.46%	-	-
HME1-Empty (+Dox)	0.56%	0.09%	0.06	0.26

Figure 24. No distinct increase in polyploid cells, including tetraploid cells, were found in ACLY-overexpressing specimens. HME1-ACLY and HME1-Empty cells were treated with doxycycline for 72hrs. Polyploidy also includes tetraploid cells that have 4N DNA content similar to G₂ and mitotic cells, but low cyclin B1 levels seen in G₀ and G₁ cells. To detect tetraploid cells, HME1-ACLY (-Dox), HME1-ACLY (+Dox), and HME1-Empty (+Dox) were further stained against cyclin B1. Three biological repeats were performed (n=3), each consisting of three replicates. No detectable increase in polyploid cells, including tetraploid cells, were found in HME1-ACLY (+Dox) cells. This was confirmed with two-tailed student *t*-tests, showing *p* values of > 0.05. (Statistical significance is represented by **p* < 0.05, ***p* < 0.01, and ****p* < 0.001)

3.6. Elevated levels of DNA double stranded breaks were present in ACLY-overexpressing cells

The presence of DNA damage was determined in ACLY-overexpressing cells. HME1-ACLY and HME1-Empty cells were treated with doxycycline for 48hrs, 72hrs, and 5 weeks. There, HME1-ACLY (+Dox), and HME1-Empty (+Dox) specimens were immunostained against a known DNA damage marker, namely phosphorylated H2AX Ser139 (γ H2AX). As a positive control, the cells were exposed to VP16 to induce DNA damage. Specimens were prepared in three biological repeats ($n=3$), where approximately 200 cells were analysed in each specimen. There, the number of γ H2AX foci were counted in each nucleus. Two-tailed z -tests were then performed to determine any significant differences in γ H2AX foci formation between specimens. At 48hr time-point, HME1-ACLY (+Dox) cells showed a mean γ H2AX foci count of 17.67 per nucleus, which appeared to be more than HME1-Empty (+Dox) cells that showed a mean γ H2AX foci count of 12.46 per nucleus (Fig. 25). The observed elevated levels of γ H2AX foci in HME1-ACLY (+Dox) cells was significant, with p value < 0.001 . At 72hr time-point, HME1-ACLY (+Dox) cells also appeared to have a higher mean γ H2AX foci count at 10.45 per nucleus, compared to HME1-Empty (+Dox) that displayed a mean γ H2AX foci count of 7.40 per nucleus (Fig. 25). This difference was statistically significant, showing p value < 0.01 . After prolonged overexpression of ACLY for 5 weeks, HME1-ACLY (+Dox) cells still remained to display higher mean γ H2AX foci count of 5.55 per nucleus when compared to HME1-Empty (+Dox) cells that displayed a mean γ H2AX foci count of 0.88 per nucleus (Fig. 25). Hence, ACLY overexpression was found to elevate γ H2AX foci formation, indicating the presence of DNA damage. In addition, the

presence of DNA damage was persistent even after prolonged ACLY overexpression of up to 5 weeks. However, the levels of DNA damage seen at all three time-points were comparatively lower than VP16-treated cells that showed a mean γ H2AX foci count of 59.82 per nucleus (Fig. 25).

Figure 25. ACLY-overexpressing cells exhibited elevated levels of γ H2AX foci formation. HME1-ACLY and HME1-Empty cells were treated with doxycycline for 48hrs, 72hrs, and 5 weeks (5 Wk). As positive control, cells were treated with VP16. Cells were then fixed and stained against phosphorylated H2AX (Ser139) (γ H2AX). Three biological repeats were performed ($n=3$), where ~ 200 cells per specimen were sampled. HME1-ACLY (+Dox) cells were found to have statistically higher mean γ H2AX foci count at 48hr, 72hr, and 5-week time-points when compared to HME1-Empty (+Dox) cells, displaying p values < 0.01 according to two-tailed z -tests. However, γ H2AX foci formation in HME1-ACLY (+Dox) cells were lower compared to VP16-treated cells. (Statistical significance is represented by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$)

To confirm the presence of DNA damage, the neutral comet assay was performed. This assay is sensitive in detecting the presence of DNA double stranded breaks (Olive and Banáth, 2006). HME1-ACLY cells were treated with or without doxycycline for 48hrs, 72hrs, and 5 weeks. As a positive control, the cells were exposed to VP16 to induce DNA damage. A total of approximately 300 cells were analysed from each specimen prepared, where levels of DNA damage were quantified by olive tail moments (Olive and Banáth, 2006). Two-tailed *z*-tests were performed to detect any significant differences in olive tail moments between the comets sampled from HME1-ACLY (-Dox) and HME1-ACLY (+Dox) specimens. In HME1-ACLY (-Dox) specimens, a statistically significant reduction in DNA damage was observed from 48hr to 72hr time-points, where mean olive tail moments were reported to be 2.45 and 1.93 respectively with *p value* < 0.001 (Fig. 26). However, at the 5-week time-point, DNA damage reached at levels similar to those at the 48hr time-point, displaying a mean olive tail moment of 2.18 with *p value* = 0.0861 (Fig. 26). As such, it was assessed that the levels of DNA damage in HME1-ACLY (-Dox) cells remained relatively constant at all three time-points. When ACLY was overexpressed in HME1-ACLY (+Dox) cells for 48hrs, the cells exhibited a mean olive tail moment of 3.60, which was higher compared to HME1-ACLY (-Dox) cells (Fig. 26). This difference was significant, showing *p value* < 0.0001. At 72hrs, HME1-ACLY (+Dox) cells also displayed a higher mean olive tail moment than HME1-ACLY (-Dox) cells with a value of 4.75, showing *p value* < 0.0001 (Fig. 26). Even after prolonged overexpression of ACLY for 5 weeks, HME1-ACLY (+Dox) cells remained to display statistically higher levels of DNA damage compared to HME-ACLY (-Dox) cells, showing a mean olive tail moment of

4.17 with p value < 0.0001 (Fig. 26). Upon examining the HME1-ACLY (+Dox) specimens, it was determined that there was a gradual increase in DNA damage at 48hr and 72hr time-points, showing p values < 0.0001 (Fig. 26). However, DNA damage levels did not increase further at the 5-week time-point, but instead reduced slightly from a mean olive tail moment of 4.75 (72hr time-point) to 4.15, with p value $= 0.0378$ (Fig. 26). This suggests that upon the induction of ACLY overexpression, HME1-ACLY (+Dox) cells experienced an initial acceleration of DNA damage. The rate of DNA damage then reached a plateau and remained sustained over prolonged periods of ACLY overexpression at levels significantly higher than HME-ACLY (-Dox) cells. Nevertheless, ACLY overexpression was concluded to cause DNA damage in cells through raising levels of DNA double stranded breaks. Despite that, the amount of DNA double stranded breaks was still comparatively lower than VP16-treated cells that showed a higher mean olive tail moment at 11.94 (Fig. 26).

Therefore, ACLY overexpression has a role in inducing DNA damage in cells through the infliction of DNA double stranded breaks, as shown by the increase in γ H2AX foci formation and olive tail moments (Fig. 25, 26). These results can account of the reported presence of nuclear and chromosomal abnormalities (Fig. 19A-C, 20A-C), chromatin bridges (Fig. 21A-B), and centrosome amplifications (Fig. 22). However, it should be noted that levels of DNA damage were not as extensive compared to VP16-treated cells.

Figure 26. ACLY-overexpressing cells displayed elevated levels of DNA double stranded breaks. To verify the presence of DNA double stranded breaks in ACLY-overexpressing cells, neutral comet assay was performed on HME1-ACLY cells treated with doxycycline for 48hrs, 72hrs, and 5 weeks (5 Wk). As positive control, HME1-ACLY cells were treated with VP16. In addition, HME1-ACLY cells were left untreated for 48hrs, 72hrs, and 5 weeks (5 Wk) to serve as negative controls. On each specimen, ~300 cells were analysed, where olive tail moments were calculated to quantify levels of DNA double stranded breaks. Levels of DNA double stranded breaks remained constant in HME1-ACLY (-Dox) cells throughout all three time-points. In contrast, HME1-ACLY (+Dox) cells displayed higher levels of DNA double stranded breaks compared to HME1-ACLY (-Dox) cells at all three time-points. In addition, levels of DNA damage in HME1-ACLY (+Dox) cells increased initially at 48hrs and 72hr time-points, which then maintained up to the 5-week time-point. However, damage levels experienced in these cells were not as extensive compared to VP16-treated cells. Olive tail moments are represented in arbitrary units (A.U.). (Statistical significance is represented by **p* < 0.05, ***p* < 0.01, and ****p* < 0.001)

3.7. DNA double stranded breaks in ACLY-overexpressing cells were not caused by oxidative stress

ACLY may accelerate metabolic processes in the cell that could drive the increased formation of metabolic by-products, one of which is reactive oxygen species (ROS) that has been known to induce DNA damage. To determine changes in ROS levels in cells after ACLY overexpression, a commercially available CellROX™ reagent (Invitrogen) was used that has the ability to exhibit an excitation and emission spectra when it is oxidised by ROS. HME1-ACLY and HME1-Empty cells were treated with doxycycline for 48hrs and 72hrs before the CellROX™ reagent was added. As a positive control, cells were treated with tert-Butyl hydroperoxide (tBHP) to induce oxidative stress.

At both 48hr and 72hr time-points, there was no discernable increase in fluorescent signals in HME1-ACLY (+Dox) specimens when compared to HME1-Empty (+Dox) specimens (Fig. 27). This indicates that there was no increase in ROS levels in HME1-ACLY (+Dox) specimens. As such, ACLY overexpression did not induce oxidative stress, and it can be concluded that the elevated levels of DNA double stranded breaks observed (Fig. 25, 26) was not caused by ROS.

Figure 27. DNA double stranded breaks in ACLY-overexpressing cells was not caused by oxidative stress. HME1-ACLY and HME1-Empty cells were treated with doxycycline for 48hrs and 72hrs. Cells were exposed with CellROX® Green reagent, an agent that becomes oxidised by reactive oxygen species (ROS) and binds with DNA to exhibit an absorption/emission spectrum of ~485/520nm. As positive control, HME1-ACLY cells were treated with tert-Butyl hydroperoxide solution (tBHP) at 200μM for 2hrs. HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells did not display increased ROS levels at both 48hr and 72hr time-points. This shows that ACLY overexpression did not lead to oxidative stress (Scale bar = 50 μm).

3.8. ACLY overexpression deregulated ATR pathway, and may have disrupted ATM signalling

DNA damage can be caused by the cell's inability to respond to replication stress, which results in the collapse of stalled replication forks to form DNA double stranded breaks. In addition, the cell's inability to respond appropriately to DNA double stranded breaks could result in DNA fragmentation or DNA misrepair, which may provide a possible explanation to the formation of CENPA (-ve) MN structures (Fig. 17C, 18C), NPBs (Fig. 18A-B), and chromatin bridges (Fig. 19). ACLY overexpression may deregulate DNA damage responses necessary to respond to replication stress and DNA double stranded breaks. To determine this, phosphorylation status of ATR, ATM, Chk1, Chk2, and Nbs1 in ACLY-overexpressing cells was analysed over time periods of 24hrs, 48hrs and 72hrs. Furthermore, to determine if ACLY overexpression affects the expression of DNA-damage response proteins, total proteins of ATR, ATM, Chk1, RPA32, Chk2, and Nbs1 were analysed in these cells over the same time periods. HME1-ACLY and HME1-Empty cells were treated doxycycline for 24hrs, 48hrs, and 72hrs. As baseline controls, untreated HME1-ACLY (-) and HME1-Empty (-) specimens were included. Two positive controls were added in the analysis, they were namely VP16 and HU that are known to induce DNA damage and replication stress respectively. Fold change ratios in total protein levels between HME1-ACLY (+Dox) and HME1-Empty (+Dox) specimens at each time-point were calculated. The fold change ratios were then transformed with binary logarithm ($\log_2 n$, where n is the fold change ratio) to best display the increase and decrease of protein levels in symmetrical manner (Quackenbush, 2002). Three biological repeats were performed altogether ($n=3$), where the data generated were subjected to two-tailed student t -tests.

3.8.1. ACLY overexpression was found to reduce levels of phosphorylated ATR but not phosphorylated ATM

First, phosphorylation status and total protein levels of ATR and ATM were analysed (Fig. 28A). HME1-ACLY (+Dox) cells displayed a gradual decrease in phosphorylated ATR (Thr1989) (pATR) over time, where levels at the 72hr time-point were significantly lower compared to those at the 24hr time-point, showing p value = 0.0407 (Fig. 28B). On the other hand, pATR levels in HME1-Empty (+Dox) cells seemed to remain unaltered up to the 72hr time-point, showing p value = 0.0703 (Fig. 28B). This allowed HME1-ACLY (+Dox) cells to display a significantly lower amount of pATR compared to HME1-Empty (+Dox) cells at the 72hr time-point, showing p value = 0.005 (Fig. 28B). In addition, the levels of pATR observed in both HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells were comparatively lower than VP16-treated cells (Fig. 28B). Little differences in total ATR proteins were found between HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells at 24hr, 48hr, and 72hr time-points, as shown by small fold change values of 0.15, 0.02, and 0.51 respectively (Fig. 28C).

Next, HME1-ACLY (+Dox) cells displayed a slight increase in phosphorylated ATM (Ser1981) (pATM) at the 72hr time-point with p value = 0.0409. However, there was no distinct differences in pATM levels between HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells at all time-points, where p values > 0.05 (Fig. 28B). As such, it was determined that there were no significant changes in pATM levels when ACLY was overexpressed. Furthermore, pATM levels in HME1-ACLY (+Dox) cells were significantly lower compared to VP16-treated cells (Fig. 28B). Lastly, little fold changes were observed in total ATM proteins between these two specimens (Fig. 28C).

Figure 28A. Western blotting was performed to visualise changes in phosphorylation and total protein levels of ATR and ATM kinases in ACLY-overexpressing cells. HME1-ACLY and HME1-Empty cells were treated with doxycycline for 24hrs, 48hrs, and 72hrs. For positive controls, HME1-ACLY cells were either treated with VP16 and HU, which are known to induce DNA damage and replication stress respectively. Asynchronous cells of all specimens were harvested and lysed for western blotting. Three biological repeats were performed (n=3), where the data was subjected to two-tailed student *t*-tests.

Figure 28B. Levels of phosphorylated ATR were decreased, but levels of phosphorylated ATM remained unchanged. ATR phosphorylation (Thr1989) in HME1-ACLY (+Dox) cells decreased over time, and was distinctively lower compared to HME1-Empty (+Dox) cells at the 72hr time-point, with p value < 0.005. On the other hand, no significant differences in ATM phosphorylation (Ser1981) between HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells at all time-points, as shown by p values > 0.05. Normalised signal intensities relative to baseline controls are represented in arbitrary units (A.U.). (Statistical significance is represented by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$)

Figure 28C. Levels of ATR and ATM proteins remained unchanged in ACLY-overexpressing cells. Total ATR levels in HME1-ACLY (+Dox) cells were maintained throughout all time-points, where small fold changes were reported when compared to HME1-Empty (+Dox) cells. On the other hand, no significant differences in total ATM levels between were observed between HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells at all time-points, as shown by small fold change values.

3.8.2. ACLY overexpression did not cause any significant changes in the phosphorylation status of ATR and ATM downstream substrates, but caused a reduction in total Chk1 levels

Downstream substrates of ATR, namely Chk1 and RPA32 were then analysed (Fig. 29A). No distinct differences in phosphorylated Chk1 (Ser345) (pChk1) levels were observed between HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells at all time-points, where *p values* > 0.05 (Fig. 24B). Moreover, pChk1 levels in these two specimens were comparably lower than VP16- and HU-treated cells (Fig. 24B). There was a gradual decrease in total Chk1 proteins in both HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells (Fig. 24A). However, at the 72hr

time-point, there was a distinct reduction in Chk1 levels in HME1-ACLY (+Dox) than HME1-Empty (+Dox) cells, reporting an average fold change value of -2.27 (Fig. 24C). As for RPA32, its protein levels remained unchanged throughout all time-points in both HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells, with small fold change values reported (Fig. 24C).

Figure 29A. Western blotting was performed to visualise changes in phosphorylation and total protein levels of ATR downstream substrate Chk1 in ACLY-overexpressing cells. HME1-ACLY and HME1-Empty cells were treated with doxycycline for 24hrs, 48hrs, and 72hrs. For positive controls, HME1-ACLY cells were either treated with VP16 and HU, which are known to induce DNA damage and replication stress respectively. Asynchronous cells of all specimens were harvested and lysed for western blotting. Three biological repeats were performed (n=3). There, phosphorylation and total protein levels of Chk1, and total protein levels of RPA32, were analysed where the data was subjected to two-tailed student *t*-tests.

Figure 29B. No distinct changes in Chk1 phosphorylation was observed in ACLY-overexpressing cells. No observable changes in phosphorylated-Chk1 (Ser345) (pChk1) were observed between HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells, where levels remained low at all time-points compared to VP16- and HU-treated cells. Normalised signal intensities relative to baseline controls are represented in arbitrary units (A.U.). (Statistical significance is represented by * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$)

Figure 29C. ACLY-overexpressing cells displayed reduced levels of Chk1 proteins, but RPA32 protein levels remained unaltered. Total Chk1 levels decreased gradually in both specimens, but more distinctively in HME1-ACLY (+Dox) cells compared to HME1-Empty (+Dox) cells at the 72hr time-point, where a fold change value of -2.27 was reported. On the other hand, no distinct differences in total RPA32 proteins were found between both specimens at all time-points as shown by low fold change values.

Next, downstream substrates of ATM, namely Chk2 and Nbs1, were analysed (Fig. 30A). Both HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells displayed an increase in phosphorylated Chk2 (Thr68) (pChk2), where levels at the 72hr time-point were comparably higher than those at the 24hr time-point, showing *p values* < 0.05 (Fig. 30B). Despite these increments, pChk2 levels were still distinctively lower than VP16-treated cells. Between HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells, no significant difference in pChk2 levels were seen at 24hr and 48hr time-points. However, at the 72hr time-point, HME1-ACLY (+Dox) cells appeared to display lower pChk2 levels than HME1-Empty (+Dox) cells, showing *p value* = 0.0491 (Fig. 30B). Levels of phosphorylated Nbs1 (Ser343) (pNbs1) in both HME1-ACLY (+Dox) and HME-Empty (+Dox) cells remained unchanged at all three time-points, and were significantly lower compared to VP16- and HU-treated cells (Fig. 30B). Lastly, for total Chk2 and Nbs1 levels, they remained unchanged in both specimens at all three time-points, as shown by low fold change values (Fig. 30C).

Figure 30A. Western blotting was performed to visualise changes in phosphorylation and total protein levels of ATM downstream substrates, Chk2 and Nbs1, in ACLY-overexpressing cells. HME1-ACLY and HME1-Empty cells were treated with doxycycline for 24hrs, 48hrs, and 72hrs. For positive controls, HME1-ACLY cells were either treated with VP16 and HU, which are known to induce DNA damage and replication stress respectively. Asynchronous cells of all specimens were harvested and lysed for western blotting. Three biological repeats were performed (n=3). There, phosphorylation and total protein levels of Chk2 and Nbs1 were analysed where the data was subjected to two-tailed student *t*-tests.

Figure 30B. No distinct increase in Chk2 and Nbs1 phosphorylation levels in ACLY-overexpressing cells. No discernable differences in phosphorylated-Chk2 (Thr68) (pChk2) levels were found between HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells at 24hr and 48hr time-points. However, at the 72hr time-point, pChk2 levels in HME1-ACLY (+Dox) were lower compared to HME1-Empty (+Dox) cells, displaying p value = 0.0491. Although an increase in pChk2 levels was observed in both specimens (showing p values < 0.05), they were still comparatively lower than VP16-treated cells. Lastly, levels of phosphorylated Nbs1 (Ser343) (pNbs1) remained low in both specimens with no distinct differences detected at all three time-points. Normalised signal intensities relative to baseline controls are represented in arbitrary units (A.U.). (Statistical significance is represented by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$)

Figure 30C. No distinct changes in Chk2 and Nbs1 protein levels were found in ACLY-overexpressing cells. Low fold change values reported at all three time-points for total Chk2 and Nbs1 proteins indicate that their levels remained unchanged in ACLY-overexpressing cells.

3.8.3. Phosphorylated ATR and Chk1 were found to be down-regulated

Lastly, to determine how ATR and Chk1 activity were down-regulated, an experiment was performed where HME1-ACLY (+Dox) cells were subjected to treatment with either MG132 or NH₄Cl. MG132 and NH₄Cl are known inhibitors of proteosomal degradation and autophagy respectively. MG132 treatment led to a recovery of total Chk1 proteins and pATR, but not total ATR proteins (Fig. 31). There was also increased levels of pChk1 in MG132-treated cells. On the other hand, NH₄Cl treatment did not exhibit the same effect, where levels of pATR, pChk1, and total Chk1 remained low similar to Figures 23 and 24 (Fig. 31). Hence, the decrease in Chk1 proteins and pATR seemed to be attributed to their degradation through the proteosomal-dependent degradation pathway.

Figure 31. MG132 treatment led to a recovery of phosphorylated ATR and total Chk1 proteins in ACLY-overexpressing cells. HME1-ACLY (+Dox) cells were treated with MG132 (proteasome inhibitor) or NH₄Cl (autophagy inhibitor) to determine the mechanisms of which ATR and Chk1 are down-regulated. MG132 treatment was able to rescue total Chk1 levels, but not NH₄Cl. MG132 treatment was also able to recover phosphorylated ATR. On the other hand, NH₄Cl treatment did not recover levels of phosphorylated ATR and total Chk1 proteins.

3.8.4. Summary of observations

Control cells (HME1-Empty) were found to experience low baseline levels of pATR especially at the 48hr and 72hr time-points compared to VP16- and HU-

treated cells (Fig. 28B), which translated into low levels of pChk1 (Fig. 29B). In ACLY-overexpressing cells, levels of pATR were found to be lower than those seen in control cells especially at the 72hr time-point, where a *p value* = 0.005 was reported (Fig. 28B). Since pChk1 levels were already low in control cells (Fig. 29B), it was difficult to discern if levels were significantly lower in ACLY-overexpressing cells. Interestingly, total Chk1 levels were reduced by about fourfold, showing a fold change value of -2.27 (Fig. 29C). Further works then revealed that pATR and Chk1 were degraded through the proteosomal degradation pathway in ACLY-overexpressing cells (Fig. 31). Results seemed to suggest that ACLY overexpression causes cells to be more vulnerable to prolonged replication fork stalling in the event DNA damage occurs from endogenous sources.

With the ATR pathway deregulated, it was predicted that there would be a rise in levels of DNA double stranded breaks as a result of the consequential collapse of replication forks. However, levels of pATM in ACLY-overexpressing cells did not increase relative to control cells (Fig. 28B). Although pChk2 levels were reportedly lower in ACLY-overexpressing cells than control cells at the 72hr time-point (Fig. 30B), this difference was considerably marginal, with *p value* = 0.0491. As such, the results were inconclusive on the role of ACLY-overexpression in negatively affecting Chk2 phosphorylation. As for pNbs1, levels remained unaltered in ACLY-overexpressing cells (Fig. 30B). Furthermore, phosphorylation levels of ATM, Chk2, and Nbs1 detected in both ACLY-overexpressing and control cells did not reach levels seen in VP16-treated cells (Fig. 30B). Hence, ACLY overexpression may play a role in disrupting

ATM signalling, which require more substantive evidence. Lastly, ACLY overexpression did not seem to play a significant role in regulating the gene expression of ATR, ATM, RPA32, Chk2, and Nbs1, as shown by small fold changes throughout the experiment (Fig. 28C, 29C, 30C).

3.9. Elevated 53BP1 activity found in ACLY-overexpressing cells

NHEJ is the predominant repair mechanism throughout the cell cycle (Chang *et al.*, 2017; Charkirabarty *et al.*, 2016). 53BP1 is activated by ATM to facilitate DNA repair through the NHEJ pathway. To determine if 53BP1 is down-regulated or active in ACLY-overexpressing cells, western blotting and immunostaining were performed.

For western blot analysis, HME1-ACLY and HME1-Empty cells were treated with doxycycline for 24hrs, 48hrs and 72hrs. As baseline controls, untreated HME1-ACLY (-) and HME1-Empty (-) specimens were included. As positive controls, cells were treated with VP16 and HU. Levels of total 53BP1 proteins in HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells were analysed at each time-point (Fig. 32A). Both specimens expressed similar levels of 53BP1 at all time-points, as shown by low fold change values (Fig. 32B). This indicates that ACLY overexpression did not down-regulate 53BP1 expression. Also, VP16-treated cells were discovered to have lower 53BP1 levels (Fig. 32A). This could likely be caused by an increased recruitment of 53BP1 at the damaged foci, which could not be extracted using RIPA buffer. This dramatic decrease in 53BP1 was not seen in HME1-ACLY (+Dox) cells, which may suggest that there was little or no recruitment of 53BP1 at DNA damage sites.

A)

B)

Figure 32. No distinct changes in 53BP1 protein levels were found in ACLY-overexpressing cells. (A) HME1-ACLY and HME1-Empty cells were treated with doxycycline for 24hrs, 48hrs and 72hrs. As positive control, cells were treated with VP16. All specimens were harvested and lysed for western blotting. **(B)** No significant changes in total 53BP1 levels were observed in HME1-ACLY (+Dox) cells when compared to HME1-Empty (+Dox) cells.

To determine if there was an elevation of 53BP1 foci formation in ACLY-overexpressing cells, immunostaining was performed. HME1-ACLY and HME1-Empty cells were treated with doxycycline for 48hrs and 72hrs. The specimens were then fixed and stained against 53BP1, where the amount of

53BP1 foci in each nucleus were counted. Specimens were prepared in two biological repeats (n=2), where approximately 200 cells were analysed each (Fig. 33A). Two-tailed *z*-tests were subsequently performed to determine differences in 53BP1 foci formation between specimens. At 48hr and 72hr time-points, the mean 53BP1 foci count in HME1-Empty (+Dox) cells was 3.55 and 3.42 per nucleus respectively (Fig. 33B). However, these values were significantly lower compared to HME1-ACLY (+Dox) cells, displaying *p values* < 0.001 (Fig. 33B). HME1-ACLY (+Dox) cells were found to exhibit a mean 53BP1 foci count of 6.27 and 6.49 per nucleus at 48hr and 72hr time-points respectively (Fig. 33B). Hence, ACLY overexpression has led to elevated levels of 53BP1 foci compared to control cells (HME1-Empty). However, levels of 53BP1 foci formation in HME1-ACLY (+Dox) were comparatively lower than VP16-treated cells that showed a mean 53BP1 foci of 28.40 per nucleus (Fig. 33B).

Taken together, although 53BP1 was not down-regulated, elevated levels of 53BP1 foci were found in ACLY-overexpressing cells. These results suggest the presence of DNA double stranded breaks, which is consistent with the data according to Figure 25.

Figure 33A. Levels of 53BP1 foci formation was determined in ACLY-overexpressing cells. HME1-ACLY and HME1-Empty cells were treated with doxycycline for 48hrs and 72hrs, where they were subsequently fixed and stained against 53BP1. Two biological repeats were prepared (n=2), where ~200 cells per specimen were analysed for the presence of 53BP1 foci, marked in red boxes (Scale bar = 10 μ m). As a positive control, cells were treated with VP16.

Figure 33B. Elevated levels of 53BP1 foci formation was found in ACLY-overexpressing cells. HME1-ACLY (+Dox) cells exhibited higher levels 53BP1 foci formation than HME1-Empty (+Dox) cells at 48hr and 72hr time-points, showing *p values* of < 0.001 according to two-tailed z-tests. However, 53BP1 foci formation in HME1-ACLY (+Dox) cells did not reach at levels similar to VP16-treated cells. (Statistical significance is represented by **p* < 0.05, ***p* < 0.01, and ****p* < 0.001)

3.10. ACLY overexpression did not promote apoptosis and may not play a role in deregulating apoptotic mechanisms

Bax and PUMA are pro-apoptotic proteins that facilitates the initiation of the intrinsic apoptosis pathway upon apoptotic stimulus. ACLY overexpression may alter expression levels of these two proteins, inhibiting cells to undergo apoptosis, thereby enabling cells to acquire and accumulate DNA damage. To determine this, western blot was performed to determine levels of these proteins (Fig. 34A). HME1-ACLY and HME1-Empty cells were treated with doxycycline for 24hrs, 48hrs, and 72hrs. As baseline controls, both cells were not treated with doxycycline (-). As positive controls, cells were treated with VP16 and HU. Between HME1-ACLY (+Dox) and HME1-Empty (+Dox) specimens, there was little or no discernable change in PUMA and Bax proteins at all time-points, as shown by small fold change values (Fig. 34B). At 72hr time-point, PUMA levels were slightly lower in HME1-ACLY (+Dox) cells than HME1-Empty (+Dox) specimens, displaying a low fold change value of -0.40 (Fig. 34B). This slight difference may not have a huge impact on the cell's ability to undergo apoptosis.

Figure 34. No distinct changes in Bax and PUMA protein levels were found in ACLY-overexpression cells. (A) HME1-ACLY and HME1-Empty cells were treated with doxycycline for 24hrs, 48hrs, and 72hrs, where levels of Bax and PUMA were analysed. **(B)** Little fold changes in PUMA and Bax levels were observed in HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells.

Next, with data indicating the presence of DNA double stranded breaks, it was determined if they were sufficient to induce apoptosis in ACLY-overexpressing cells. The TUNEL assay was performed to detect cells that are at the late stage of the apoptosis pathway, where extensive DNA fragmentation typically occurs (Fig. 35). HME1-ACLY and HME1-Empty cells were treated with doxycycline for 48hrs and 72hrs. In addition, a positive experimental control was prepared by treating the fixed cells with DNaseI. There was no discernable increase in fluorescent signals in HME1-ACLY (+Dox) specimens compared to HME1-Empty (+Dox) specimens (Fig. 35). Moreover, both specimens displayed little or low levels of fluorescent signals (Fig. 35).

Taken together, ACLY overexpression did not down-regulate the expression of Bax and PUMA proteins. Although both proteins were present in the cells, it was not known if their activity were impaired in the presence of an apoptotic stimulus. Furthermore, despite the presence of DNA double stranded breaks in ACLY-overexpressing cells as reported in previous experiments (Fig. 25, 26), low apoptotic activity was observed. Results may also suggest that the levels of DNA damage were insufficient to cause extensive DNA fragmentation.

Figure 35. Extensive DNA fragmentation was not detected in ACLY-overexpressing cells. HME1-ACLY and HME1-Empty cells were treated with doxycycline for 48hrs and 72hrs, where TUNEL assay was subsequently performed to detect apoptotic cells. DNaseI was used as a positive experimental control. Both HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells did not show an increase in fluorescent signals, thus indicating little or no cells undergoing apoptosis. Furthermore, the results may suggest that the presence of DNA double stranded breaks previously reported did not result in extensive DNA fragmentation. (Scale bar = 20 μ m)

3.11. Cell growth was sustained in ACLY-overexpressing cells

The presence of DNA double stranded breaks should result in checkpoint activation to enable time for appropriate DNA damage responses to take effect. A delay in cell growth was therefore expected in cells that were overexpressing ACLY. To determine this, an MTT proliferation assay was performed (Fig. 36). The assay was done in three biological repeats (n=3), where specimens were prepared in triplicates each. HME1-ACLY and HME1-Empty cells were seeded in 96-well culture plates, where they were treated with or without doxycycline over a period of 24hrs, 48hrs, and 72hrs. At each time-point, the MTT assay was applied to measure the amount of viable cells and subsequently determine the growth rate of each specimen. ACLY overexpression in HME1-ACLY (+Dox) cells did not change the rate of cell growth when compared HME1-ACLY (-Dox) cells. This was shown by similar levels of viable cells between the two specimens at all time-points (Fig. 36). In addition, doxycycline treatment did not alter the growth rate of HME1-Empty (+Dox) cells when compared to HME1-Empty (-Dox) (Fig. 36). This means that there were no off-target effects of doxycycline on cell growth.

Figure 36. Growth rate of ACLY-overexpressing cells was not slowed. HME1-ACLY and HME1-Empty cells were treated with doxycycline for 24hrs, 48hrs, and 72hrs. MTT assay was performed in three biological repeats (n=3) to determine cell growth in both HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells. As negative controls, HME1-ACLY and HME1-Empty cells were allowed to incubate without doxycycline (-Dox). No significant difference in growth rate was observed between HME1-ACLY (-Dox) and HME1-ACLY (+Dox) specimens, and this was similar between HME1-Empty (-Dox) and HME1-Empty (+Dox) specimens.

Next, it was examined if ACLY overexpression would down-regulate the expression of CDK inhibitor $p21^{Cip1}$ (Fig. 37A). HME1-ACLY and HME1-Empty cells were treated with doxycycline for 24hrs, 48hrs, and 72hrs. As baseline controls, both cells were not treated with doxycycline (-). As positive controls, cells were treated with VP16 and HU. No discernable differences in $p21^{Cip1}$ levels were observed at each time-point between HME1-ACLY (+Dox) and HME1-Empty (+Dox) specimens, as shown by the low fold change values (Fig. 37B). Hence, ACLY overexpression did not influence the expression of $p21^{Cip1}$. This means that cell cycle arrest was not promoted, which is consistent with previous findings that displayed little changes in the growth rate of HME1-ACLY (+Dox) cells (Fig. 36).

Figure 37. No distinct changes in p21^{Cip1} protein levels were found in ACLY-overexpressing cells. (A) HME1-ACLY and HME1-Empty cells were treated with doxycycline for 24hrs, 48hrs, and 72hrs. These specimens were harvested and lysed for western blotting, where levels of cell cycle inhibitor p21^{Cip1} were analysed. **(B)** No distinct differences in p21^{Cip1} levels were observed between the two specimens at all three time-points.

At the S phase of the cell cycle, it is essential for cells to effectively initiate checkpoint activation in the presence of DNA damage. This grants sufficient time for DNA damage repair and thereby maintaining genome integrity as cells divide. To determine if ACLY overexpression promotes cell cycle progression, HME1-

ACLY and HME1-Empty cells were incubated with or without doxycycline for 72hrs before performing a double thymidine block to synchronise the cells at the G₁-S transition phase. After the cells were released from thymidine block, their progression through the cell cycle from S phase to G₂/M phase was monitored at time intervals of 2hrs, for a total of 10hrs (Fig. 38A). Three biological repeats were performed altogether (n=3). HME1-ACLY (+Dox) cells progressed through the cell cycle at a similar pace compared to HME1-ACLY (-Dox) cells. This can be shown by similar amounts of G₀/G₁ phase cells, S phase cells and G₂/M phase cells in both HME1-ACLY (+Dox) and HME1-ACLY (-Dox) cells at each time-point (Fig. 38B). On the other hand, doxycycline treatment in HME1-Empty cells did not alter their rate of progression through the cell cycle. This can be shown by similar progression trends in both HME1-Empty (+Dox) and HME1-Empty (-Dox) cells (Fig. 38B). Hence, there were no off-target effects of doxycycline on cell cycle progression.

Altogether, cell cycle arrest was not promoted in ACLY-overexpressing cells. Instead, cell cycle progression was still sustained in these cells despite the presence of DNA double stranded breaks (Fig. 25, 26), which could potentially result in unrepaired or misrepaired DNA.

Figure 38A. Cell cycle progression patterns of ACLY-overexpressing cells were determined using flow cytometry. HME1-ACLY and HME1-Empty cells were treated with doxycycline for three days, after which they were synchronized at G₁-S transition phase via double thymidine block, where progression of cells through the S phase up to G₂/M phase was monitored by flow cytometry analysis. As negative controls, HME1-ACLY and HME1-Empty cells were left to incubate without doxycycline (-Dox) for three days, where they were subsequently subjected to double thymidine block. Three biological repeats were performed (n=3).

Figure 38B. Cell cycle progression in ACLY-overexpressing cells was not slowed. HME1-ACLY (+Dox) cells progressed through the S phase at a similar pace compared to HME1-ACLY (-Dox) cells. This was similar for both HME1-Empty (+Dox) and HME1-Empty (-Dox) cells. As such, there were no off-target effects of doxycycline on cell cycle progression. Also, ACLY overexpression did not delay or increase the rate of cell cycle progression from the S phase to G₂/M phase.

3.12. ACLY overexpression did not induce genome-wide histone acetylations

ACLY metabolic enzyme catalyses citrate to release Acetyl-CoA in the nuclear-cytoplasmic fraction. It was discovered that elevation in Acetyl-CoA levels induces genome-wide histone acetylations through modulating HATs activity (Lee *et al.*, 2014). As such, levels of histone acetylations were determined in ACLY-overexpressing cells. The presence of such epigenetic modifications may modulate the expression of DNA damage response proteins.

HME1-ACLY cells were allowed to incubate for 48hrs and 72hrs with or without doxycycline. As a positive control, cells were incubated with sodium butyrate (NaB), a known HDAC inhibitor, for 24hrs to induce histone acetylations. Histones from all specimens were acid extracted and analysed through Coomassie blue staining and western blot. To determine acetylation levels of all histones (H2A, H2B, H3 and H4), a broad detecting antibody that binds to acetylated lysine residues was used.

NaB-treated cells showed elevated levels of H3 and H4 histone acetylations. Unexpectedly, in HME1-ACLY (+Dox) specimens, there was no discernable increase in H3 and H4 histone acetylations at all time-points when compared to HME1-ACLY (-Dox) specimens (Fig. 39). Furthermore, both specimens showed little or undetectable levels of histone acetylations (Fig. 39).

As such, ACLY overexpression did not seem to promote genome-wide histone acetylations. This shows that there was no permanent acquisition of histone epigenetic marks to down-regulate expression of DNA damage response

proteins, which could be supported by findings showing little changes in the expression several DNA damage response proteins observed (ATR, ATM, RPA32, Chk2, Nbs1, 53BP1, Bax, PUMA, and p21^{Cip1}) (Fig. 28C, 29C, 30C, 32B, 34B, 37B).

Figure 39. ACLY overexpression did not induce genome-wide histone acetylations. HME1-ACLY cells were induced to overexpress ACLY by treatment with doxycycline for 48hrs and 72hrs. As positive control, HME1-ACLY cells were treated with 2mM of sodium butyrate (NaB), a histone deacetylase (HDAC) inhibitor, for 24hrs. Histones from all specimens were acid-extracted before running Western blot and Coomassie blue staining. Presence of histone acetylation was determined by using antibodies that binds to acetylated-lysine residues on histones. NaB treatment led to a discernable increase in H3 and H4 lysine acetylation. However, both HME1-ACLY (-Dox) and HME1-ACLY (+Dox) cells did not show any increase in histone acetylations even at 72hr time-point.

3.13. DNA damage caused by ACLY overexpression may have contributed to the acquired ability of cells to grow under anchorage-independent conditions

Anchorage-independent growth is a characteristic of metastatic cells, where they are able to remain viable without the need to be attached to the extracellular matrix, and able to proliferate uncontrollably (Guadamillas *et al.*, 2011). DNA damage that was occurring in ACLY-overexpressing cells may predispose them to acquire random mutations with some enabling the ability to promote anchorage-independent growth. To determine this, ACLY-overexpressing cells were subjected to the soft agar colony formation assay. MCF-7 (ER+ and PR+) (estrogen receptor positive and progesterone receptor positive) breast cancer cell line was used as a positive indicator of anchorage-independent growth.

To determine the exact colony size threshold to include colonies in the count that display anchorage-independent growth, MCF-7 cells were grown in soft agar for a duration of 4 weeks. Weekly replenishments of media were done by overlaying new layers of agarose containing fresh complete media. Colonies above the size of 0.01 mm^2 (equivalent to $112 \text{ }\mu\text{m}$ in diameter) were included into the colony count. There, the size of each colony was determined. In total, 2241 colonies were picked up, where they displayed a mean colony size of 0.022 mm^2 (Fig. 40). As such, a colony size threshold of 0.02 mm^2 was applied to determine if ACLY-overexpressing cells exhibited an increase in anchorage-independent proliferative fitness.

Figure 40. Soft agar colony formation assay was performed using the MCF-7 breast cancer cell line to determine the colony size threshold that would best identify cells with an enhanced anchorage-independent proliferative fitness. MCF-7 cells (a double positive aggressive breast cancer cell line) were grown in soft agar over a period of 4 weeks. NBT was used to stain viable cells and colonies were counted under brightfield microscopy. Colonies > 0.01 mm² in area were analysed. A total of 2241 colonies were detected with mean size of 0.022 mm² in area, an equivalent to an average of 265 µm in radius. As such, colonies with a size of > 0.02mm² in area were counted that closely represents the enhanced ability of cells to grow under anchorage-independent conditions. (Scale bar = 34 mm).

HME1-ACLY and HME1-Empty cells were treated with doxycycline for one week before plating them in soft agars in triplicates on 6-well plates. A total of three biological repeats were performed ($n=3$). The cells were then grown in soft agar for a total of 4 weeks, with weekly replenishments by overlaying new layers of agarose containing fresh medium with doxycycline. Colonies were counted from each specimen and subjected to two-tailed student *t*-tests (Fig. 30B). HME1-ACLY (-Dox) and HME1-Empty (+Dox) specimens produced similar levels of colonies, displaying a *p value* > 0.05 (Fig. 30A). As such, no off-targets of doxycycline on anchorage-independent growth were observed. On the other hand, HME1-ACLY (+Dox) specimens showed a significant increase in colony formation by more than twofold compared to HME1-ACLY (-Dox) and HME1-Empty (+Dox) specimens, displaying *p values* < 0.05 (Fig. 30A). Hence, ACLY overexpression promoted cells to acquire the ability for anchorage-independent growth.

Figure 41A. ACLY-overexpressing cells were seeded into soft agar cultures to examine their ability to grow under anchorage-independent conditions.

HME1-ACLY and HME1-Empty cells were treated with doxycycline for 7 days total before seeding them into soft agar in triplicates in six-well culture plates. HME1-ACLY (-Dox), HME1-ACLY (+Dox), HME1-Empty (+Dox)

cells were left to incubate in soft agar for 4 weeks, with weekly nutrient replenishment. For analysis, nitro blue tetrazolium chloride (NBT) was added to soft agar cultures to stain for viable cells, after which colonies with sizes > 0.02mm² were counted under brightfield microscopy. Three biological repeats were prepared (n=3). (Scale bar = 34 mm)

Figure 41B. ACLY-overexpressing cells displayed an increase in anchorage-independent proliferative fitness. HME1-ACLY (+Dox) cells were able to form significantly more colonies than HME1-ACLY (-Dox) and HME1-Empty (+Dox), showing p values < 0.05 according to two-tailed student t -tests. Data suggests that ACLY expression promoted anchorage-independent growth. (Statistical significance is represented by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$)

3.14. ACLY overexpression was found to deregulate p53 phosphorylation (Ser15) and p21^{Cip1} expression at early time-points after tBHP-induced oxidative stress

ACLY-overexpressing cells were treated with tert-Butyl hydroperoxide (tBHP) to determine if DNA damage responses were impaired in the presence of oxidative stress. tBHP treatment was used to mimic conditions similar to elevated levels of reactive oxygen species (ROS). HME1-ACLY and HME1-Empty cells were first treated with doxycycline for 24hrs. After which, the cells were incubated with 200 μ M of tBHP for 2hrs before the media was replaced with fresh complete media containing doxycycline. Cells were subsequently harvested at 4hrs, 8hrs, 12hrs, and 24hrs post-treatment to analyse activity of downstream ATR and ATM substrates, namely Chk1, Chk2, Nbs1, and p53 (Fig. 42A, 43A, 44A). Levels of p21^{Cip1} expression were also monitored to determine if growth arrest was initiated (Fig. 45A).

Phosphorylation levels of ATR substrate Chk1 in both HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells were increased initially with no discernable differences, where they peaked at the 4hr time-point before gradually decreasing in a time-dependent manner (Fig. 42B). Also, no distinct changes in total Chk1 levels were observed in HME1-ACLY (+Dox) cells before treatment and all time-points post-treatment (Fig. 42B). However, it was noted that there was a time-dependent decrease in total Chk1 levels in both specimens after tBHP treatment (Fig. 42B).

Figure 42. No distinct changes in phosphorylation and protein levels of ATR substrate Chk1 in ACLY-overexpressing cells before and after tBHP treatment. (A) HME1-ACLY and HME1-Empty cells were treated with doxycycline for 24hrs, and were subsequently treated with tert-Butyl hydroperoxide solution (tBHP) at 200 μ M for 2hrs. Cells were then incubated in fresh media and harvested at 4hr, 8hr, 12hr, and 24hr time-points. Specimens were lysed for western blotting to observe activity of Chk1. (B) Both HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells were able to respond to the presence of tBHP-induced oxidative stress by increasing pChk1 levels, where they peaked at the 4hr time-point before decreasing gradually for the remainder of the experiment. Both specimens displayed a gradual decrease in total Chk1 levels after they were released from tBHP treatment. Normalised signal intensities are represented in arbitrary units (A.U.).

Next, phosphorylation levels of ATM downstream substrates Chk2 and Nbs1 were examined. Chk2 phosphorylation patterns in both HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells were similar with no distinct differences, where pChk2 levels peaked at the start point when tBHP was removed (0hr time-point), and gradually decreased in a time-dependent manner post-treatment (Fig. 43B). No differences in Nbs1 phosphorylation patterns were also observed between HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells, where both specimens displayed a gradual increase of pNbs1 levels initially, reaching a plateau from the 4hr time-point to the 12hr time-point before decreasing subsequently (Fig. 43C). For most of the experiment, there was no discernable changes in total Chk2 and Nbs1 protein levels in HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells (Fig. 43B-C). Although HME1-ACLY (+Dox) cells initially displayed lower levels of total Nbs1 than HME1-Empty (+Dox) cells before tBHP-treatment, it did not significantly affect the ability of HME1-ACLY (+Dox) cells to phosphorylate Nbs1 in the presence of oxidative stress (Fig. 43C).

Figure 43A. Phosphorylation and total protein levels of ATM substrates, Chk2 and Nbs1, were examined in ACLY-overexpressing cells before and after tBHP treatment. HME1-ACLY and HME1-Empty cells were treated with doxycycline for 24hrs, and were subsequently treated with tert-Butyl hydroperoxide solution (tBHP) at 200 μ M for 2hrs. Cells were then incubated in fresh media and harvested at 4hr, 8hr, 12hr, and 24hr time-points. Specimens were lysed for western blotting to observe activity of Chk2 and Nbs1.

Figure 43B. No distinct changes in Chk2 responses were found in ACLY-overexpressing cells after tBHP treatment. No differences in Chk2 phosphorylation levels in response to tBHP-induced oxidative stress were observed between HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells. In both specimens, an increase in pChk2 levels was found that peaked at the 0hr time-point. Subsequently, pChk2 levels decreased in a gradual manner for the remainder of the experiment. Also, total Chk2 proteins in HME1-ACLY (+Dox) cells remained relatively unaltered when compared to HME1-Empty (+Dox) cells. Normalised signal intensities are represented in arbitrary units (A.U.).

Figure 43C. No distinct changes in Nbs1 responses were found in ACLY-overexpressing cells after tBHP treatment. No differences in Nbs1 phosphorylation levels in response to tBHP-induced oxidative stress were observed between HME1-ACLY (+Dox) and HME1-Empty (+Dox) cells. In both specimens, pNbs1 levels increased initially that plateaued from the 4hr time-point to the 12hr time-point. Nbs1 phosphorylation levels subsequently started to fall at the 24hr time-point. Total Nbs1 proteins remained unchanged in HME1-ACLY (+Dox) cells throughout most of the experiment. Although total Nbs1 levels were found to be lower in HME1-ACLY (+Dox) cells prior to tBHP treatment, it did not affect the cells' ability to respond to oxidative stress. Normalised signal intensities are represented in arbitrary units (A.U.).

Phosphorylation and total protein levels of p53 were also observed since it has been known to be a substrate of both ATR and ATM (Meek, 2009). Interestingly, HME1-ACLY (+Dox) cells displayed lower p53 phosphorylation than HME1-Empty (+Dox) cells at all time-points after tBHP treatment (Fig. 44B). This was more distinct at early time-points, namely 0hr and 4hr post-tBHP treatment (Fig. 44A-B). Furthermore, HME1-ACLY (+Dox) cells had lower levels of total p53 proteins than HME1-Empty (+Dox) prior to tBHP treatment (Fig. 44B). Even though a gradual increase in total p53 levels was detected in both specimens after tBHP-treatment, HME1-ACLY (+Dox) cells still presented lower levels of it compared to HME1-Empty (+Dox) cells at all the time-points (Fig. 44B).

Figure 44. Levels of p53 phosphorylation and total proteins were found to be lower in ACLY-overexpressing cells. (A) HME1-ACLY and HME1-Empty cells were treated with doxycycline for 24hrs, and were subsequently treated with tert-Butyl hydroperoxide solution (tBHP) at 200 μ M for 2hrs. Cells were then incubated in fresh media and harvested at 4hr, 8hr, 12hr, and 24hr time-points. Specimens were lysed for western blotting to observe activity of p53. **(B)** HME1-ACLY (+Dox) cells displayed lower p53 phosphorylation and total protein levels compared to HME1-Empty (+Dox) cells at all time-points after tBHP treatment. Furthermore, prior to tBHP treatment, HME1-ACLY (+Dox) cells were found to display lower p53 proteins than HME1-Empty (+Dox) cells. Normalised signal intensities are represented in arbitrary units (A.U.).

Next, total p21^{Cip1} proteins were analysed in both HME1-ACLY (+Dox) and HME1-Empty (+Dox) specimens (Fig. 45A). At the 4hr time-point after tBHP treatment, HME1-ACLY (+Dox) cells were found to display lower p21^{Cip1} expression compared to HME1-Empty (+Dox) cells (Fig. 45B). Interestingly, at 8hr and 12hr time-points, HME1-ACLY (+Dox) cells displayed a band shift toward a higher molecular weight than HME1-Empty (+Dox) cells (Fig. 45B). This band shift may indicate the presence of post-translational modifications. However, the impact of such modifications on the ability of HME1-ACLY (+Dox) cells to initiate cell cycle arrest is unknown.

Hence, ACLY overexpression disrupted p53 responses to the presence of oxidative stress, especially at the early time-points. This may account for the lower p21^{Cip1} expression levels displayed in HME1-ACLY (+Dox) cells, since activated p53 has been known to induce its expression. The lack of timely p53 phosphorylation could be attributed to decreased levels of total p53 proteins, which suggest that p53 was down-regulated.

Figure 45. Levels of total p21^{Cip1} proteins did not increase promptly after tBHP treatment in ACLY-overexpressing cells. (A) HME1-ACLY and HME1-Empty cells were treated with doxycycline for 24hrs, and were subsequently treated with tert-Butyl hydroperoxide solution (tBHP) at 200 μ M for 2hrs. Cells were then incubated in fresh media and harvested at 4hr, 8hr, 12hr, and 24hr time-points. Specimens were lysed for western blotting to observe levels of p21^{Cip1} expression. **(B)** HME1-ACLY (+Dox) cells were found to express lower levels of p21^{Cip1} expression at the 4hr time-point after tBHP treatment compared to HME1-Empty (+Dox) cells. Levels of p21^{Cip1} between the two specimens remained the same for subsequent time-points. In addition, HME1-ACLY (+Dox) cells displayed a band shift toward a slightly higher molecular weight at 8hr and 12hr time-points when compared to HME1-Empty (+Dox) cells. This was likely caused by unknown post-translational modifications on p21^{Cip1} due to ACLY overexpression. The impact of these p21^{Cip1} band shifts on cell cycle progression remains to be investigated. Normalised signal intensities are represented in arbitrary units (A.U.).

4. DISCUSSION

4.1. The presence of micronuclei structures, nuclear budding structures and nucleoplasmic bridges in cells indicate the presence of DNA damage and thereby genome instability

As mentioned in the introduction, it is presently known that somatic mutations on the genome of the cell contributes to cancer development and even progression (See Section 1.2.2.). Somatic mutations can come in various forms that include point mutations, chromosomal mutations and gene copy number variations (Clancy, 2008), of which all of them results in genome instability. These mutations could occur randomly over time, resulting in an accumulation of them that ultimately activates oncogenes and down-regulate tumour suppressor genes. This fits the model of Clonal Evolution as theorised by Nowell (See Section 1.3.2.) (Greaves and Maley, 2012; Nowell, 1976). Cells are then able to acquire characteristics that is described by Weinberg as the ten hallmarks of cancer (Hanahan and Weinberg, 2011). Although genome instability is a listed hallmark of cancer according to the literature, it should also be considered as the main driver of tumourigenesis and progression toward malignancy. The study here was interested in understanding if metabolic reprogramming by overexpression of a metabolic enzyme, ACLY, was able to cause genome instability. hTERT-HME1 (HME1) and hTERT-RPE1 (RPE1) cells were used in this study as a model to overexpress ACLY. These cell lines were selected as they closely resemble normal phenotype of human epithelial cells.

To visualise the presence of genome instability, the presence of micronuclei structures (MN), nuclear budding structures (NBUD), and nucleoplasmic bridges (NPB) were used as positive indicators, since they have been well described to

be closely associated with DNA damage (Fenech *et al.*, 2011). Transfection was performed to introduce the pXJ40-FLAG-ACLY plasmid into HME1 and RPE1 cells to induce ectopic ACLY expression. Although expression was achieved within 24hrs post-transfection in these cells (Fig. 15A-B), they became unstable at 48hrs and mostly not viable at the 72hr time-point. As such, the long-term impact of ACLY overexpression could not be carefully studied. Due to this limitation, a stable doxycycline-inducible cell line was generated from the HME1 cell line to enable stable ACLY expression, thus eliminating the toxic effects of transfection that could otherwise contaminate experimental findings. Although RPE1 cells displayed a higher degree of MN formation compared to HME1 cells (Fig. 16B), HME1 cells were selected over RPE1 cells to create the doxycycline-inducible system. Since ACLY overexpression has been reported in most breast cancer tissues, and was found to be associated with poor prognosis of breast cancers (Wang *et al.*, 2017), it was assessed that HME1 cells (of breast epithelial origin) would be a more relevant model to study the impact of ACLY overexpression on genome stability. Furthermore, little research has linked ACLY overexpression to cancers associated with the retina. Thus, findings derived from RPE1 cells would not have much significance.

A third generation lentiviral system was used to safely integrate the ACLY coding sequence into HME1 cells. This produces a stable HME1-ACLY cell line where the expression of ACLY was initiated through the addition of doxycycline in complete media, thereby establishing a Tet-On system. With the consideration of any off-target effects of doxycycline or doxycycline-rtTA interactions, another stable cell line was generated by integrating an empty lentiviral expression vector

into the cell, producing the HME1-Empty cell line. With the HME1-ACLY cell line, expression of ACLY was achieved up to 72hrs (Fig. 18), where the presence of the aforementioned nuclear abnormalities was determined. VP16 treatment was used as a positive indicator of DNA double stranded breaks. Studies have found its role in inhibiting topoisomerase II α (TopoII α) by stabilising its interaction with the DNA. This impedes the ability of TopoII α in re-ligating DNA breaks (Ong *et al.*, 2013), resulting in unrepaired DNA. Cells were treated with low concentrations of VP16 (5 μ M) for 2hrs to sufficiently induce DNA double stranded breaks (DSBs).

ACLY overexpression was found to induce the formation of MN structures in transfected HME1 and RPE1 cells, although there were background formations of them (Fig. 16A-B). This was later verified using stable HME1-ACLY cells, where similar findings were made (Fig. 19A-B). Moreover, results from the cytokinesis block micronucleus cytochrome assay (CBMN) assay were consistent to the formation of these structures in once-divided cells (Fig. 20A-B). MN structures were then further examined if they originated from lagging chromosomes, acentric chromosomes, or acentric DNA fragments. Since lagging chromosomes have centromeres, while acentric chromosomes and acentric DNA fragments do not, MNs were stained against a centromere marker CENPA (centromere protein A). Most of the MN structures examined in ACLY-overexpressing cells were CENPA negative (Fig. 19C, 20C), showing that they were derived from either acentric chromosomes or acentric DNA fragments that broke off from the parent nucleus (Fenech *et al.*, 2011). As such, it was determined that ACLY overexpression did not disrupt mechanisms involved in

kinetochore-microtubule attachment and the activation of the spindle assembly checkpoint. This conclusion was further substantiated by findings that showed no significant increase in lagging chromosomes in both anaphase and telophase cells (Fig. 21A-B). Three possible explanations could account for the formation of centromere-negative MNs. First, aberrant chromosomal rearrangements may have occurred, which can be caused by the misrepair of DSBs through the error prone non-homologous end-joining pathway, resulting in the formation of acentric chromosomes (Fenech *et al.*, 2011). Second, DNA fragmentation could occur through increased levels of DSBs, allowing acentric DNA fragments to break off. This was demonstrated in cells treated with VP16 (Fig. 19A-B, 20A-B). Lastly, simultaneous excision repair events may have occurred, which is the cell's intrinsic mechanism to remove damaged DNA from the parent genome in a form of acentric DNA fragments (Fenech *et al.*, 2011). Nevertheless, the presence of centromere-negative MN structures in ACLY-overexpressing cells indicate the increased presence of DNA damage. To determine if aberrant chromosomal translocations had occurred, individual chromosomes from ACLY-overexpressing cells have to be analysed through cytogenetic testing using the fluorescent in-situ hybridisation method.

Next, ACLY overexpression led to an increase in the formation of NBUD structures (Fig. 19A-B). The CBMN assay also showed similar findings in once-divided cells (Fig. 20A-B). Furthermore, the percentage frequency of cells with NBUD structures were similar to VP16-treated cells. Hence, the data seem to suggest that the NBUD structures observed in ACLY-overexpressing cells were likely caused by DSBs. However, NBUD structures have been found to originate

through other mechanisms (Fenech *et al.*, 2011). As such, even though a study has shown how DNA damage through γ -irradiation (Haaf *et al.*, 1999), and how VP16-induced DNA damage (Fig. 19A-B, 20A-B) can contribute to the formation of NBUD structures, it does not necessarily mean that the formation of such structures in ACLY-overexpressing cells are primarily caused by DNA damage. One study discovered that gene amplification, induced under folic acid deficiency conditions, can account for the formation of NBUD structures (Crott *et al.*, 2001). In another study using a colon cancer cell line, it was found that these NBUD structures comprise of amplified DNA, which was reported to be released subsequently through budding as double minute chromosomes (Shimizu *et al.*, 1998). Moreover, one paper theorised that NBUD structures could be formed transiently as a by-product of NPB breakage (Fenech *et al.*, 2011). Hence, it remains unknown if the presence of NBUD structures in ACLY-overexpressing cells were caused by DSBs or gene amplification. Nevertheless, the results suggest that the genome was unstable. Further works are required to address if the NBUD structures in ACLY-overexpressing cells were caused by gene amplification. Gene amplification is also a type of somatic mutation that is seen in some cancers (Albertson, 2006; Clancy, 2008). Several oncogenes have been identified to be amplified in cancers, which include *c-MYC*, *n-MYC*, *EGFR*, and *ERBB2* (HER2/neu) (Albertson, 2006; Shimizu *et al.*, 1998).

Lastly, the CBMN assay revealed an increased percentage of binuclear cells bearing NPBs in specimens where ACLY was overexpressed (Fig. 20A-B). Results were consistent when anaphase and telophase cells were analysed, which showed an increased presence of chromatin bridges (Fig. 21A-B). These NPBs

can contribute to the formation of MN structures and transient presence of NBUD structures as a by-product of their breakage after cytokinesis (Fenech *et al.*, 2011). NPB and chromatin bridges are caused by dicentric chromosomes (Fenech *et al.*, 2011), which can be formed through various mechanisms. Firstly, dicentric chromosomes can be formed by aberrant chromosomal translocations as a consequence of DSBs and their misrepair (Fenech *et al.*, 2011). Although DNA repair through the non-homologous end-joining pathway (NHEJ) is the predominant repair mechanism throughout the cell cycle (Chang *et al.*, 2017; Charkirabarty *et al.*, 2016), it is down-regulated during the S phase where the homologous recombination (HR) repair pathway is essential during DNA replication to maintain genome integrity (Daley and Sung, 2014). Unregulated activation of the error-prone NHEJ pathway and down-regulation of the HR pathway during S phase results in the increased propensity of cells to incorrectly join the ends of DNA strand breaks, causing chromosomal translocations. In another mechanism, dicentric chromosomes have been found to be formed as a result of telomere end fusions (Fenech *et al.*, 2011). Several studies revealed that the shortening of telomeres over repeated cellular divisions result in the formation of uncapped telomere ends. DNA repair mechanisms would recognise these sites as DSBs and initiate repair to introduce unwanted telomere end fusions, which consequently lead to the formation of dicentric chromosomes seen in cancers (Mackinnon and Campbell, 2011). Furthermore, telomere end fusion events can predispose the cells to breakage-fusion-bridge cycles (Gisselsson *et al.*, 2000; Fenech *et al.*, 2011), where chromosomes involved in telomere end fusions can break off and make new fusions with other chromosomes. Breakage-fusion-bridge cycles enable chromosomal mutations in the cell, which studies

have found to promote within-tumour heterogeneity or intra-tumour heterogeneity (Gisselsson *et al.*, 2000). In this study, the possible ways these NPBs originated from in ACLY-overexpressing cells were not extensively studied. However, with data showing the appearance of centromere-negative MNs that could comprise of acentric chromosomes (Fig. 19B-C, 20B-C), and the appearance of NPBs that are made of dicentric chromosomes (Fig. 20B), it became more apparent that chromosomal translocations had occurred in these cells. Indeed, studies have shown that chromosomal translocations as a result of error-prone DNA ligations can consequentially yield both acentric and dicentric chromosomes (Fenech *et al.*, 2011). In addition, it was noted that the percentage frequency of binuclear cells with NPBs in ACLY-overexpressing specimens did not increase dramatically compared to controls (Fig. 20B). Nevertheless, these results seemed to suggest that ACLY-overexpressing were experiencing elevated levels of DNA damage.

Next, an investigation was performed to determine if ACLY overexpression induces centrosome amplification. Centrosome amplification is an aberrant formation of more than two centrosomes that encourage the formation multi-polar spindles. As a consequence, chromosomes are pulled toward multiple poles, resulting in chromosomal mis-segregation. Studies have observed this phenomenon in most types of cancer, and it has been noted to promote cancer progression with increased malignancy (Fukasawa, 2005). With this, the study was interested if multi-polar spindle formation could occur in ACLY-overexpressing cells, which may provide insights in how MN structures were likely formed. To detect centrosomes, fixed specimens were probed against a

centrosome marker, namely pericentrin. Pericentrin is a component of the pericentriolar material in centrosomes where it participates in microtubule organisation events throughout the cell cycle (Delaval and Doxsey, 2010). Although there was an increased percentage of mitotic cells displaying centrosome amplification in specimens overexpressing ACLY (Fig. 22), it did not promote aneuploidy as shown by little or no increase in percentage of sub-G₁ and polyploid cells (Fig. 23, 24). In one study, it was described that centrosome amplification is a mechanism to maintain genome integrity of the cells (Löffler *et al.*, 2013). When DNA damage becomes irreparable, cells would initiate centrosome amplification to induce mitotic catastrophe and subsequently cell death (Löffler *et al.*, 2013). As such, the presence of centrosome amplification in ACLY-overexpressing cells clearly suggests the presence of DNA damage.

Altogether, the increased presence of MN, NBUD, and NPB structures therefore indicates that ACLY overexpression causes genome instability, which was caused by elevated levels of DNA damage. This was consistent by findings showing an increased presence of DNA damage-associated centrosome amplifications. No off-target effects of doxycycline on the formation of these nuclear abnormalities were detected. Furthermore, few studies have reported the genotoxic effects of low doxycycline treatment on genome stability.

4.2. Nuclear abnormalities observed in ACLY-overexpressing cells were caused by DNA double stranded breaks

Experiments were then performed to verify the presence of DNA damage, particularly DNA double stranded breaks since they have been associated with many forms of chromosomal aberrations. In this study, it was found that VP16

can induce the formation of acentric MN structures, which were likely formed as a result of DNA fragmentation events (Fig. 19B-C, 20B-C). It was therefore appropriate to use VP16-treatment as a positive control to demonstrate the formation of DNA double stranded breaks.

To detect for the presence of DNA double stranded breaks, fixed specimens were probed against phosphorylated H2AX (Ser139) (γ H2AX). In the presence of DNA double stranded breaks, H2AX are recruited from the nuclear soluble fraction toward damage sites (Atsumi *et al.*, 2015). There, they are phosphorylated by ATM kinase at Ser139, which is necessary for the recruitment of downstream DNA damage response proteins to facilitate DNA repair through the HR or NHEJ repair pathway (Daley and Sung, 2014; Kozlov *et al.*, 2011). As such, DSBs could be visualised by discrete foci formation in the nucleus using fluorescence microscopy. ACLY overexpression was found to elevate levels of γ H2AX foci formation when compared to control cells (HME1-Empty), displaying higher mean γ H2AX foci count per nucleus at 24hr, 48hr, and 5-week time-points (Fig. 25). However, the mean γ H2AX foci count was lower compared to VP16-treated cells, suggesting that the magnitude of DNA damage occurring was not extensive.

To verify the aforementioned findings, the neutral comet assay was performed. This assay has been known to detect DSBs with high sensitivity. In this study, the experimental procedures employed was claimed in a literature to measure between 50-10,000 breaks per cell (Olive and Banáth, 2006). The assay is therefore able to detect DNA fragmentations, which was the case for VP16-

treated cells (Fig. 26). ACLY overexpression was found to induce DSBs, as shown by higher mean olive tail moments at 48hr, 72hr, and 5-week time-points when compared to control cells (HME1-ACLY untreated cells) (Fig. 26). DNA damage was not found to increase exponentially over time as a result of prolonged ACLY-overexpression. Rather, they were sustained with little changes in damage levels as evident between 72hr and 5-week time-points (Fig. 26). However, the mean olive tail moments reported in ACLY-overexpressing cells were much lower than VP16-treated cells, suggesting a low magnitude of DNA fragmentation (Fig. 26). The results were consistent with immunostaining data that showed a slight elevation of γ H2AX foci formation compared to control cells (Fig. 25). Low levels of DNA fragmentation detected in the neutral comet assay were also consistent with results from the TUNEL assay that showed little or no fluorescent signals in ACLY-overexpressing cells (Fig. 35). Since the TUNEL assay is particularly sensitive to high levels of DNA fragmentation that are mostly in apoptotic cells, the results were expected (Crowley *et al.*, 2016).

Hence, (1) elevated levels of γ H2AX foci and (2) the increase in mean olive tail moments in ACLY-overexpressing cells were indicative of an increased presence of DSBs. In addition, levels of DNA damage did not increase exponentially in a time-dependent manner, but were sustained in tandem with stable ACLY overexpression. Furthermore, the magnitude of DNA damage occurring was not extensive. Findings therefore ascertained that the nuclear and chromosomal abnormalities observed were mainly caused by DNA damage through means of DSBs.

4.3. ACLY overexpression deregulated DNA damage responses not by epigenetic silencing, but through other mechanisms

Next, the study was interested in determining the sources of these DSBs reported (Fig. 25, 26). Two possibilities were explored, which were (1) the increase in reactive oxygen species and (2) the deregulation of DNA damage response pathways. ACLY-overexpressing cells were compared with control cells (HME1-Empty) that were treated with equal amounts of doxycycline. Any distinct differences between the two cell lines were considered as effects attributed by ACLY overexpression.

4.3.1. DNA double stranded breaks were not caused by oxidative stress

In actively respiring cells, reactive oxygen species (ROS) are released as by-products of normal cellular metabolism. Besides being able to damage lipids and proteins as mentioned (See 1.7.3.), they are also able to damage both nuclear and mitochondrial DNA (Cooke *et al.*, 2003). ROS have been shown to damage DNA by reacting chemically with deoxyguanosine to form 8-Hydroxydeoxyguanosine (8-oxodG) (Sova *et al.*, 2010). If left unrepaired, it introduces base substitutions from guanosine (G) to thymidine (T), which may drive oncogenic transformation of cells. Besides causing point mutations in cells, ROS-induced DNA damage has been found to coincide with the formation DSBs. In one study, treatment at various doses of hydrogen peroxide on cardiomyocytes were found to increase levels of γ H2AX foci formation (Ye *et al.*, 2016). In another study on a mice model, oxidative stress was found deplete glutathione in the cell. The depletion of this cellular anti-oxidant was found to predispose the cells to DNA deletions through double stranded breaks (Sharma *et al.*, 2016). As such, levels of intracellular ROS were determined in ACLY-overexpressing cells. Instead of

performing a southern blot to detect levels of 8-oxodG as an indicator of intracellular ROS levels, a commercially available CellROX™Green reagent (Invitrogen, C10444) was used. As mentioned, oxidation of this reagent in the presence of ROS enables it to display an absorption and emission spectra of 485/520 nm, thus enabling users to qualitatively and quantitatively determine levels of ROS in live or fixed cells.

Extended expression of ACLY was not found to increase levels of intracellular ROS, as shown by little or no fluorescent signals from the reagent (Fig. 27). Hence, the results were conclusive that ACLY did not induce DNA damage through the generation of ROS. This means that the presence of DSBs could be caused by the deregulation of DNA damage responses essential to maintain genome integrity.

4.3.2. ACLY overexpression has no role in inducing permanent histone acetylations to regulate expression of DNA damage response proteins

Akt-induced metabolic programming was found to modulate HATs activity, resulting in genome-wide histone acetylations to enrich genes associated with cell growth and proliferation (Lee *et al.*, 2014). Based on this research, it was theorised that ACLY overexpression would result in HAT-directed histone acetylations that might down-regulate the expression of tumour suppressor genes. Findings were hoped to reveal a holistic understanding on the opposing effects of NAD⁺ and Acetyl-CoA metabolic cofactors on genome stability. To determine if histone acetylations were present in ACLY-overexpressing cells, an experiment was designed to extract total histones (H2A, H2B, H3 and H4) using

the acid extraction method, where they were analysed for the presence of acetylations.

However, ACLY overexpression unexpectedly did not increase levels of H3 and H4 histone acetylations even up to the 72hr time-point (Fig. 39), showing no indication of permanent acquisition of genome-wide histone acetylations. As such, ACLY has no role in inducing histone acetylations to down-regulate the expression of tumour suppressor genes. This could be supported by results in this study that showed little or no changes in the expression of DNA damage response proteins, namely ATR, ATM, RPA32, Chk2, Nbs1, 53BP1, Bax, PUMA and p21^{Cip1} (Fig. 28C, 29C, 30C, 32B, 34B, 37B).

In the aforementioned study that discovered the correlation of genome-wide histone acetylations to oncogenic activation, a mix of human glioblastoma (SF188 and LN229) and human prostate cancer (PC-3) cell lines were used (Lee *et al.*, 2014). These cancer cell lines have acquired mutations and aberrant epigenetic marks that enable them to display malignancy. For example, the LN229 cell line was found to have mutations in p53 (Van Meir *et al.*, 1994), deletions of p16 and p18 tumour suppressor genes (Wiedemeyer *et al.*, 2008), and DNA methylations that epigenetically silence expression of tumour suppressor STAT6 (Park *et al.*, 2019). There are other more somatic mutations and epigenetic alterations in these cell lines that will not be described here. Nevertheless, these somatic mutations and epigenetic alterations may contaminate the experimental findings that were meant to understand the role of Akt-induced metabolic reprogramming on tumourigenesis. As such, the results

shown in the study may not be a good representative on the actual events that promote tumourigenesis. Furthermore, genome-wide histone acetylations observed in the study may have likely been transient in response to a short-term glucose and acetate treatment in the media. Such responses are expected to occur as cancer cells would naturally exploit the increase in nutrient availability to enrich the expression of genes necessary for cell growth.

Taken together, based on the present results, ACLY overexpression did not induce genome-wide histone acetylations. As such, it remains inconclusive if the aberrant acquisition of permanent histone acetylation marks has a role in tumourigenesis.

4.3.3. ATR pathway was deregulated in ACLY-overexpressing cells, suggesting their susceptibility to replication stress

The presence of DSBs (Fig. 25, 26) could be a result of collapsed stalled replication forks due to prolonged periods of replication stress. Replication fork stalling typically occurs during the S phase of the cell cycle (Zeman and Cimprich, 2014). This can be attributed to the presence of DNA lesions caused by endogenous sources that include metabolic by-products, repetitive sequences, common fragile sites, and misincorporation of nucleotide bases (Hakem, 2008; Mazouzi, 2014; Pray, 2008; Zeman and Cimprich, 2014). Hence, DNA replication is often met with obstacles that require timely and appropriate DNA damage responses to maintain genome integrity. The ATR kinase is responsible for triggering downstream responses in response to replication stress to induce cell cycle arrest and initiate repair of DNA lesions (Awasthi *et al.*, 2015) (Refer to Section 1.7.4.). As such, the present study was interested to examine if ATR-

mediated DNA damage responses have been deregulated in ACLY-overexpressing cells. Experiments performed did not take into consideration of DNA damage occurring from exogenous sources. As a positive control, cells were treated with hydroxyurea (HU) as it has been known to promote replication fork stalling by depleting nucleotides (Petermann *et al.*, 2010).

Phosphorylation of ATR at Thr1989 (pATR), and Chk1 at Ser345 (pChk1), were expected to be elevated above baseline levels for extended periods of time, which can demonstrate that ACLY overexpression leads to prolonged replication fork stalling. However, a decrease in pATR was observed below levels seen in control cells, where differences were especially distinct at the 72hr time-point, showing $p\text{ value} = 0.005$ based on two-tailed student *t*-tests (Fig. 28B). With data showing that the control cells were experiencing small basal amounts DNA damage, as shown by lower pATR levels compared to HU-treated cells (Fig. 28B), it was determined that ACLY overexpression was responsible for reducing ATR phosphorylation. This effect could not be discernibly translated into differences in pChk1 phosphorylation between ACLY-overexpressing and control cells (Fig. 29B). Since pChk1 levels were already reportedly low in control cells compared to HU-treated cells (Fig. 29B), any possible reduction in pChk1 levels in ACLY-overexpressing cells would be too small to be detected. Interestingly, total Chk1 levels were found to be down-regulated when compared to control cells (Fig. 29C). Further works on ACLY-overexpressing cells through MG132 treatment subsequently revealed a recovery of pATR and Chk1 levels (Fig. 31), suggesting that they were likely degraded via the proteasome-dependent pathway. MG132-treatment also led to the recovery of pChk1 (Fig. 31) that corresponded to the

increased recovery of pATR. It could therefore be inferred that the significant reduction of pATR in ACLY-overexpressing cells reported in Figure 28B was likely caused by its degradation through the proteosome-dependent pathway.

Few studies have shown how pATR is specifically degraded through the proteosomal degradation pathway, despite extensive studies characterising its role in initiating checkpoint activation and HR repair in response to replication stress (Awasth *et al.*, 2015). Furthermore, few studies have shown how MG132 treatment could lead to off-target effects on ATR phosphorylation. Irrespective of the possibilities, the increased presence of phosphorylated ATR at Thr1989 due to MG132-treatment was a novel finding. The molecular mechanism on how ACLY was able to deregulate pATR should be investigated.

On the other hand, there was insufficient evidence to support that the decrease in Chk1 levels was caused by epigenetic silencing, since no works were done to quantify its expression levels through quantitative polymerase chain reaction (q-PCR). However, Chk1 proteins were more likely degraded through the ubiquitin-proteosomal dependent pathway. Several studies have reported how Chk1 can be degraded. In one study, Chk1 degradation was found to be induced through glucose deprivation (Kim *et al.*, 2011). In another study, Chk1 autophosphorylation at Thr378 and Thr382 marks it for rapid proteosomal degradation to enable cell cycle progression through the G₂ phase (Gong *et al.*, 2018). Lastly, treatment of cells with various anti-cancer agents have been found to induce Chk1 phosphorylation at Ser345 to mark for its degradation through the proteosome degradation pathway, allowing for cell cycle arrest and

subsequent cell death (Feng *et al.*, 2008; Zhang *et al.*, 2005). With these studies, ACLY can be postulated to induce glucose deprivation more efficiently than control cells since it could accelerate cellular metabolic processes in the cell, leading to the *Warburg effect*. Also, ACLY could in a novel mechanism, induce Chk1 degradation through promoting Thr378 and Thr382 phosphorylation. These two possibilities, remains to be investigated.

In addition, since (1) RPA32 did not show dramatic fold changes in expression levels (Fig. 29C), (2) Chk1 was found to be down-regulated through the proteosomal degradation pathway (Fig. 31), and that (3) no H3 and H4 histone acetylations were detected (Fig. 39), it was clear that ACLY has no role in inducing permanent changes in histone epigenetic marks to down-regulate the expression of DNA damage response proteins.

Taken together, ACLY overexpression was found to deregulate ATR-mediated DNA damage responses by the proteosomal-dependent degradation of pATR and Chk1 proteins. However, evidence was insufficient to ascertain the mechanisms ACLY exerts to achieve these outcomes, thereby requiring further investigation. In addition, the design of the experiment made it challenging to detect any difference in Chk1 phosphorylation between ACLY-overexpressing cells and control cells. Nevertheless, results suggest that ACLY-overexpressing cells were vulnerable to prolonged stalling of replication forks and consequently DSBs, which could be supported by findings showing elevated levels of γ H2AX foci and increased mean olive tail moments (Fig. 25, 26).

4.3.4. *ACLY overexpression may have disrupted ATM signalling*

Since ACLY overexpression was found to deregulate the ATR pathway, and the presence of DSBs was detected, it was expected to see an increased activity of the ATM pathway. The study then examined the activity of ATM and its downstream substrates, namely Chk2 and Nbs1. In the presence of DSBs, the ATM kinase is activated to orchestrate various pathways to induce cell cycle arrest, promote recruitment of DNA repair proteins at damage sites, and even induce apoptosis (Daley and Sung, 2014; Kozlov *et al.*, 2011; Wu *et al.*, 2000) (Refer to Section 1.7.4.). These events mediated by ATM are critical to maintain genome integrity. As a positive control, cells were treated with VP16 since it has been known consistently to induce DSBs as already demonstrated here (Fig. 25, 26). Unexpectedly, no significant increase in pATM levels was observed in ACLY-overexpressing cells when compared to control cells (Fig. 28B). Findings were similar for pChk2 and pNbs1, with the exception of a marginal decrease in pChk2 levels at the 72hr time-point (Fig. 30B). Furthermore, phosphorylation levels of ATM, Chk2, and Nbs1 were comparably lower than VP16-treated cells (Fig. 28B, 30B). Based on these results, it was postulated that ACLY could be involved in deregulating ATM signalling when the cells were experiencing elevated amounts of DSBs. The deregulation of ATM pathway can cause the failure of cells to initiate DNA repair. Such failure is detrimental especially for cells in the S phase of the cell cycle. This is so as the inability to initiate HR pathway will cause cells to rely more on error prone NHEJ pathway to fix broken DNA ends, which consequently promote DNA misrepair. The experimental design was insufficient to conclude any possible interference of ATM signalling by ACLY overexpression, thus requiring additional studies.

Next, elevated levels of 53BP1 foci formation were found (Fig. 33A-B), which was consistent with findings showing an increased presence of DSBs (Fig. 25, 26). Since ATM has been well established to induce γ H2AX and 53BP1 foci formation, its phosphorylation levels was expected to be elevated in ACLY-overexpressing cells, along with its downstream substrates. However, this was not the case as western blot analysis revealed no discernable increase in pATM, pChk2, and pNbs1 when compared to control cells (Fig. 28B, 30B). As such, these results seemed to display a discrepancy on the activity of ATM kinase. A few studies have shown how phosphorylation of H2AX at Ser139, and how 53BP1 recruitment at DNA strand breaks can occur independently of ATM activity. In one study, it was discovered that DNA-PK can induce phosphorylation of H2AX at Ser139 (Stiff *et al.*, 2004). Other studies using mouse models have reported ATM-independent phosphorylation of H2AX upon exposure to ionising radiation (Koike *et al.*, 2007; Koike *et al.*, 2008). Lastly, it was discovered that 53BP1 could function upstream of ATM kinase in the presence of DNA damage (Mochan *et al.*, 2004). These possible alternative pathways where 53BP1 and H2AX can be activated to form discrete foci independently of ATM kinase activity were not extensively studied. As such, further investigations are required to resolve the discrepancy, which could then bring clarity on whether ATM signalling was deregulated.

Next, small fold changes in ATM, Chk2, Nbs1, and 53BP1 were found (Fig. 28C, 30C, 32B), which further affirms that there was no epigenetic silencing of DNA damage response proteins. This supports the notion that ACLY overexpression

has no role in inducing permanent changes in histone acetylation marks that down-regulate expression of proteins vital in DNA damage responses.

Taken together, results seem to point the possible involvement of ACLY in the deregulation of ATM-mediated DNA damage responses, which could account for the elevated levels of DSBs occurring (Fig. 25, 26). The design of the experiment allowed DNA damage from endogenous sources to occur naturally, which was found to be considerably low in both doxycycline-treated and untreated HME1-ACLY cells compared to VP16-treated cells (Fig. 26). Furthermore, the magnitude of DNA damages occurring in ACLY-overexpressing cells were small, where mean olive tail moments were reportedly increased by 1.5-, 2.5-, and 1.9-fold at 48hr, 72hr, and 5-week time-points respectively (Fig. 26). These factors could potentially pose difficulties in assessing whether ATM signalling was deregulated, since any possible reduction in ATM, Chk2, and Nbs1 phosphorylation would not be easily detected on Western blots. Changes in the experimental design was therefore required. On the other hand, it was clear that ACLY overexpression did not epigenetically silence the expression DNA damage response proteins examined here.

4.3.5. Results were insufficient to determine if apoptotic activity in ACLY-overexpressing cells were impaired

The presence of irreparable DSBs would cause cells to initiate apoptosis through the intrinsic pathway. The expression of two pro-apoptotic proteins, namely Bax and PUMA, have been known to be induced by activated p53 (Miyashita and Reed, 1995; Nakano and Vousden, 2001). Increased levels of PUMA expression acts to sequester inhibitory effects of pro-survival proteins on Bax (Yu and

Zhang, 2008). This promotes Bax recruitment and oligomerisation at the outer mitochondrial membrane to induce permeabilisation and consequential release of cytochrome c, where it acts further downstream to initiate the apoptotic pathway (See Section 1.7.5.) (Stephen, 2010). Hence, the study was interested to examine if ACLY overexpression down-regulate Bax and PUMA activity. The down-regulation of both of these proteins would reduce the susceptibility of cells to undergo apoptosis, thereby promoting tolerance to somatic DNA mutations.

Bax and PUMA proteins were found to be neither up-regulated nor down-regulated, as shown by small fold change values (Fig. 34B). These findings were indicative that these proteins were not epigenetically silenced by ACLY. In addition, ACLY-overexpressing cells were not found to be undergoing apoptosis as shown by TUNEL assay (Fig. 35). This suggests that the cells remained viable despite the presence of DNA double stranded breaks.

However, the design of the experiment was not sufficient to determine whether Bax and PUMA activity were actually deregulated ACLY-overexpressing cells. Interestingly, in one study, increased glucose metabolism through the overexpression of Glut1 glucose transporter was found to impair Bax activation, thereby promoting cell survival (Rathmell *et al.*, 2003). This finding shows that it is possible for metabolic reprogramming to modulate the activity of key proteins involved in the apoptotic pathway. Further works should be done to determine if the activity of Bax and PUMA were impaired by ACLY-induced metabolic alterations in the presence of an apoptotic stimulus.

4.3.6. Cell cycle progression was promoted despite the presence of DNA double stranded breaks

In the presence of DSBs, cells would usually induce cell cycle arrest through the activation of Chk1 or Chk2 kinases. These kinases initiate cell cycle arrest by inhibiting CDC25 family of phosphatases, preventing them from activating cyclin dependent kinases (CDKs) (Falck *et al.*, 2001; Patil *et al.*, 2013). In addition, Chk2 phosphorylate p53, activating it to induce the expression of CDK inhibitor p21^{Cip1} (Abbas and Dutta, 2009). As previously mentioned, p21^{Cip1} can function to inhibit CDK1, CDK2, and PCNA, all of which have been shown to result in cell cycle arrest (Abbas and Dutta, 2009) (See Section 1.7.5.). As such, the study was interested to investigate if ACLY-overexpressing cells were able to respond to the presence of DSBs by initiating cell cycle arrest.

First, cell growth of ACLY-overexpressing cells continued to be sustained up to the 72hr time-point (Fig. 36). A close examination on ACLY-overexpressing cells revealed that they were able to progress through the cell cycle from the G₁/S phase transition to M phase without any delay (Fig. 38A-B). It was then discovered that p21^{Cip1} expression levels remain unaltered instead of being increased (Fig. 37B). This could be attributed to the lack of proper Chk2 responses, as shown by little changes in its phosphorylation status (Fig. 30B). As a consequence, ACLY-overexpressing cells were able to grow normally despite the presence of DSBs, which may have contributed to the formation of nuclear and chromosomal abnormalities. Although the results suggest the possible role of ACLY in deregulating ATM pathway, the experimental design was insufficient to provide conclusive evidence on it.

4.3.7. Phosphorylation of p53 was deregulated by ACLY overexpression in the presence of tBHP-induced oxidative stress

It became increasingly clear that there were limitations in the experimental design to provide substantive evidence on whether ATR- and ATM-mediated DNA damage responses were deregulated in ACLY-overexpressing cells. Although ACLY has been found to be responsible for decreasing ATR phosphorylation (Fig. 28B), the corresponding decrease in pChk1 levels could not be sufficiently detected on Western blots since its basal phosphorylation levels were already significantly low (Fig. 29B). In addition, basal levels of naturally-occurring DSBs were found to be considerably low in doxycycline-untreated HME1-ACLY compared to VP16-treated cells based on neutral comet assays (Fig. 26). Even when ACLY-overexpressing cells were displaying increased levels of DSBs, the magnitude of the damages were not extensive with only small-scale changes reported (Fig. 26). As such, any significant effects of ACLY on ATM, Chk2, and Nbs1 phosphorylation would potentially be difficult to capture on Western blots. Due to these limitations, it was difficult to be certain on the cells' predisposition to DNA misrepair, unregulated cell growth, and survival in the presence of DNA damage. Although it has been widely accepted that cells are vulnerable to endogenous sources of DNA damage (Hakem, 2008), levels of such damage are minimal as reflected by low basal levels of DNA damage responses. Furthermore, it is highly improbable for a cell to experience high levels of endogenous DNA damage. This means that the frequency of endogenous DNA damage occurring in a large population of cells would be very low, and any initiation of DNA damage responses would either be too minute to be detected, or translated into small fold changes.

To address these concerns, changes were made to the experimental design. An experiment was performed that involved treating ACLY-overexpressing cells with 200 μ M of tBHP, pushing most cells to undergo oxidative stress. This would serve to amplify the effects of ROS-induced DNA damage that cells experience as a by-product of active respiration. With this experimental design, any deregulation of DNA damage responses by ACLY could be easily detectable. It should be noted that tBHP treatment occurred after cells were induced to overexpress ACLY for only 24hrs. Any effects ACLY may have exerted on the cells at 48hr and 72hr time-points were not taken in consideration.

The activity of ATR- and ATM-mediated DNA damage responses were then determined over Western blots by examining the phosphorylation status of Chk1 (Ser345), Chk2 (Thr68), Nbs1 (Ser343), and also p53 (Ser15). In a similar trend as control cells (HME1-Empty), ACLY-overexpressing cells were found to display an increased phosphorylation of Chk1, Chk2, and Nbs1 (Fig. 42B, 43B, 43C). These results suggest that ATR and ATM kinases were likely active in ACLY-overexpressing cells to respond to oxidative-induced replication stress and DSBs. This is so as ATR is known to phosphorylate Chk1 at Ser345 in the presence of replication stress (Patil *et al.*, 2013), and that ATM is known to phosphorylate Chk2 at Thr68 and Nbs1 at Ser343 in the presence of DSBs (Cai *et al.*, 2009; Wu *et al.*, 2000; Zhang *et al.*, 2004) (Refer to Section 1.7.4.).

However, p53 phosphorylation seemed to be deregulated by ACLY especially at the early time-points after tBHP treatment, and levels remained lower than control cells up till the 24hr time-point (Fig. 44B). This could be caused by the

down-regulation of total p53 in ACLY-overexpressing cells, where levels were found to be lower than control cells throughout the experiment (Fig. 44B). Quantitative PCR was not performed to determine if transcriptional activity of p53 was reduced, thus the possible role of ACLY in modulating expression of p53 remains to be examined. On the other hand, p53 could be down-regulated through the ubiquitin-dependent proteosomal degradation pathway. Under low stress conditions, p53 has been characterised to be regulated through an orchestrated balance of its expression and degradation (Chao, 2015). Degradation of p53 is mediated by several E3 ligases, which include MDM2 and ARF-BP1 (Chao, 2015). These E3 ligases mediate the polyubiquitination of p53, targeting it for 26S proteosomal degradation (Chao, 2015). The degradation of p53 has been shown to be advantageous for tumourigenesis. Studies on various sarcoma samples identified the presence of *MDM2* gene amplification, thus contributing to MDM2 overexpression and subsequent loss of p53 activity (Oliner *et al.*, 2016). Hence, through an unknown mechanism, ACLY overexpression could promote p53 degradation, resulting in lower levels of phosphorylation in the presence of oxidative stress. Few studies have shown how metabolic reprogramming could down-regulate p53. Instead, extensive studies have characterised the role of p53 in modulating carbohydrate metabolism by (1) decreasing gene expression of glucose transporters, namely GLUT1, GLUT2, and GLUT3, and (2) suppressing glycolysis (Napoli and Flores, 2017). It could be possible that the decrease in p53 levels caused by ACLY overexpression (Fig. 44B) could promote the *Warburg effect* by increasing carbon flow through the glycolytic pathway. Nevertheless, it is important for further works to determine if p53 was degraded through the ubiquitin-dependent proteosomal pathway.

The deregulation of p53 phosphorylation corresponded to lower levels of p21^{Cip1} expression at 4hr the time-point (Fig. 45B). Furthermore, there was likely post-translational modifications occurring on p21^{Cip1} at 8hr and 12hr post-tBHP treatment, which was shown by slight band shifts on the western blots (Fig. 45A). These band shifts were demonstrated in one study, where p21^{Cip1} was found to be hyperphosphorylated to promote G₂/M phase progression (Dash and El-Deiry, 2005). In that study, it was reported that p21^{Cip1} hyperphosphorylation led to a band shift to an approximate molecular weight of 23 kDa on western blots. Through an unknown mechanism, it is possible for ACLY to induce p21^{Cip1} phosphorylation to promote cell cycle progression in the presence of oxidative DNA damage. Hence, further investigation is required to determine if ACLY was able to promote cell cycle progression in the presence of oxidative stress by impairing p21^{Cip1} activity.

The deregulation of p53 responses may have other implications on the cell's ability to promote DNA damage repair and induce apoptosis. First, activation of p53 has been shown to promote DNA repair through nucleotide excision repair (NER), base-excision repair (BER), and mismatch repair (MM), all of which can repair DNA lesions caused by oxidative stress (Sengupta and Harris, 2005). Second, as mentioned, activated p53 can promote apoptosis through the transcriptional activation of pro-apoptotic proteins, which include Bax and PUMA (Miyashita and Reed, 1995; Nakano and Vousden, 2001; Pitolli *et al.*, 2019). Hence, further investigations could be made to determine if the deregulation of p53, in the presence of oxidative stress, would lead to a decreased

expression of its target genes. Several target genes could be explored, which include (1) p48^{DDB2} and XPC involved in NER, (2) MSH2 involved in MM, and (3) PUMA, NOX and Bax involved in apoptosis (Pitolli *et al.*, 2019; Sengupta and Harris, 2005).

However, the results presented here seemed to be contrary to earlier findings that suggests the likely deregulation of ATR pathway, as shown by low ATR phosphorylation and total Chk1 levels (Fig. 28B, 29C). This could be attributed to other unknown effects induced by prolonged ACLY overexpression, where cells were made to overexpress ACLY for up to 72hrs. These effects were not fully replicated in this experiment as cells were induced to overexpress ACLY for only 24hrs prior to tBHP treatment. Hence, these results should not disregard earlier findings that showed the role of ACLY overexpression in deregulating ATR pathway. Furthermore, the possible involvement in ACLY in disrupting ATM signalling in the presence of DSBs should still be considered and investigated. Further works should be done by inducing oxidative stress on ACLY-overexpressing cells at 48hr and 72hr time-points under similar conditions represented in Figure 28A, Figure 29A, and Figure 30A.

4.3.8. Metabolic by-products likely produced from ACLY overexpression may have impaired DNA damage responses

As previously described, ACLY catalyses the production of Acetyl-CoA from citrate released from the mitochondria (Bauer *et al.*, 2005). This nuclear-cytoplasmic pool of Acetyl-CoA is not only utilised by HATs to facilitate histone acetylations, it also feeds into the fatty acid biosynthesis and mevalonate pathways to synthesize complex lipids and isoprenoids respectively (Zaidi *et al.*,

2012). The production of these metabolic products are essential as building blocks for cell growth (Buhaescu and Izzedine, 2007; Zaidi *et al.*, 2012).

A by-product of the fatty-acid synthesis pathway, namely long-chain fatty acid palmitate (palmitic acid), was found to deregulate p53 phosphorylation in mouse embryonic fibroblast cells. In that study, palmitic acid impaired p53 phosphorylation at Ser15 in cells that were either irradiated, or treated with hydroxyurea and doxorubicin (Zeng *et al.*, 2008). As a result, the expression of p21 and Bax could not be induced. Other saturated fatty acids such as stearic acid and myristic acid were found to induce similar effects (Zeng *et al.*, 2008). Although the modulation of DNA damage responses by saturated fatty acids were found in mouse embryonic fibroblast cells, this phenomenon could likely occur in HME1 human breast epithelial cells that were used in this study. Hence, it is plausible that the reduced levels of p53 phosphorylation found in ACLY-overexpressing cells (shown in Figure 44A-B) could be caused by an increased synthesis of saturated fatty acids.

The same study also discovered how palmitate could impair ATR and Chk1 phosphorylation when the cells were irradiated or treated with doxorubicin (Zeng *et al.*, 2008). With this finding, it is possible that the accumulation of palmitate after prolonged ACLY-expression could lead to reduced levels of ATR phosphorylation as observed (Fig. 28B). As such, further investigation is required to bring clarity on whether ACLY-induced metabolic reprogramming, through the elevation of the fatty acid synthesis pathway, deregulates the ATR pathway in the presence of oxidative stress.

Altogether, ACLY overexpression could facilitate the increased production of saturated fatty acids through the fatty acid synthesis pathway, which could impair ATR pathway activation and p53 responses in the presence of oxidative DNA damage. As such, further works could be done to measure levels of saturated fatty acids, particularly palmitate, in ACLY-overexpressing cells.

4.3.9. Summary of findings

Having considered all of the data, it was apparently clear that ACLY overexpression did not epigenetically silence the expression of DNA damage response proteins. This could be shown by undetectable changes in H3 and H4 histone acetylations (Fig. 39), and the small fold changes in total protein levels in most of the DNA damage response proteins examined here, namely, ATR, ATM, RPA32, Chk2, Nbs1, 53BP1, Bax, PUMA, and even p21^{Cip1} (Fig. 28C, 29C, 30C 32B, 34B, 37B).

Next, the ATR pathway was found to be deregulated by ACLY as shown by the decrease in ATR phosphorylation and total Chk1 levels from basal levels (Fig. 28B, 29C). It was determined that ACLY, through an unknown mechanism, promoted the degradation of phosphorylated ATR and total Chk1 proteins via the proteasome-dependent pathway (Fig. 31). These events could cause cells to be susceptible to replication stress and consequently DSBs. ACLY overexpression may have caused the accumulation of saturated fatty acids to sufficiently impair ATR phosphorylation. These saturated fatty acids could be involved in targeting phosphorylated ATR, along with Chk1, to be degraded via the proteasome-

dependent pathway. Although this proposed mechanism presents an unlikely link between the by-products of metabolic pathways (saturated fatty acids) and the deregulation of ATR pathway, it is worthy of further investigation.

Since ATR phosphorylation was found to be decreased below basal levels, cells were expected to experience prolonged replication stress and consequentially DSBs. This was the case where increased levels of DSBs were found in neutral comet assays (Fig. 26), and an increased formation of γ H2AX and 53BP1 foci were observed (Fig. 25, 33). However, there was no significant increase in the corresponding activity of the ATM pathway (Fig. 28B, 30B). Interestingly, a statistically significant reduction of phosphorylated Chk2 was observed in ACLY-overexpressing cells. These results seemed to suggest that ATM activation and signalling toward downstream substrates, specifically Chk2 and Nbs1, were likely disrupted. Given the nature of the experimental design that only allowed small amounts DNA damage from endogenous sources to occur naturally, it could potentially be difficult to detect any significant changes in levels of ATM, Chk2, and Nbs1 phosphorylation in ACLY-overexpressing cells on Western blots. Further investigations are therefore required to verify whether ATM pathway was deregulated, and if γ H2AX and 53BP1 foci formation occurred independently from it.

ACLY overexpression was also found to deregulate p53 activation in the presence of oxidative stress, as shown by reduced levels of p53 phosphorylation (Fig. 44A-B). This could be attributed to the down-regulation of total p53 (Fig. 44B). Through unknown mechanisms that need to be verified in future studies,

ACLY could act to transcriptionally repress p53 expression or induce its degradation through the ubiquitin-dependent proteosomal pathway. On the other hand, p53 phosphorylation could also be impaired by the possible increase in fatty acid synthesis, since a study had discovered this phenomenon and ACLY is known to be metabolically linked to the fatty acid synthesis pathway (Bauer *et al.*, 2005; Zaidi *et al.*, 2012; Zeng *et al.*, 2008).

Due to the deregulation of p53 responses, timely expression of p21^{Cip1} was disrupted in the presence of tBHP-induced oxidative stress (Fig. 45B). The lack of p53 responses could also account for the little changes in Bax, PUMA and p21^{Cip1} levels observed in Western blots (Fig. 34B, 37B), and low apoptotic activity observed in the TUNEL assay (Fig. 35). As a result, cell growth was sustained (Fig. 36, 38A-B), allowing elevated levels of DSBs to persist and remain constant for prolonged periods of time up to the 5-week time-point (Fig. 26). This encouraged DSBs to be left unrepaired or misrepaired, leading to the formation of nuclear and chromosomal abnormalities (Fig. 19-21).

Therefore, further investigations should be made to provide mechanistic insights on how ACLY deregulates the ATR pathway and p53 in the presence of oxidative stress. In addition, future works can identify whether p21^{Cip1} was hyperphosphorylated, and if found to be so, to establish the mechanisms behind it. Lastly, the possible impairment of Bax activation could be examined in the presence of an apoptotic stimulus. Findings will hopefully bring clarity on the role of ACLY-induced metabolic alterations on impairing various DNA damage response mechanisms to promote genome instability.

Figure 46. ACLY overexpression deregulated DNA damage responses in the presence of DNA damage from endogenous sources. ACLY overexpression was found to deregulate ATR pathway by promoting the degradation of phosphorylated ATR (Thr1989) and total Chk1 proteins through the proteasome-dependent pathway. Furthermore, ACLY overexpression was found to reduce p53 phosphorylation at Ser15 in the presence of oxidative stress. The deregulation of the ATR pathway could have caused the cells to be susceptible to replication stress, and consequentially contribute to the increased presence of DNA double stranded breaks. Elevated levels of γ H2AX and 53BP1 foci formation were detected, but activity of the ATM pathway remained unchanged. This led to the postulation that γ H2AX foci formation was mediated by DNA-PK, while 53BP1 foci formation occurred upstream of the ATM pathway, both of which were not investigated. Next, the reduced levels of p53 phosphorylation could have prevented the expression of its target genes, namely p21^{Cip1}, PUMA, and Bax. As a result, growth was sustained in the presence of DNA double stranded breaks, causing them to be left unrepaired or misrepaired. This tolerance to the persistent presence of DNA damage, coupled with low apoptotic activity, could have resulted in the formation of nuclear and chromosomal abnormalities. In addition, ACLY overexpression may impair p21^{Cip1} and Bax activity, where future works will seek to bring clarity.

4.4. ACLY overexpression may have produced other metabolic by-products to promote anchorage-independent growth

Elevated levels of DSBs, coupled by the ability to sustain cell growth, leads to increased tolerance to DNA damage. This can account for the formation of nuclear and chromosomal abnormalities as described (Fig. 19-21). The presence of these structures could favour the accumulation of somatic mutations in ACLY-overexpressing cells, increasing the probability of malignant transformation. One of the characteristics of cancers is the ability to grow under anchorage-independent conditions, which is often associated with increased metastatic potential (Guadamillas *et al.*, 2011).

The soft agar colony formation was thereby performed to determine if these cells have acquired the ability metastasize. This was found to be true as shown by an increased amount of colonies formed by specimens that were overexpressing ACLY (Fig. 41A-B). In addition, the increase in colonies formed in ACLY-overexpressing specimens seemed to correspond to sustained elevated levels of DSBs, as shown by higher mean γ H2AX foci formation and olive tail moment at the 5-week time-point (Fig. 25, 26). However, the study did not examine the somatic DNA mutation patterns in these cells, and how they might enhance anchorage-independent proliferative fitness. As such, it cannot be concluded with certainty that the presence of nuclear and chromosomal abnormalities seen this study contributed to the malignant transformation of ACLY-overexpressing cells. Large scale mutation screens could be performed on these cells by amplifying all exons according to the consensus coding DNA sequence database (CCDS) through polymerase chain reactions (PCR) (Sjöblom *et al.*, 2006). A mutation analysis could then be performed using DNA from control cells

(HME1-Empty) to identify patterns of somatic mutations that were occurring as a result of ACLY overexpression. There, genes that are more frequently mutated can be validated with the list candidate genes known to be associated with anchorage-independent growth (Sjöblom *et al.*, 2006).

On the other hand, ACLY overexpression could have promoted anchorage-independent growth by accelerating carbon flow through the mevalonate pathway. The mevalonate metabolic pathway synthesizes farnesyl or geranylgeranyl isoprenoids, which are then utilised to prenylate specific proteins in an enzymatic reaction known as farnesylation or geranylgeranylation (Wang and Casey, 2016). The prenylation of specific proteins are important to induce their localisation to cellular membranes due to their hydrophobic C-terminus. Increased activity of the mevalonate pathway has been shown to promote constitutive prenylation of Ras GTPase. This increases trafficking of prenylated-Ras toward the plasma membrane, resulting in sustained Ras activity (Gruenbacher and Thurnher, 2018). As a consequence, aberrant cell proliferation can be promoted (Hobbs and Der, 2019). Furthermore, constitutive Ras activity has been found to induce DNA hypermethylation to epigenetically silence the expression of *Fas* gene. The subsequent down-regulation of Fas receptor expression impairs the ability of cells to undergo anoikis under anchorage-independent conditions (Gazin *et al.*, 2007). Hence, ACLY overexpression could promote anchorage-independent growth through increasing mevalonate pathway activity. Further investigation is therefore required to examine whether the enhancement of anchorage-independent proliferative fitness seen in ACLY-

overexpressing cells were a result of genome instability or a result of increased mevalonate pathway activity.

4.5. ACLY overexpression may potentially contribute to other aberrant epigenetic modifications not investigated

There is little evidence to speculate that ACLY overexpression could cause histone methylations and DNA methylations. Thus far, a study has shown how the mevalonate pathway could promote DNA methylation to silence expression of Fas receptors (Gazin *et al.*, 2007; Gruenbacher and Thurnher, 2018). Future works may consider to determine if there are such aberrant epigenetic modifications occurring in ACLY-overexpressing cells.

Since the present study involved the use of human breast epithelial cells, investigations could examine the presence of epigenetic modifications that have been reported in several breast cancers. For example, histone methylation at H3K9, and demethylation at H3K4 has been found in several breast cancers (Bradley, 2007; Kondo *et al.*, 2008; Shi, 2007). In addition, one study has uncovered 168 genes that were altered in breast cancers as a result of DNA methylations (de Almeida *et al.*, 2019).

4.6. Conclusion

It was hoped that this study would bring about a holistic understanding on the opposing effects of Acetyl-CoA and NAD⁺ metabolism on genome stability (Fig 5). Since NAD⁺ modulates sirtuin activity to maintain genome integrity (Sivanand *et al.*, 2017), it was proposed that Acetyl-CoA could lead to an unstable genome, which consequently promote tumourigenesis. To investigate

this, the study used ATP citrate lyase (ACLY) as a mean to induce metabolic changes in the cell.

ACLY overexpression caused an increased presence of nuclear and chromosomal abnormalities, as shown by the presence of MN, NBUD, and NPB structures, and chromatin bridges (Fig. 16B, 19A-C, 20A-C). These results suggest the presence of DNA damage. In addition, the presence of NPBs and chromatin bridges were suggestive of DNA misrepair (Fig. 20A-B, 21A-B), where they can lead to aberrant chromosomal translocations. These abnormalities were caused by the presence of DSBs, as shown by increased γ H2AX and 53BP1 foci formation, increased mean olive tail moments, and the presence of DNA damage-associated centrosome amplifications (Fig. 22, 25, 26, 33B).

Next, ACLY overexpression did not induce the generation of intracellular ROS to cause these DSBs (Fig. 27), but was found to impair p53 phosphorylation (Fig. 44B) and promote degradation of phosphorylated ATR and Chk1 through the proteasome degradation pathway (Fig. 28B, 29C, 31). Furthermore, ACLY did not induce genome wide histone acetylations (Fig. 39), which could explain the unaltered expression of most DNA damage response proteins examined, specifically ATR, ATM, RPA32, Chk2, Nbs1, 53BP1, Bax, PUMA, and even p21^{Cip1} (Fig. 28C, 29C, 30C, 32B, 34B, 37B). The deregulation of ATR pathway could cause cells to be susceptible to prolonged stalling of replication forks, where they subsequently collapse to form DSBs. Although this should have led to an increase in ATM pathway activity, this was not clearly seen (Fig. 28A-B, 30A-B). Coupled by the lack of p53 responses to the presence of DNA damage,

these events could have resulted in the inability of cells to initiate cell cycle arrest, and even apoptosis (Fig. 35, 38A-B). Growth was sustained in these cells (Fig. 36), which may have caused DSBs to be left unrepaired or misrepaired. All of these could have promoted the formation of nuclear and chromosomal abnormalities (Fig. 19-21).

Lastly, the presence of these nuclear and chromosomal aberrations could have resulted in somatic mutations to favour anchorage-independent growth (Fig. 41A-B). However, it should be noted that ACLY overexpression could also modulate the mevalonate pathway to promote anchorage-independent growth.

Therefore, ACLY-induced metabolic reprogramming contributes to genome instability (Fig. 47). This is attributed by the inability of DNA damage responses to faithfully signal for the repair of naturally-occurring DNA damage from endogenous sources (Fig. 47). Future studies will need to provide further mechanistic insights on how ACLY was able to deregulate the activity of these DNA damage responses. Lastly, due to the instability of the genome, it may have promoted the acquisition of somatic DNA mutations that ultimately encourage malignant transformation of cells from a normal phenotype.

Figure 47. Summary of the contribution of ACLY to genome instability by deregulating DNA damage responses

4.7. Future works

Future studies should attempt to address if ACLY overexpression caused an increased synthesis of saturated fatty acids, and whether an accumulation of these metabolic by-products impaired ATR and ATM pathway activation and p53 phosphorylation in the presence of replication stress. The possible role of ACLY in down-regulating p53 levels in the cell, either through ubiquitin-dependent proteosomal degradation pathway or transcriptional repression, should be investigated as well.

Under oxidative stress conditions, ACLY-overexpressing cells could be further examined if they are able to initiate cell cycle arrest, DNA repair and even apoptosis. Expression levels of several p53 target genes involved in DNA damage repair and apoptosis could be analysed, which include (1) p48^{DDB2} and XPC involved in nucleotide excision repair, (2) MSH2 involved in mismatch repair, and (3) PUMA, NOX and Bax involved in apoptosis. Besides observing DNA repairs mediated by 53BP1, the activity of other DNA repair mechanisms could be closely examined, which include homologous recombination repair, nucleotide excision repair, base excision repair, and mismatch repair. In addition, further works could address if p21^{Cip1} was hyperphosphorylated, and if found to be so, to determine the mechanisms ACLY induced it. Since a study has demonstrated how metabolic alterations induced by GLUT1 could impair Bax activation (Rathmell *et al.*, 2006), the possible role of ACLY in deregulating Bax activity in the presence of an apoptotic stimulus could be explored. Findings will hopefully bring clarity on the origins of these nuclear abnormalities seen in this study (Fig. 16A-B, 19-20).

As mentioned, the presence of NBUD structures seen in ACLY-overexpressing cells may not be primarily caused by DNA damage (Fig. 19A-B, 20A-B). Instead, they could be a result of gene amplifications, which is a phenotype seen in several cancers (Albertson, 2006; Clancy, 2008). As such, these cells could be further tested for the presence of an array of amplified oncogenes, which include *c-MYC*, *EGFR*, and *ERBB2* (HER2/neu) that are found in breast cancers (Albertson, 2006; Hyman *et al.*, 2002; Shimizu *et al.*, 1998).

Further works could also determine if the presence of these nuclear abnormalities were able to promote the acquisition of somatic DNA mutations that favours tumourigenesis. In this study, ACLY overexpression was found to increase anchorage-independent proliferative fitness (Fig. 41), but no evidence was gathered to identify somatic mutations that could have promoted the phenomenon. As mentioned, large scale mutation screens could be performed to identify patterns of somatic mutations and the genes that are frequently mutated, which could be compared with the established list of candidate genes associated with cancers (Sjöblom *et al.*, 2006). Besides using the soft agar assay to examine the malignancy of ACLY-overexpressing cells, other established assays could be performed such as the focus formation assay, and the cell migration and invasion assay (Justus *et al.*, 2014).

Hence, these additional studies would hopefully affirm the role of metabolic alterations in tumourigenesis. Future therapeutics could be directed at modulating cellular metabolism to reduce the probability of cells from acquiring further

random somatic mutations that may promote the onset and progression of cancers.

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