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ATP-citrate lyase links cyclin E to cellular metabolism in breast cancer

Kim Lucenay

ATP-CITRATE LYASE LINKS CYCLIN E TO CELLULAR METABOLISM IN BREAST CANCER

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By

Kimberly S. Lucenay, B.A.
Houston, Texas

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DEDICATION

This dissertation is dedicated to my husband and parents:

Charles M. Lucenay

and

Alan and Peggy Szymanski

For their unconditional love, support and sacrifices they made for me, I am eternally grateful.

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ATP-CITRATE LYASE LINKS CYCLIN E TO CELLULAR METABOLISM IN BREAST CANCER

Kimberly S. Lucenay, B.A.
Advisory Professor: Khandan Keyomarsi, Ph.D.

Cyclin E is altered or overexpressed in approximately one-third of tumors from patients with invasive breast cancer and is a powerful independent predictor for survival in women with stage I-III breast cancer. Full-length cyclin E (EL) is post-translationally cleaved into two low-molecular-weight isoforms, LMW-E (T1) and LMW-E (T2). LMW-E have been shown to exhibit greater binding affinity for cyclin-dependent kinase 2 (CDK2) , cyclin dependent kinase inhibitors (CKIs), p21 and p27, but are resistant to p21 and p27 inhibition. In addition, transgenic mice expressing LMW-E have increased mammary tumor development and metastasis compared to EL transgenic mice. Therefore, LMW-E are more aggressive in cell cycle abrogation and mammary tumor development. The LMW-E isoforms are tumor specific and accumulate in the cytoplasm due to lack of a nuclear localization sequence (NLS). Therefore, we hypothesized that aberrant localization of LMW-E isoforms leads to molecular events that ultimately contribute to LMW-E breast cancer tumorigenicity. To address this hypothesis, we used a retrovirus-based protein complementation assay (RePCA) to identify LMW-E (T1) protein-protein interactions in breast cancer. Using this methodology, we found ATP-citrate lyase (ACLY) as a novel interacting protein of LMW-E (T1) in the cytoplasm. ACLY is a 125kDa homotetrameric enzyme that catalyzes cytoplasmic citrate to acetyl-CoA and oxaloacetate in the *de novo* lipogenesis

pathway. End products of this pathway consist of complex fatty acids that fuel membrane production of highly proliferating cells and lipid-based post-translational modifications that mediate protein-protein interactions. Additionally, we found that LMW-E upregulates ACLY enzymatic activity which leads to lipid droplet formation; thereby providing cells with essential building blocks to support growth. ACLY is also required for LMW-E mediated transformation, migration and invasion *in vitro*, as well as tumor growth *in vivo*. Taken together these data suggest a novel interplay between LMW-E and ACLY and how metabolic pathways and the cell cycle are linked in breast cancer tumorigenesis.

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ABBREVIATIONS

ACC	acetyl-CoA carboxylase
ACLY	ATP-citrate lyase
ALDO-A	aldolase A
AP-MS	affinity purification-mass spectrometry
BiF	bimolecular fluorescence
BL1	basal-like 1
BL-2	basal-like 2
BLIA	basal-like immune activated
BLIS	basal-like immune suppressed
CDK	cyclin dependent kinase
CKI	cyclin dependent kinase inhibitor
CSC	cancer stem cell
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA binding domain
DFS	disease free survival
DSS	disease specific survival
DTT	DL-dithiothreitol
EMT	epithelial-to-mesenchymal transition
ER	estrogen receptor
ERM	enhanced retroviral mutagen
FACS	fluorescence activated cell sorting
FASN	fatty acid synthase
FDG-PET	^{18}F fluorodeoxyglucose positron emission tomography
FRET	fluorescence resonance energy transfer
GFP	green fluorescence protein
GST	glutathione-S-transferase
HA	hemagglutinin
HER2	human epidermal growth factor receptor
HMEC	human mammary epithelial cell
IFPC	intense fluorescent protein C-terminus
IFPN	intense fluorescent protein N-terminus
IM	immunomodulatory
IPTG	isopropyl-β-D-1thiogalactopyranoside
LAR	luminal androgen receptor
LDs	lipid droplets
LMW-E	low molecular weight cyclin E
M	mesenchymal
MES	mesenchymal
MSL	mesenchymal stem-like
NLS	nuclear localization sequence
ORR	overall response rate
OXPHOS	oxidative phosphorylation
PCA	protein complementation assay
PMSF	phenylmethylsulfonyl fluoride

PPP	pentose phosphate pathway
PR	progesterone receptor
RePCA	retrovirus-based protein complementation assay
RTKs	receptor tyrosine kinases
SA	splice acceptor
SD	splice donor
TAD	transcriptional activation domain
TCA	tricarboxylic acid cycle
TDCs	tumor derived cells
TNBC	triple negative breast cancer
TTP	time to progression
Y2H	yeast-2-hybrid

CHAPTER 1: **INTRODUCTION TO THE LINK BETWEEN BREAST CANCER AND THE CELL CYCLE**

1.1 OVERVIEW OF BREAST CANCER

1.1a. Breast cancer statistics

Breast cancer is the most frequently diagnosed cancer in women with 1 in 8 women developing the disease over the course her lifetime and an estimated 231,840 new cases in 2015 alone [1]. Moreover, breast cancer in women continues to be a leading cause of cancer death, falling second to cancer of the lung and broncus [1]. Breast cancer develops when normal, healthy mammary cells acquire mutations, or abnormal changes that lead to uncontrolled growth. However, a genetic predisposition is responsible for only 5-10% of all breast cancers leaving 85-90% of breast cancers to arise sporadically [2]. Significant advances have been made in the identification of the genetic drivers of hereditary breast cancer, including BRCA1, BRCA2, CHEK2, ATM PALB2, p53 and PTEN mutations [2-5], but identification of the molecular mechanisms underlying sporadic breast cancer remain largely elusive for this complex disease.

1.1b. Molecular subtypes of breast cancer

Breast cancer is a heterogeneous disease composed of several molecular subtypes harboring distinct prognosis and outcomes. Clinical variables, on their own, such as tumor grade, tumor size and lymph node status are not sufficient to determine prognosis, therefore, determination of the molecular state of the tumor is required to drive treatment decisions [6]. A patient's overall prognosis is largely dependent on the molecular profile of the tumor itself. Studies analyzing gene expression using DNA

microarrays were able to segregate and differentiate breast cancers based on their unique gene expression profile [7]. Data from this seminal paper lead to our current system in subtyping breast cancer. At this time, there are six molecular subtypes of breast cancer: Luminal A, Luminal B, HER2-enriched, Basal-like (which includes triple-negative breast cancer "TNBC" and its relative subtypes), Claudin-low and normal-like.

Luminal A breast cancer originates in the inner (luminal) cells of the mammary ducts and is the most common breast cancer subtype [8, 9]. It is characterized by estrogen receptor positivity (ER+), and/or progesterone receptor positivity (PR+) and negative expression of human epidermal growth factor receptor 2 (HER2-) (Table 1). Luminal A tumors have a low proliferation rate as seen by $Ki67^{-/low}$ staining [7, 10]. The prevalence of luminal A breast cancer is 28-31% and while the relapse rate is 27.8%, overall, patients have a good prognosis with a 5-year survival rate of 95%, which is the highest of all the intrinsic subtypes [8, 11, 12]. Treatment for luminal A tumors usually requires only endocrine therapy. Interruption of the estrogen-signaling pathway has proven to be the most effective treatment for luminal A breast cancer. Selective estrogen receptor modulators (SERMs) such as tamoxifen; which inhibit the function of ER are given to premenopausal and postmenopausal patients and selective estrogen receptor down-regulators (SERDs) such as fulvestrant are given to postmenopausal patients [13, 14]. Additionally, postmenopausal patients may be given aromatase inhibitors such as anestrozole, exemestane or letrozole which block estrogen production or inactivate ER [8, 15]. However, luminal A tumors often become resistant to endocrine-based therapy and alternate treatment strategies are being developed [12]. The insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor

Table: 1 The six molecular subtypes of breast cancer

Subtype	Expression profile	Prognosis	Prevalence	Treatment
Luminal A	ER+ and/or PR+ HER2- Low Ki67	Good	28-31%	Hormone therapy
Luminal B	ER+/or PR+ HER2+/or HER2- with Hi Ki67	Poor	19-23%	Hormone therapy and/or chemotherapy
HER2-enriched	ER-/PR- HER2+ Hi KI67	Poor	12-21%	Targeted therapy (Trastuzumab, Lapatinib)
Basal-like	ER-/PR-HER2- EGFR Cytokeratin5/6	Poor	11-23%	Combination of surgery, radiation and chemotherapy
Claudin-low	ER-/PR-HER2- Ki67 low Claudin3,4,7 low CD44+/CD24 /-low EpCam-/low	Poor	7-14%	Chemotherapy
Normal-like	N/A	Good	3-10%	N/A

(+): positive
(-): negative

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(VEGF) signaling pathways have been shown to contribute to resistance and treatments using an anti-VEGF antibody, bevacizumab, along with the chemotherapeutic, paclitaxel, has been effective [16-18]. Additional therapies targeting VEGFR1, VEGFR2, KIT and PDGFR combined with tamoxifen treatment have been shown to effectively reduce tumor volume [19].

Luminal B breast cancer is similar to luminal A breast cancer in that it originates from the luminal cells of the mammary duct and is ER+ and/or PR+. However, luminal B breast tumors can either be HER2- or HER2+ and display an elevated proliferation rate as seen by high Ki67 staining [7, 8]. The prevalence of luminal B breast cancer is 19-23% and has a higher recurrence rate than luminal A breast cancer [11]. Overall, luminal B breast cancer is more aggressive and leads to a worse prognosis for patients due to its high proliferative capacity and higher histological grade [8].

Current treatment strategies for luminal B breast cancer rely on chemotherapy, endocrine therapy and aromatase inhibitors, but deciding on a treatment regimen can often be convoluted due to the heterogeneity of the tumor, innate chemotherapy insensitivity and resistance to endocrine therapy. Therefore, there are a few diagnostic tools that have been developed over the years that are commercially available to help drive treatment decisions. One type of diagnostic tool is Onco*type* DX. Onco*type* DX was developed by Genomic Health, Inc. and is used for women with early stage ER+ breast cancer [20]. This test helps estimate the likelihood of disease recurrence and also helps define benefits from chemotherapy [20]. Onco*type* DX analyzes gene expression levels from 16 cancer-related genes and normalizes their expression to five background genes in order to determine a Recurrence Score between 0-100 for the

patient [20]. For example, Paik et al. and colleagues found that node-negative ER+ breast cancer patients with a high Recurrence Score were likely to benefit from a chemotherapy regimen such as cyclophosphamide/methotrexate/fluorouracil (CMF), while a low Recurrence Score signified minimal benefit [21]. Therefore, diagnostic tools such as Onco*type* DX provide valuable information for clinicians in designing treatment strategies for ER+ breast cancer patients.

Luminal B tumors are also subject to becoming endocrine resistant [11]. Indeed, a study conducted by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) found that 33% of breast cancer patients treated with tamoxifen over five years are likely to develop recurrent disease within 15 years [22, 23]. Endocrine resistance develops by multiple mechanisms including loss of ERα protein expression, expressing truncated forms of either ERα or ERβ, modifications made to ERα post-translationally, or degregulation of co-activator proteins [22]. For example, overexpression of nuclear receptor co-activator 3 (NCOA3), a co-activator of ERα, results in constitutive ERα transcription and leads to estrogen resistance *in vitro* as well as *in vivo* [22, 24, 25].

Since endocrine resistance is a challenge, there is a focus to identify other biomarkers and molecular pathways that drive luminal B tumors. IGF-1R (Insulin growth factor 1 receptor) monoclonal antibodies are currently in phase I and II clinical trial either alone as a single agent or coupled with chemotherapy [26]. Other targets being investigated for luminal B breast cancer are FGF, PI3K and cyclin D1 [26]. Cyclin D1 is an attractive target for luminal B tumors due to its ability to drive proliferation through interaction with CDK4/6 [27].

Human epidermal growth factor receptor 2 or HER2 is a component of the epidermal growth factor receptor family [28]. HER2-enriched breast cancer is characterized by amplification of the HER2 gene located in the 17q12 chromosome and subsequent enrichment of the HER2 protein. It is also characterized by negative expression of ER and PR, but presents positivity for Ki67 [7, 8]. The prevalence of HER2-enriched breast cancer is 12-21% and has a poor prognosis due to positive lymph node status, poor tumor grade, early and frequent recurrence of the tumor, as well as metastasis to the liver and lungs [11, 29]. Additionally, women are diagnosed at an earlier age compared to luminal A or luminal B breast cancer and up to 40% have p53 mutations [8, 9].

Treatment for HER2 positive breast cancer relies mainly on chemotherapy and targeted therapies such as trastuzumab (Herceptin) and lapatinib (Tykerb). Trastuzumab is a humanized monoclonal antibody that targets domain IV in the extracellular domain of HER2 and prevents signal transduction [12, 30]. The combination of trastuzumab plus chemotherapy resulted in increased response and overall survival in HER2 positive metastatic breast cancer patients. Specifically, the overall survival rate increased from 20.3 months to 25.1 months under combination treatment vs. chemotherapy alone [31]. These data along with increased overall response rates (ORR) and time to progression (TTP) rates made this regimen a first-line treatment for HER2 positive metastatic breast cancer in 1998 [31].

A derivative of trastuzumab, pertuzumab, is a monoclonal antibody that targets domain II of HER2 and prevents dimerization [31]. Presently, the first-line treatment for metastatic HER2 postive breast cancer resides from the results of the CLEOPATRA

trial which combined trastuzumab plus docetaxel with or without pertuzumab [31]. Patients in the pertuzumab group displayed significant progression free survival and overall survival [31].

Additional treatment strategies being used to target HER2+ breast cancer is the use of antibody-drug conjugates which specifically target tumor cells to deliver cytotoxic agents [12, 32]. Trastuzumab-emtansine (T-DM1) is currently being used in clinical trials and combines trastuzumab and DM1, a microtubule inhibitor [33, 34]. Patients given T-DM-1 showed an overall response rate of 25.9 to 34.5% [35].

One of the on-going challenges with HER2+ breast cancer is resistance to HER2-targeted therapies, such as trastuzumab. It has been shown that HER2+ breast cancer patients with metastatic disease do quite well initially with trastuzumab treatment, but exhibit progression of the disease within at least one year [36]. There are multiple mechanisms attributed to trastuzumab resistance, including increased activation of PI3K/Akt pathway either by activation of other growth factor receptors like IGF1R or by mutations in the tumor suppressor, PTEN [36]. Moreover, steric hindrance by mucin-4 (MUC4) potentially blocks trastuzumab and HER2 from interacting [36]. Therefore, potential targets bring investigated to treat HER2 positive breast cancer with trastuzumab resistance include inhibitors toward mTOR and PI3K pathways [30].

Another treatment option for HER2+ breast cancer patients are anti-HER2 vaccines [37]. Treating cancer with a vaccine has the potential to induce a long-lasting immune response that could be manufactured against multiple antigens. Moreover, with the help of memory cells within the immune system, cancer vaccines would eliminate risk of recurrence [37]. HER2-based vaccines are currently in phase II clinical

trial [38]. A peptide-based method, called E75, targets amino acids 369-377 in the extracellular domain of HER2. In this trial, 195 patients were given up to six doses, with about half receiving additional immunizations [38]. In the end, although DFS was not statistically significant between treated and non-treated patients, risk of recurrence was down 48% in the treated group [38]. Moreover, in node-positive patients, the differences were greater. Specifically, patients treated with the E75 vaccine had a 53% decrease in recurrence risk along with 24 month DFS rate of 90.2% compared to 79.1% for untreated patients [38].

Basal-like breast cancer is a very aggressive subtype of breast cancer that arises from the basal cells or outer cells surrounding the mammary duct. Basal-like breast tumors exhibit histological features of basal cells including positivity for high molecular weight cytokeratins such as cytokeratin 5, 6, and 17 [8, 39]. These tumors also express P-cadherin, caveolin 1 and 2, nestin, CD44, and EGFR, but do not express ER, PR or HER2 and are often referred to as “triple negative” [8, 29]. However, basal-like breast cancer and triple-negative breast cancer (TNBC) are not synonymous due to a discordance rate of up to 30% [8, 40]. A basal-like IHC profile has been identified and has been coined the “Basal Core Group” and is based on the expression of five markers: ER, PR, HER2, EGFR, and CK5/6. This group of markers has 100% specificity and 76% sensitivity when identifying basal-like breast tumors [8, 41].

TNBC, on the other hand, was subtyped in 2011 by Lehmann et al. by gene expression profiling from 21 breast cancer data sets and found that TNBC can be stratified into 6 subtypes: basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory

(IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR) [42]. The BL1 and BL2 subtype displayed a gene expression signature consisting of cell cycle and DNA damage response genes and the M and MSL subtype displayed a gene expression signature for genes involved in the epithelial-mesenchymal transition and growth factor pathways. The LAR subtype displayed expression of genes in the androgen receptor signaling pathway. More recently, Berstein et al. also subtyped TNBC tumors from 198 patients and found only four subtypes: Luminal–AR (LAR), mesenchymal (MES), Basal-like immune suppressed (BLIS) and Basal-like Immune-Activated (BLIA) [43]. These two studies provide overlap for the LAR and MES TNBC subtypes, but lack agreement on the other four Lehmann subtypes. Nevertheless, the ongoing efforts to classify TNBC provide a foundation for molecular-targeted therapies and treatment strategies for patients afflicted with TNBC.

The prevalence of basal-like breast cancer is 11-23% and is more common in premenopausal women and women of African decent [9, 11]. Unfortunately, prognosis for basal-like breast cancer is poor due to large tumor size at presentation, high histological grade and proliferation rate, lymph node positivity, and lack of targeted treatment strategies [8, 44]. Surgery, chemotherapy and radiation are the main treatment strategies for basal-like breast tumors [45]. Basal-like tumors have a high rate of p53 mutations as well as BRCA1 mutations, both of which function in DNA damage repair [8]. Thus, these tumors initially respond very well to chemotherapy, however, the relapse rate is common within the first one to three years [46, 47]. In patients with BRCA1 mutations, inhibition of PARP-1 (poly-ADP-ribose-polymerase- 1),

which also aids in DNA repair, leads to cell death due to the accumulation of DNA damage; a concept known as synthetic lethality. One study showed that 47% of breast cancer patients with BRCA1 and BRCA2 mutations responded to treatment using the PARP-1 inhibitor, Olaparib (AZD2281), and 63% showed clinical benefit from the drug [48].

In addition to PARP inhibitors as a targeted therapy to treat basal-like breast cancer, inhibition of EGFR is also promising due to its overexpression in TNBC [45]. However, the efficacy of the EGFR monoclonal antibody, cetuximab, is modest. A randomized phase III trial evaluating cisplatin with and without cetuximab showed a response rate of 20% and progression free survival extended about 2 months [49]. Other studies have investigated the efficacy of small molecule tyrosine kinase inhibitors toward EGFR as a monotherapy with little success and may be beneficial to combine these small molecule inhibitors with a chemotherapy regimen [50]. Lastly, other targets being evaluated for treatment of basal-like breast cancer include c-Kit, EGFR, VEGFR, mTOR, αβ-crystallin, Src, HDAC inhibitors, HSP90 and the JAK/STAT signaling pathway [51, 52].

The claudin-low subtype was first discovered in 2007 by Herschkowitz et al. and has a similar gene expression pattern to the basal-like subtype including negativity for ER, PR and HER2, but differs from the basal-like group in that claudin-low tumors display low expression of tight junction genes claudin 3, 4, 7 and occludin and the intercellular adhesion gene, E-cadherin [8]. Furthermore, genes involved in the immune response expressed by T- and B-lymphoid cells that infiltrate the tumor are unique to the claudin-low group [53, 54]. Additionally, claudin-low tumors express

genes related to the cancer stem cell (CSC) phenotype such as $CD44^{+/high}/CD24^{-/low}$ along with mesenchymal features such as vimentin and N-cadherin expression [11]. The prevalence of claudin-low breast cancer is 7-14% and, overall, has a poor prognosis [11, 29]. Generally, the poor prognosis of claudin-low tumors is due to radiation and chemotherapy insensitivity, and tumor relapse due to a residual CSC population [11]. Specifically, *in vitro* studies evaluated patient breast tumor biopsies before and after neoadjuvant chemotherapy and showed that chemotherapy increased the percentage of $CD44^{+/high}/CD24^{-/low}$ cells and these cells were more capable of mammosphere formation; both characteristics of CSCs [55]. Therefore, identification of signaling pathways that regulate self-renewal and thus, compounds that target these pathways would provide a treatment strategy for claudin-low tumors. Lastly, normal-like tumors have a prevalence rate of 3-10%, a gene expression profile that resembles the normal breast and the prognosis overall is very good [11].

1.2 THE CELL CYCLE AND CANCER

1.2a. The cell cycle

The mammalian cell cycle is a series of synchronized events a single cell must accomplish in order to divide into two daughter cells and consists of two major parts: interphase and mitosis [56]. Interphase encompasses G1, S and G2 phases of the cell cycle and is a period for cell growth (schematic of the mammalian cell cycle Figure 1). G1 is the gap phase between mitosis and DNA replication in which cells grow in

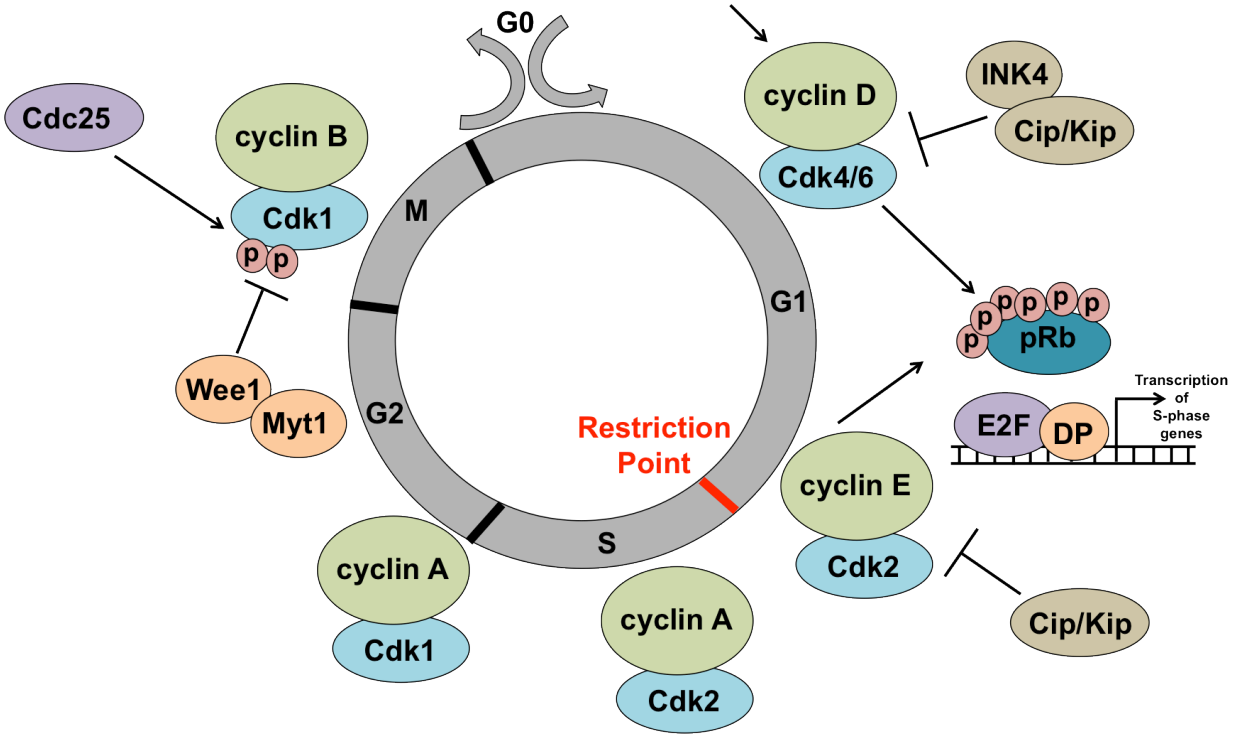

Figure 1. Schematic of the mammalian cell cycle

The mammalian cell cycle consists of 5 phases: G0, G1, S, G2 and M. Progression though one cycle first requires growth factor stimulation and subsequent activation of cyclin/CDK complexes. At the restriction point, the cell is fully committed to the cell cycle and can proceed in the absence of growth factors. G1 cyclin/CDK complexes are controlled by Cip/Kip and INK4 families of inhibitors, while cyclin B/CDK1 are tightly controlled by phosphorylation and dephosphorylation by Cdc25 and Wee1/Myt1, respectively.

volume, analyze growth signals and prepare for DNA replication [57]. DNA synthesis occurs in S phase and G2 is the gap phase between S phase and M phase in which the cell prepares for cell division. Mitosis, or M phase, occurs just prior to cell division when chromosomes condense, align, sister chromatids separate and cytokinesis occurs to form two daughter cells [57]. Cells can reversibly exit the cell cycle at G0 phase and enter a quiescent or non-proliferative state when nutrients are lacking or cell adhesion is inhibited. When conditions become more favorable quiescent cells are able to re-enter the cell cycle [57-59]. In fact, the majority of the non-proliferating cells in the human body are in G0 [60].

Our knowledge of the mammalian cell cycle stemmed from studies using single cell eukaryotes and marine invertebrates [57]. Progression through the mammalian cell cycle occurs mainly through phosphorylation events facilitated by cyclins and their partners; cyclin-dependent kinases (CDKs) [56]. Cyclins, as their name implies, oscillate in expression during the cell cycle and are synthesized and degraded in a highly orchestrated fashion, while CDK protein expression remains stable [61]. To date, there are as many as 29 cyclin or cyclin-related proteins (A1, A2, B1, B2, B3, C, D1, D2, D3, E1, E2, F, G1, G2, H, I, J, K, L1, L2, M1, M2, M3, M4, O, T1, T2, Cables 1, Cables 2) and are related to one another by 150 conserved amino acid residues called ‘the cyclin box’. In addition there are at least 13 CDKs (CDK1-13) [62, 63]. However, only specific cyclin/Cdk complexes have the required kinase activity to drive the cell cycle forward in a timely fashion (Table 2) [62, 63].

Unlike other cyclins, D-type cyclins levels do not oscillate and are controlled by the presence or absence of growth factors [57]. Mouse knockout studies of

Table: 2. Biological functions of cyclins and CDKs in the mammalian cell cycle

Cyclin or CDK	Biological function	Cell cycle binding partner	Knockout phenotype	Viability
Cyclin A	Cell cycle (G1-S) and (G2-M)	CDK1 and CDK2	-A1: males are sterile due to arrest in meiotic prophase -A2: Defective mitosis	A1: Viable A2: Embryonic lethal before E5.5
Cyclin B	Cell cycle (G2-M)	CDK1	-B1: Embryonic lethal -B2: Develop normally, males and females are fertile.	B1: Lethal at E10.5 B2: Viable
Cyclin C	Cell cycle (G0-S) Transcription	CDK3 and CDK8	ND*	ND*
Cyclin D	Cell cycle (G0-S)	CDK4 and CDK6	-D1: Small body size, hypoplastic retinopathy, defective breast development during pregnancy, neuropathy -D2: Defective ovarian granulosa cell development and female sterility. Males have hypoplastic testes but are fertile. Impaired proliferation of peripheral B-lymphocytes. -D3: Hypoplastic thymus with loss of T-cell maturation from (CD4-,CD8-) to (CD4+, CD8+) cells due to cytokine-independent defects in pre-TCD signaling. -D2 and D3: Severe megaloblasticanemia -D1 and D3: Neuropathy leading to meconium	D1: Viable D2: Viable D3: Viable D2 and D3: Embryonic lethal before E18.5 D1 and D3: Death at P1, but few survive to 2 months D1 and D2: Viable, but die within 3 weeks D1, D2, D3:

			aspiration. Survivors fail to thrive and exhibit hypoplastic retinas. -D1 and D2: Retarded growth and impaired coordination. Inhibited postnatal cerebellar development and hypoplastic retinas. -D1,D2,D3: Severe hematopoietic deficits and death due to anemia and defects of heart development.	Embryonic lethal at E16.5
Cyclin E	Cell cycle (G1-S)	CDK2	-E1: Normal -E2: Hypoplastic testes, reduced sperm count, and male infertility -E1 and E2: Cardiac abnormalities, reduced endoduplication in megakaryocytes	E1: Viable E2: Viable E1 and E2: Embryos dead by E11.5
Cyclin F	Cell cycle (S, G2, M)	ND*	Embryonic lethal due to defects in placental development.	Lethal at E10.5
Cyclin G	DNA damage response	CDK5	ND*	ND*
Cyclin H	CDK7	CDK activation, transcription, DNA repair	ND*	ND*
Cyclin L	CDK12 and CDK13	Transcription, splicing	ND*	ND*
Cyclin K	CDK12 and CDK13	Transcription, CDK activation	ND*	ND*
Cyclin T	CDK9	Transcription	ND*	ND*

CDK1	Cell cycle (G2-M)	Cyclin B	Embryonic lethal due to defects in first cell divisions	Lethal
CDK2	Cell cycle (G1-S)	Cyclin E and Cyclin A	Sterility in males and females due to defects in meiosis; no effect on mitotic cells	Viable
CDK3	Cell cycle (interphase)	Cyclin C	Mutation inducing premature stop codon results in a normal phenotype; most laboratory strains carry this mutation	Viable
CDK4	Cell cycle (G0-S)	Cyclin D	-CDK4: Small body size. Males are sterile due to hypoplastic tested and low sperm counts. Female sterility due to defects in hypothalamus and pituitary, abnormal estrus and failure of corpus luteum. Abnormal development of pancreatic β-islet cells and insulin-dependent diabetes. -CDK4 and CDK6: Small embryos, partial failure of hematopoiesis resulting from multipotential progenitors and multilineage deficits.	CDK4: Viable CDK4 and CDK6: Embryonic lethality at E14.5 and few live pups die soon after birth
CDK5	Neuronal cell cycle and differentiation	Cyclin G	Defective development and structure of the nevous system	Perinatal lethality
CDK6	Cell cycle (G0-S)	Cyclin D	-CDK6: Thymic and splenic hypoplasia. Mild defects in hematopoiesis. T-lymphocytes have delay in S-phase entry. -CDK4 and CDK6: Small embryos, partial failure of hematopoiesis resulting from multipotential	CDK6: Viable CDK4 and CDK6: Embryonic lethality at E14.5 and few live pups die soon after birth

			progenitors and multilineage deficits.	
CDK7	Phosphoylate and activate CDKs. Controls transcription by phosphorylating TFIIH and CTD of RNA polymerase II	Cyclin H	ND*	ND*
CDK8	Transcription	Cyclin C	ND*	ND*
CDK9	Transcription	Cyclin T	ND*	ND*
CDK10	Cell cycle (G2-M) and transcription	ND*	ND*	ND*
CDK11	Cell cycle (G2-M) and transcription	Cyclin L	Essential for peri-implantation of embryos and defects in mitosis such as centrosome maturation, spindle formation, sister chromatid cohesion and cytokinesis	Lethal at E3.5
CDK12	Splicing regulation	Cyclin K	ND*	ND*
CDK13	Splicing regulation	Cyclin K	ND*	ND*

*Abbreviations:
ND, Not Determined

Adapted from:

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cyclin D1, D2 and D3, collectively referred to as cyclin D, have been shown to be functionally redundant, but have tissue specificity. Studies in mice revealed that knockout of any two D-type cyclins results in dealth, highlighting the functional importance this cyclin [57, 64]. Upon mitogenic stimulation cyclin D binds and activates CDK4 and CDK6 [57, 63]. The cyclin D/CDK4/6 complex partially phosphorylates pocket proteins Rb, p107 and p130. Rb, retinoblastoma tumor suppressor protein, is the primary substrate of the cyclinD/CDK4/6 complex [57]. These pocket proteins are bound to transcription factors E2F1 and DP1 that positively regulate transcription of cell cycle genes cyclin A, cyclin E, CDK1 and cdc25 [57, 60]. The binding of Rb to E2F1 and DP1 restricts E2F1 transcriptional activity and complete inhibition of Rb and dissociation from E2F1 is achieved by hyperphosphorylation by cyclin E/CDK2. This results in a positive feedback loop and the ability of the cell to progress to S phase [57, 63]. In late G1 the cell reaches the restriction point, “R” or “START”, in which the cell is no longer responsive to growth factors; and indicates that the cell is fully committed to the cell cycle [60, 65].

Cyclin A1 and cyclin A2 (collectively referred to as cyclin A) function in S phase to promote DNA replication. Upon entry into S phase, cyclin A first binds to CDK2 to phosphorylate substrates involved in DNA synthesis and to drive progression through S phase [66]. Then, cyclin A dissociates from CDK2 and binds CDK1 during G2 to promote entry to M phase. Cyclin A degradation occurs at the end of G2 and the onset of mitosis begins [66].

Cyclin B1 and B2 are both present in dividing mammalian cells, however cyclin B1 is referred to as the mitotic cyclin translocates to the nucleus and binds to CDK1 to

drive the cell through mitosis [63, 67, 68]. The stages of mitosis are prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis. Cyclin B/CDK1 aids the mitotic process by phosphorylating more than 70 substrates involved in initiating centrosome separation, nuclear envelope breakdown and chromosome condensation [63]. At first, in order to prevent premature cell division, Wee1 and Myt1 kinases phosphorylate CDK1 at tyrosine 15 and threonine 14 to inhibit its activity; then Cdc25C dephosphorylates CDK1 at these same sites leading to its activation and allowing the cell to progress through mitosis [60, 65]. A complete cell cycle ends with the ubiquitination of cyclin B by the anaphase-promoting complex/cyclosome (APC/C) leading to its degradation [57, 63]. Depending on mitogenic signals, the cell will either go through another cell cycle or enter quiescence.

Regulation of cell cycle CDKs occurs by two families of CDK inhibitors (CKIs): INK4 and Cip/Kip. The INK4 family consists of $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$ and $p19^{INK4d}$ and function by preventing CDK4 and CDK6 binding to cyclin D [57, 60]. The INK4 proteins display approximately 40% sequence homology that comes from four sequential ankyrin motifs [57]. p16 was initially identified in SV40 T antigen transformed cells and found to be a binding protein and inhibitor of CDK4, but can also inhibit CDK6 in a similar fashion [69]. Importantly, Rb can inhibit p16 gene expression via negative feedback inhibition thus controlling cell proliferation [57]. In contrast, p15 expression is not related to Rb status, rather is induced by the growth inhibitory cytokine, transforming growth factor- β (TGF-β) [57].

The Cip/Kip family consists of $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ and mainly function to inhibit cyclin/Cdk complexes, but also inhibit kinases not related to the cell cycle [57,

60, 65]. p21 inhibits cyclin D/CDK4, cyclin E/CDK2 and cyclin A/CDK2, and inhibition of cyclin/CDK complexes may require more than one p21 molecule [69]. Additionally, proliferating cell nuclear antigen (PCNA) and other components of the DNA replication machinery are inhibited by p21, and are therefore arrested prior to S phase to prevent premature DNA replication [69]. p21 is transcriptionally regulated by p53, a tumor suppressor protein, and is essential for p53-mediated arrest upon DNA damage [57, 69].

The other Cip/Kip proteins, p27 and p57, are similar to p21 in that they share a conserved N-terminus required for cyclin/CDK inhibition [57]. Specifically, p27 controls cell proliferation by facilitating anti-growth signals from TGF- β and proteins involved in cell contact [57]. Whereas p21 and p27 are ubiquitously expressed, p57 is tissue specific and plays a specialized role in controlling cell proliferation [57].

The integrity of the mammalian cell cycle is maintained by two main cell cycle checkpoints: DNA damage checkpoint at G1/S and DNA damage checkpoint at G2/M [63]. Genotoxic agents or environmental factors that cause DNA damage are recognized and evaluated at the G1/S checkpoint. Here, the cell induces cell cycle arrest in a p53-dependent manner to repair damage or if damage is too severe, progress to apoptosis [63, 70]. The induction of p53 causes the upregulation of genes involved in cell cycle arrest (p21), regulation of p53 (Mdm2) and apoptosis (Bax, Fas) [60]. There are also mechanisms in place if damage occurs during DNA replication, but these mechanisms are still being elucidated [60, 71]. Studies have shown that the replication fork stalls due to p21 sequestering PCNA or PCNA being degraded though ubiquitination by Rad6 [70]. The G2/M checkpoint monitors the cell for DNA damage

before mitosis. Here, cell cycle arrest occurs through Wee1 and Myt1 kinase upregulation and well as downregulation of Cdc25C [63, 70]. In addition, cyclin B/CDK1 is sequestered in the cytoplasm by Cdc25A/14-3-3 proteins [70]. Lastly, there is a checkpoint during mitosis call the 'spindle assembly checkpoint' [60, 63]. The spindle assembly checkpoint ensures proper alignment and segregation of the chromosomes [60, 63]. Ultimately, loss of checkpoint control can lead to genetic abnormalities and overall contribute to tumorigenic processes in mammalian cells.

1.2b. Cell cycle deregulation in cancer

Cancer is largely a disease of improper cellular proliferation, but the underlying mechanisms that permit aberrant proliferation in cancer cells are complex and encompass decades of research [72]. Observations that the cell cycle is deregulated in cancer resulted from the early work of Theodor Boveri. In 1889, observed that complementary chromosomes within the nucleus are required for proper embryonic development and reasoned that aberrant chromosomes may lead to malignant transformtion [73]. Later, in 1971, Alfred Knudson demonstrated retinoblastoma could be caused by as little as one mutation in each of the alleles of the retinoblastoma gene (RB1) [74]. This important observation lead to the "two-hit hypothesis" which states that individuals would develop familial retinoblastoma if they first have a hereditary mutation in one RB1 allele and acquire an additional mutation in the second allele by an environmental source. In cases of sporadic retinoblastoma, both mutations would be acquired. Knudson's studies were integral in establishing the idea that cancer arises from genetic changes in either oncogenes, which acquire gain-of-function

mutations, or tumor-suppressor genes, in the case of RB1, which acquire loss-of-function mutations [72].

Since then, molecular studies have shown that the cell cycle machinery is often deregulated in human cancers and alterations in genes encoding proteins involved in G1 and S phases of the cell cycle are the most common (Table 3) [75]. For example, cyclin D1, a G1 cyclin, is amplified and overexpressed in a wide variety of human neoplasias (Table 3). Cyclin D1, originally called PRAD1, was first identified as playing a role in parathyroid tumorigenesis and thought to function during the cell cycle [76]. The causative role for cyclin D1 came in 1994 when Wang et al. and colleagues overexpressed cyclin D1 in the mammary cells of transgenic mice. They found that cyclin D1 overexpression in the mammary tissue resulted in increased proliferation and induced the development of mammary adenocarcinomas [77]. Additionally, cyclin D2 and D3 are also overexpressed in some cancers, but their oncogenic role is not well established [69]. Other cyclins are overexpressed or amplified in human neoplasias as well. For example, cyclin E is amplified, overexpressed or both, in carcinomas of the breast, colon, lung, and leukemia as well as a number of other cancers (Table 3), while cyclin A has been shown to be altered in liver cancer by providing an insertion site for hepatitis B virus (HBV) [57, 60, 69]. The integration of HBV at the CCNA locus produces a chimeric protein in which the N-terminal cyclin box is replaced by the virus. Consequently, cyclin A can no longer be degraded [69].

Moreover, cyclin D kinase partners, CDK4 and CDK6, are overexpressed in many human cancers as a result of gene amplification, but also harbor mutations or

Table 3: Cell cycle proteins deregulated in cancer

Deregulated Protein	Molecular Alteration	Cancer Type
cyclin D1	Gene amplification, overexpression	Breast, lung, pancreas, gastrointestinal, endometrium, bladder, bone marrow (leukemia), head and neck, lymphoma, melanoma, liver, pituitary, prostate, testis/ovary, other sarcomas
cyclin D2	Overexpression	Gastrointestinal, lymphoma, testis/ovary
cyclin D3	Overexpression	Pancreas, pituitary, lymphoma
cyclin E1	Gene amplification, overexpression	Glioblastoma, breast, lung, gastointestinal, endometrium, bladder, bone marrow (leukemia), lymphoma, melanoma, liver, prostate, testis/ovary, bone, other sarcomas
cyclin A	Overexpression, altered	Liver
CDK2	Overexpression	Gastrointestinal, liver
CDK4	Gene amplification, overexpression, mutations resulting in lack of CKI binding	Glioblastoma, breast, lung, endometrium, bone marrow (leukemia), head and neck, liver, testis/ovary, bone
CDK6	Gene amplification, overexpression, mutations/translocation resulting in lack of CKI binding	Glioblastoma, lymphoma, other sarcomas
Rb	Deletion, missense mutation	Glioblastoma, breast, lung, endometrium, bladder, bone marrow (leukemia), lymphoma, liver, pituitary, prostate, testis/ovary, bone
$p15^{INK4B}$	Deletion	Glioblastoma, lung, bone marrow (leukemia), lymphoma, liver
$p16^{INK4A}$	Deletion, mutation	Glioblastoma, lung, pancreas,

		gastrointestinal, endometrium, bladder, bone marrow (leukemia), head and neck, melanoma, lymphoma, liver, pituitary, prostate, testis/ovary, bone, other sarcomas
$p27^{KIP1}$	Deletion	Glioblastoma, breast, lung, pancreas, gastrointestinal, endometrium, bladder, bone marrow (leukemia), head and neck, lymphoma, liver, pituitary, prostate, testis/ovary, other sarcomas
Cdc25B	Overexpression	Breast

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translocations rendering them insensitive to inhibition by CKIs (Table 3) [57]. Specifically, in neuroblastoma cell lines, mutations found in CDK6 prevent $p16^{INK4A}$ interaction, but preserve its kinase activity, leading to unrestricted phosphorylation of pRb [78].

Cyclin dependent kinase inhibitors are an important class of tumor suppressors and are often either mutated or deleted entirely in human tumors (Table 3). Interestingly, the $p16^{INK4A}$ locus is a close second to p53 in terms of genetic aberrations [57, 60]. Mutated p16 is linked to familial melanoma syndrome and deletions have also been described in nearly 50% of gliomas and mesotheliomas, 40-60% gastrointestinal cancers and 20-30% acute lymphoblastic leukemias [57, 60]. Located in close proximity to the p16 locus, is p15 and is often deleted concurrently [60]. p27 is also found to be deleted or downregulated in many human cancers, including lung, breast and bladder and its loss is implicated with poor prognosis and aggressive disease [60]. On the other hand, in colorectal cancer, p27 loss is due to increased proteolysis by the proteasome [60]. Although p21 is not directly altered in tumors, p21 is a transcriptional target of p53 and p53 is the most common mutated gene in human cancer [57, 60]. Therefore, upon DNA damage, inactive p53 cannot activate p21 to induce cell cycle arrest, thus leading to genomic instability.

The culmination of alterations in G1 and S phase proteins in human cancer highlights the importance of integrity of the cell cycle prior to the Restriction Point. As previously mentioned, the Restriction Point is defined as the threshold in G1 in which cells can proliferate in the absence of growth factors. Cells that lose function of G1 inhibitors (CKIs) and/or gain of function of G1/S drivers such as cyclin D1 or cyclin E

have unrestricted access through this critical checkpoint. Rb is the key regulator of the Restriction Point and is commonly deleted or mutated [65]. Upon inactivation of Rb by deletion, mutation or hyperphosphorylation by G1 cyclin/CDK complexes, Rb releases transcriptional repression of E2F leading to transcriptional activation of cell proliferation genes such as cyclin A, cyclin E, CDK1, Cdc25 and c-myc [57, 69]. Rb is kept in the hyperphosphorylated state by cyclin E/CDK2 creating a positive feedback loop and driving the cell through the Restriction Point and fully committing the cell to cell division [57].

1.3 CYCLIN E

1.3a. Regulation of cyclin E levels

Human cyclin E was first discovered in 1991 through screening of human cDNA libraries for genes that would substitute for mutated G1 cyclins in yeast [79]. There are two E-type cyclins, cyclin E1 and cyclin E2, and display 47% sequence homology throughout the entire gene and 75% sequence homology within the cyclin box [80]. The majority of studies on cyclin E are on cyclin E1 (generally referred to as cyclin E in this dissertation) and cyclin E2 will be addressed specifically as cyclin E2. Full length cyclin E is found on chromosome 19q12-q13, is 409 amino acids in length, and contains numerous regulatory domains (Figure 2) [81, 82]. The MRAIL motif is a hydrophobic stretch of amino acids within the cyclin box that facilitates the recognition of RXL motif containing substrates or CKIs (Figure 2) [82]. The “cyclin box” as well as the last 50 amino acids of cyclin E aid in CDK2 binding and activation. The VDCLE motif is responsible for Rb binding as well as pocket proteins p107 and p130 (Figure 3) [82].

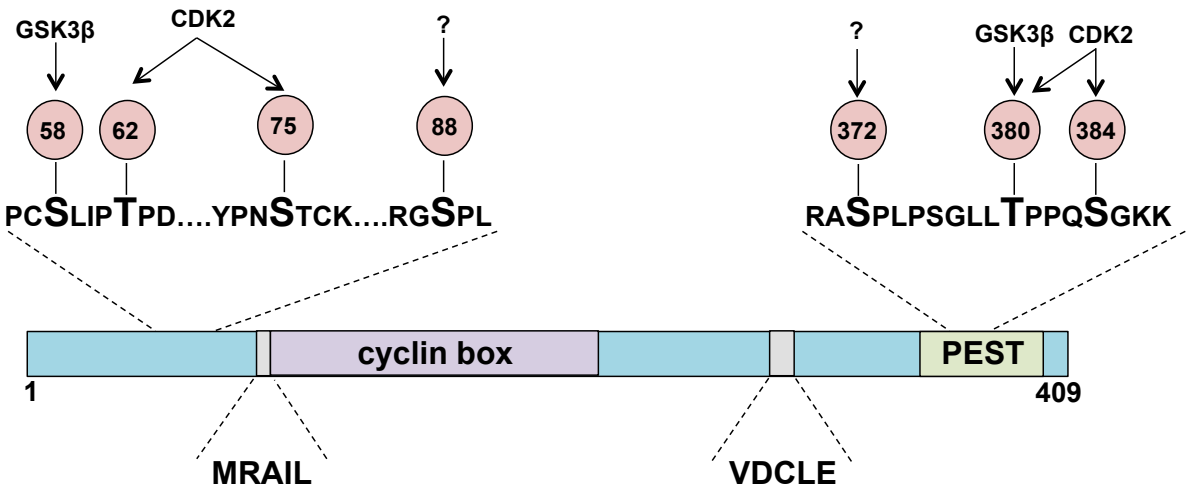

Figure 2. Schematic of human cyclin E1

The known phosphorylation sites of human cyclin E1 are shown in pink circles at their specific amino acid location along with its respective kinase. Known regulatory motifs of cyclin E such as MRAIL and VDCLE are indicated as well as the conserved cyclin box and PEST domain. *(Figure adapted from Hwang, H.C. and B.E. Clurman, Cyclin E in normal and neoplastic cell cycles. Oncogene, 2005.* ***24****(17): p. 2776-86).*

Expression of cyclin E oscillates throughout the cell cycle and peaks at the G1/S transition [81, 82]. Transcriptional control of cyclin E is mediated by E2F. As previously stated in section 1.2a, E2F transcriptional activity is repressed by pRb and pocket proteins p107 and p130. Upon mitogenic signaling, cyclin D/CDK4/6 complexes partially phosphorylate Rb, therefore activating E2F [81]. E2F activation results in the transcription of S phase genes as well as the transcription of cyclin E. The promoter of cyclin E contains an E2F binding site, and thus, cyclin E can positively regulate its own transcription through a positive feedback loop and reinforce Rb inactivation [81]. Interestingly, cyclin E transcription independent of E2F activation has also been described [83]. However, this phenomenon may be cell type specific. For example, in pancreatic and hepatic cells, cyclin E is transcribed by LRH-1 [84].

The degradation of cyclin E occurs by two different mechanisms and is based on whether cyclin E is bound to CDK2. Proteolysis of monomeric cyclin E in mediated by Cul-3, however the exact mechanisms of degradation are still being elucidated [81, 82]. The second method of cyclin E degradation is through the SCF-Fbw7 pathway and degrades the cyclin E when bound to CDK2. The SCF-Fbw7 complex is comprised of SKP-1, CDC53/Cullin, Rbx1/Roc1, CDC34 and Fbw7 [82]. Ubiquitination of cyclin E occurs in the “PEST-box” or “destruction-box” [82]. For cyclin E to be degraded by the SCF complex, cyclin E must be phosphorylated at T380 and S384 (Figure 2). The former is phosphorylated by GSK3β or CDK2, while the latter is an autophosphorylation site of cyclin E/CDK2 [81, 82]. Phosphorylation of T62 assists in binding Fbw7 to mediate degradation as well. *In vitro* studies by Koepp et al. and collegues in 2001 showed that mutation of T380 and T62 prevent Fbw7 binding to cyclin E resulting in its

stability [85]. S372 is also thought to play a role mediating cyclin E stability, but the exact mechanism is unknown and more investigation is required [81].

Additional regulation of the cyclin E/CDK2 complex comes from the CKIs such as p21 or p27. p21 and p27 inhibit the kinase activity of the cyclin E/CDK2 complex and therefore the cyclinE/CDK2 complex does not display the appropriate signal to SCF-Fbw7 for degradation [81]. Lastly, cyclin E is able to avoid degradation by localizing to alternative subcellular compartments (discussed in section 1.3d) [81]. Although not completely understood, proteolysis of cyclin E2 occurs in a similar fashion as cyclin E. Specifically, cyclin E2 is degraded by the proteasome and is stabilized by phosphorylation at T392 (T380 in cyclin E) [81].

1.3b. Cyclin E function

The primary function of cyclin E/CDK2 is to promote cell cycle progression through the Restriction Point into S phase by further phosphorylating Rb. As stated previously, full inactivation of pRb by cyclin E/CDK2 results in transcriptional activation of E2F and enables transcription of S phase related genes such as thymidine kinase, polymerase α, MCM, Cdc6, b-myb, cyclin E, cyclin A and Histone H1 [82]. Moreover, cyclin E/CDK2 is able to phosphorylate p21 and p27, thereby promoting their degradation by the proteasome pathway and releasing inhibition of cyclin A (Figure 3) [81, 82, 86, 87].

For the cell to properly progress to S phase, cyclin E also participates in DNA replication origin licensing during DNA synthesis. Cyclin E and Cdc6 work together along with PCNA and polymerase α to load MCM proteins and Cdc45 to the origins of replication (Figure 3) [81, 82]. However, these fundamental functions of cyclin E were

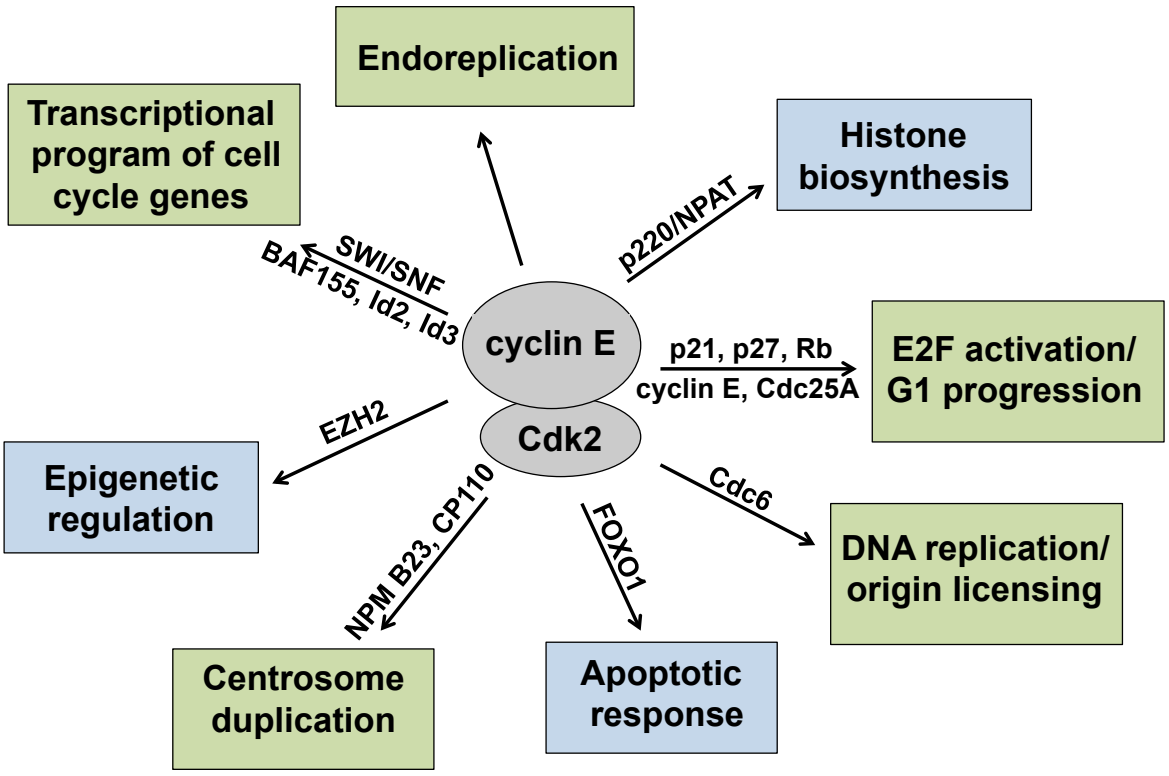

Figure 3. Functions of cyclin E and known substrates

Cyclin E functions and known substrates are shown. Cell cycle related functions are highlighted in green and non cell cycle related functions are highlighted in blue. Cyclin E/CDK2 substrates known to be associated with each respective function are shown on the arrow. (Figure adapted from: *Hwang, H.C. and B.E. Clurman, Cyclin E in normal and neoplastic cell cycles. Oncogene, 2005.* ***24****(17): p. 2776-86; Moroy, T. and C. Geisen, Cyclin E. Int J Biochem Cell Biol, 2004.* ***36****(8): p. 1424-39; Siu,K.T.,M.R. Rosner, and A.C. Minella, An integrated view of cyclin E function and regulation. Cell Cycle, 2012.* ***11****(1): p. 57-64.)*

challenged in 2003 when Geng et al. created a cyclin E knockout mouse [88]. Surprisingly, the authors showed that cyclin E is largely unnecessary for development in the mouse. However, the authors did find that cyclin E is required for endoreplication of placental trophoblast giant cells and megakaryocytes [81]. Additionally, cyclin E null mice are deficient re-entering the cell cycle from quiescence as well as defective in loading MCM proteins at the origins of replication [88].

Another function of cyclin E is centrosome duplication. Specifically, cyclin E/CDK2 phosphorylates NPM/B23 (nucleophosmini B23) on T199 to facilitate the onset of centrosome duplication (Figure3) [81]. CP110 is also phosphorylated by cyclin E/CDK2 and is involved in duplication and separation of chromosomes [87]. Moreover, cyclin E participates in the regulation of the cell cycle transcriptional program. Transcription factors known to be regulated by cyclin E/CDK2 are Id2, Id3, BAF155 and SWI/SNF [82]. Cyclin E/CDK2 also phosphorylates CBP/p300, resulting in activation of its histone acetyltransferase activity, and E2F5 to promote transcriptional activation assisting in cell cycle progression [87].

Cyclin E is also involved in non-cell cycle related functions. Cyclin E/CDK2 phosphorylates p220/NPAT which functions in histone biosynthesis in S-phase (Figure 3) [82]. Specifically, cells deficient of p220/NPAT arrest late in G1 and are unable to progress to S-phase. Another substrate of cyclin E/CDK2 is EZH2. Cyclin E/CDK2 phosphorylates EZH2 on T350 to facilitate targeting to specific loci and promote EZH2 transcriptional repression by H3K27 tri-methtylation [87]. Lastly, cyclin E is involved in the apoptotic response. Cyclin E/CDK2 phosphorylates FOXO1, a transcription factor that controls expression of pro-apoptotic genes such as Fas and Bim [87]. Specifically,

cyclin E/CDK2 phosphorylates FOXO1 on S249, thereby signaling its cytoplasmic translocation. Since cyclin E performs many cell cycle dependent and independent functions inside the cell, deregulation of any of these functions sets the stage for genomic instability and oncogenic transformation.

1.3c. The role of cyclin E in cancer

One of the first studies examining the oncogenicity of cyclin E found that overexpression of cyclin E in combination with constitutively active H-Ras in rat embryonic fibroblasts (REFs) resulted in foci that had been malignantly transformed [89]. The capability of cyclin E along with active H-Ras to transform REFs puts cyclin E in a elite group of oncogenes whose members also include Myc, SV 40 T-antigen and E1A [81]. Moreover, examination of human cyclin E in transgenic mice revealed that lactating mammary glands contained regions of hyperplasia and over 10% of the cyclin E transgenic mice developed mammary carcinomas [90]. Furthermore, in the cyclin E null mouse, Geng et al. and colleagues found that cyclin E was absolutely required for oncogenic transformation [57].

Many cancer types have been shown to overexpress cyclin E protein or amplify its mRNA transcript by as much as 64 fold including glioblastoma, breast, lung, cervical, endometrium, gastrointestinal tract, bladder, melanoma, liver, prostate, testis/ovary, bone, lymphoma, leukemia, sarcomas and adrenocortical tumors (Table 3) [75, 81, 91, 92]. However, cyclin E and its role in breast cancer has been extensively studied [93-97]. For example, patients displaying high levels of cyclin E have a worse outcome compared to patients with low levels of cyclin E and this was shown to be irrespective of the proliferation index by Ki67 staining [97]. Moreover, Keyomarsi and

colleagues analyzed cyclin E expression and survival in 395 women with stage I-IV breast cancer and found that high expression of cyclin E was an independent predictor of survival; stronger than hormone receptor status and lymph node involvement [98].

There are multiple mechanisms that lead to deregulation of cyclin E expression. First, mutations in the signaling pathways that converge upon Rb increase cyclin E levels through E2F activation and although not as common, 2-20% of endometrial, ovarian, colorectal, breast and gastric cancers contain cyclin E gene amplification that may lead to increased mRNA levels [81]. Furthermore, defects in cyclin E degradation have also been shown to lead to cyclin E overexpression. For example, mutations in Fbw7 have been found in endometrial, pancreatic and colon cancers as well as several human cancer cell lines [81].

The consequences of cyclin E overexpression include genomic instability and centrosome amplification. One of the first studies examining overexpression of cyclin E and genomic instability showed that constitutive overexpression of cyclin E, but not cyclin D1 or cyclin A in murine and human cancer cell lines caused genetic instability, specifically, defects during chromosomal duplication and segregation [99]. Genetic instability mediated by cyclin E overexpression is often linked with prolonged S-phase. During replication excess cyclin E may result in defects of MCM proteins loading at the replication fork and stalled replication forks are vulnerable to breakage [81]. Moreover, defects in the replication process may result in premature mitosis entry and chromosomes that have not been properly replicated leading to inappropriate pairing and segregation [81].

Centrosome amplification is also a consequence of cyclin E overexpression. Studies in *Xenopus laevis* confirmed the requirement of cyclin E/CDK2 in centrosome duplication during S-phase as well as multiple studies from mammalian systems [100-102]. Faithful chromosome segregation during mitosis requires the two centrosomes to form at the spindle poles. Centrosome amplification results in a multipolar spindles and vast chromosome segregation errors. *In vitro* studies have shown that cyclin E overexpression with concomitant p53 loss resulted in centrosome amplification and aneuploidy [103]. Therefore, deregulation of cyclin E causes multiple defects in the genomic integrity of the cell leading to neoplastic formation.

1.4 LOW MOLECULAR WEIGHT CYCLIN E

1.4a Generation and function of low molecular weight cyclin E

In addition to cyclin E gene amplification and protein overexpression in human cancers, cyclin E is post-translationally cleaved into low molecular weight isoforms (LMW-E) that range in size from 33-45kDa, compared to the 50kDa full length form of cyclin E (EL1) (Figure 4) [104]. LMW-E are generated from N-terminal elastase cleavage of EL at amino acids Q40-E45 to form the first isoform, LMW-E (T1), or A69-D70 to form the second isoform, LMW-E(T2) [104]. In all, there are six isoforms of cyclin E either resulting from post-translational cleavage (EL3 and EL6), alternative translational start sites (EL4) or phosphorylation events (EL2 and EL5) (Figure 4) [105].

LMW-E are present in multiple tumor types including breast, ovarian, colorectal cancers and melanomas [92, 106-108]. For example, a prospective study of 340 breast cancer patients with stage I or II disease showed tumor-specific expression of

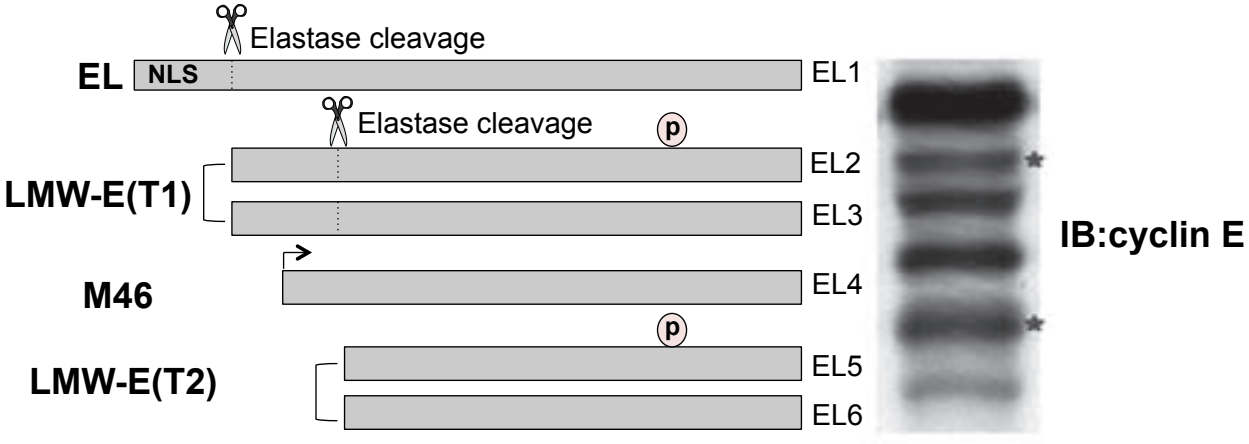

Figure 4. Schematic of the cyclin E isoforms

Schematic of the cyclin E isoforms. Full length cyclin E (EL1) is 50kDa. Upon elastase cleavage, the first truncation of LMW-E is formed LMW-E(T1) or EL3 and is 44kDa. EL2 is a phosphorylated form of EL3. EL4 is a rare alternatively translated isoform of cyclin E and is 40 kDa. Elastase further leaves EL2/3 giving rise to EL5/6. EL6 is 33kDa and EL5 is the phosphorylated form of EL5 and is 35kDa. The immunoblot is expression of the cyclin E isoforms in MDA-MB-436 cells and the stars denote the phosphorylated forms of LMW-E. (Figure adapted from: Mull, B.B., J. Cox, T. Bui, and K. Keyomarsi, *Post-translational modification and stability of low molecular weight cyclin E.* Oncogene, 2009. **28**(35): p. 3167-76).

LMW-E, while normal adjacent breast tissue showed only full-length cyclin E [109]. A causal role for LMW-E mediating tumorigenesis came from two important studies. First, generation of LMW-E transgenic mice display enhanced mammary tumor formation and metastasis compared to EL transgenic mice [110]. Specifically, 27% (34 of 124) LMW-E transgenic mice developed mammary tumors compared to 10.4% (7 of 67) EL transgenic mice [110]. Moreover, 25% of tumors from LMW-E transgenic mice presented lung metastasis compared to 8.3% of tumors from EL transgenic mice [110]. Additionally, expression of LMW-E in non-tumorigenic human mammary epithelial cells (hMECs) implanted into mice formed tumors in 74% (23 of 31) of animals, while only 7% (1 of 15) of mice formed tumors when implanted with EL expressing hMECs and LMW-E tumorigenicity was further enhanced by *in vivo* passaging [111].

LMW-E have significant biological effects with respect to the tumorigenic process. One function of the LMW-E isoforms is the deregulation of the G1/S transition [112]. Specifically, expression of LMW-E in 76NE6 cells (non-tumorigenic human mammary epithelial cells that have been immortalized by transfection of the 16E6 gene of HPV) display a shortened G1 phase paired with a 2-fold increase in time spent in S-phase compared to EL or vector expression [112]. Additionally, these cells exhibit a decreased doubling time, 28.3 to 31.1 hours for LMW-E expressing cells and 35.9-45.2 hours for EL and vector expressing cells [112].

Moreover, LMW-E are biologically hyperactive. It has been shown that LMW-E/CDK2 complexes have increased kinase activity; more than EL/CDK2 complexes and efficiently phosphorylate common cyclin E substrates such as histone H1 and Rb [112-114]. Of note, LMW-E bind as efficiently to CDK2, p21 and p27 as EL and the

increased kinase activity observed is not due to increased binding to CDK2 and/or decreased binding to the CKIs [109, 112-114].

Overexpression of LMW-E also causes resistance to anti-estrogens. Specifically, estrogen positive MCF7 cells overexpressing either EL or LMW-E have a significantly greater percentage of cells in S-phase after treatment with the pure anti-estrogen, ICI 182,780 [113]. While vector control cells displayed only 10% of cells in S-phase after anti-estrogen treatment, EL and LMW-E cells displayed 31% for EL, 40% for LMW-E(T1) and 48% for LMW-E(T2) of cells in S-phase after treatment [113].

One of the mechanisms by which LMW-E mediates tumorigenesis is through genomic instability. LMW-E overexpressing breast cancer cells have more polyploidy and chromosomal aberrations such as chromosomal fragments, chromosomal breaks, chromosomal fusions and subtelomeric chromatid breaks [109, 113]. The presence of polyploidy is not only found *in vitro*, but *in vivo* as well. Analysis of 331 stage I-III breast cancer patients show a significant correlation between cyclin E levels and polyploidy [113]. Specifically, patients with high levels of cyclin E and polyploidy have a lower 5-year DSS (Disease Specific Survival) than breast cancer patients with diploid tumors [113].

LMW-E mediated chromosomal abnormalities are due to multiple defects during mitosis. First, LMW-E overexpression leads to centrosome amplification [115]. Using an inducible model system, Bagheri-Yarmand et. al and colleagues found that inducible expression of LMW-E lead to a 2.5-fold increase in cells presenting more than two centrosomes [115]. Additionally, these cells display defective spindle formation and a number of other mitotic errors such as micronuclei, chromosome missegregation,

anaphase bridges and cytokinesis failure [115]. These mitotic abnormalities were found to be the result of deregulation of Cdc25C by cyclin E/CDK2 [116].

Another characteristic of LMW-E expression is the acquisition of a cancer stem cell (CSC) phenotype along with the ability to undergo epithelial-to-mesenchymal transition (EMT) [117]. LMW-E expressing hMECs, especially ones that have undergone *in vivo* passaging also known as TDCs (tumor derived cells), activate an EMT associated gene expression profile [117]. Epithelial genes such as E-cadherin were decreased, while mesenchymal associated genes such as Vimentin, N-cadherin, Twist and Slug all increased expression compared to EL expressing hMECs [117]. Additionally, LMW-E expressing hMECs and TDCs exhibited significantly higher levels of $CD44^{+/high}/CD24^{-/low}$ expressing cells, a characteristic marker of CSCs [117]. Interestingly, the CSC population in LMW-E expressing cells is mediated, at least in part, by a histone acetyltransferase, Hbo1. Specifically, stable knockdown of Hbo1 in cyclin E expressing hMECs significantly reduced the CSC population [117].

1.4b Low molecular weight cyclin E subcellular localization

Cyclin E has a classical nuclear localization sequence (NLS) that targets it to the nucleus via the well characterized importin-α/importin-β import pathway [118]. However, the NLS in the LMW-E isoforms is lost post-translationally due to proteolytic processing by elastase [104]. To examine the subcellular localization of LMW-E, Delk et.al and colleagues first analyzed multiple immortalized mammary epithelial cell lines as well as cancer cell lines of the breast, ovary and osteosarcoma [119]. Fractionation of whole cell lysates followed by western blot analysis for cyclin E expression revealed cytoplasmic localization of the LMW-E isoforms. Furthermore, to examine whether

LMW-E/CDK2 complexes localized to the cytoplasm, green fluorescent protein (GFP) was rationally bisected and each half was either fused to the cyclin E isoforms or CDK2. In concordance with the western blot results, LMW-E/CDK2 complexes preferentially localized to the cytoplasm, while EL/CDK2 complexes were found in the nucleus of 98% of cells examined [119]. Interestingly, while in the cytoplasm, LMW-E/CDK2 complexes were less sensitive to Fbw7 mediated degradation [119] .

Cytoplasmic localization of the LMW-E isoforms is correlated to cyclin E biological deregulation. For example, the LMW-E isoforms are linked to cells with centrosome abnormalities. Specifically, in 30 invasive breast carcinoma tissue samples, those that present high levels of cytoplasmic LMW-E also show increased levels of abnormal centrosomes with an r^2=0.35 [115]. Moreover, in 118 breast cancer patient tissue samples, patients with cytoplasmic LMW-E expression also display a significantly increased population of $CD44^{+/high}/CD24^{-/low}$ cells [117]. Together, these findings indicate that the LMW-E isoforms are biologically and spatially different from their full-length counterpart and their deregulation implies a functional role in the tumorigenic process.

1.5 GAP IN KNOWLEDGE

Deregulation of cyclin E, especially the LMW-E isoforms, leads to molecular events that are correlated with the tumorigenic process. Moreover, LMW-E expression in women with stage I-III breast cancer is an independent predictor of survival. However, understanding the mechanisms by which LMW-E predisposes the mammary gland to tumorigenesis is still required and the following questions remain:

- Do the LMW-E isoforms have protein-binding partner(s) in the cytoplasm?

- Are the function(s) of the LMW-E cytoplasmic binding partner(s) enhanced and/or suppressed?
- Are cytoplasmic LMW-E binding partners required for LMW-E mediated transformation, invasive capabilities and tumor growth?

These are the primary objectives of this dissertation and the following chapters include research data to answer these questions. The overall ***hypothesis*** of this work is that ***aberrant localization of the LMW-E isoforms leads to molecular interactions that ultimately contribute to LMW-E breast cancer tumorigenicity***. This dissertation and future studies will continue to expose tumor-specific signaling pathways in breast cancer that will help define appropriate treatment strategies and enable targeted drug design for cancer therapies.

CHAPTER 2: **RETROVIRUS-BASED PROTEIN COMPLEMENTATION ASSAY (REPCA) REVEALS NOVEL LMW-E BINDING PROTEINS**

2.1 INTRODUCTION

2.1a. Methods used to identify protein-protein interactions

The study of proteomics involves an understanding how protein structures, modifications, localization and interactions with other proteins affect how the cell functions [120]. Two categories of proteomics research dominate the field, expression proteomics and functional proteomics. Expression proteomics evaluates changes in protein expression from normal cells under different conditions such as exposure to drugs or disease state [121]. Functional proteomics, however, strives to understand protein function and evaluate their role within the cell [121].

One of the most informative methods to understand protein function is through identification of protein-protein interactions. Protein-protein interactions, either transient or stable, are essential for every biological process including building enzyme-substrate complexes for signal transduction pathways, mediating ion channels, and constructing the machinery for the cellular cytoskeleton [122]. One method to identify protein-protein interactions is through affinity purification of proteins coupled to mass spectrometry (AP-MS) (Table 4) [121, 123]. Specifically, the protein of interest is tagged with peptides such as hemagglutinin (HA), FLAG, TAP (consisting of a calmodulin-binding domain, a protease cleavage site, TEV, and a protein A tag) or glutathione-S-transferase (GST) and purified from the protein mixture by an immobilized solid support specific to the tag and/or by an antibody [121]. After binding, the protein complex is then eluted, digested into small peptides and identified by mass

Table 4: Common methods for identifying protein-protein interactions

Method	Abbreviation	Advantages	Disadvantages	High through-put capacity
Affinity purification followed by mass spectroscopy or Co-immuno-precipitation	AP-MS or Co-IP	• Specific • Simple and cost effective	• Limited to more stable interactions and high abundance proteins • Tag interference	yes
Yeast 2 hybrid	Y2H	• Time efficient • Cost effective	• High false positive rate • Dependent on subcellular localization • Interactions dependent on post-translational modifications not present in yeast	yes
Split ubiquitin system	SUS	• Suitable to identify membrane proteins	• Time consuming cloning • Protein structure must be taken into consideration • False positive readout due to unknown protease cleavage of Cub	yes
Fluorescence resonance energy transfer	FRET	• Live cell imaging • Quantitative measurement between molecules • Localization of protein complexes	• Insufficient on its own to prove protein complex formation • Photo-bleaching • Expensive equipment • Auto-fluorescence of cells	no

Bimolecular fluorescence or protein complement-ation assays	BiF or PCA	• Live cell imaging • Localization of protein complex	• Non-biological levels of protein and tag interference • Expensive equipment	no

spectroscopy; which measures the abundance of the peptides in the sample and the mass-to-charge ratio [121]. AP-MS is amendable for high-throughput methods and has high specificity, however, screening is often limited by subcellular localization and is ineffective at isolating low copy number proteins or transient interactions [124, 125]. Furthermore, the tag added to the protein of interest may affect protein structure and thus, limit protein interactions; but the use of smaller tags and varying the location of the tag between the N- and C- terminal ends of the protein may alleviate this issue [121].

Another method used to identify protein-protein interactions, though less common, is a yeast two-hybrid (Y2H) assay and was developed in 1989 by Stanley Fields and Ok-Kyu Song to confirm the interaction between two known interacting proteins in yeast; SNF1 and SNF4 [126]. The premise behind the Y2H assay relies on the activation of a downstream reporter gene by a transcription factor binding to an upstream activation sequence (Table 4) (Figure 5) [127, 128]. Essentially, the protein of interest or bait 'X' is fused to the DNA-binding domain (DBD), usually GAL4 or LEXA. Then a cDNA library of prey proteins 'Y' are fused to the transcriptional activation domain (TAD), usually GAL4 or B42 (Figure 5) [122]. The binding of 'X' to 'Y' reconstitutes a functional transcription factor and activates transcription of the reporter gene in the nucleus [122, 127]. The Y2H assay is an attractive system to study protein-protein interactions because it is time efficient and relatively inexpensive, however, the Y2H assay has several limitations [129]. First, screening from a Y2H assay results in a high false positive rate and is dependent on nuclear subcellular localization [125, 126]. The high false positive rate due to spontaneous transcriptional activation has been

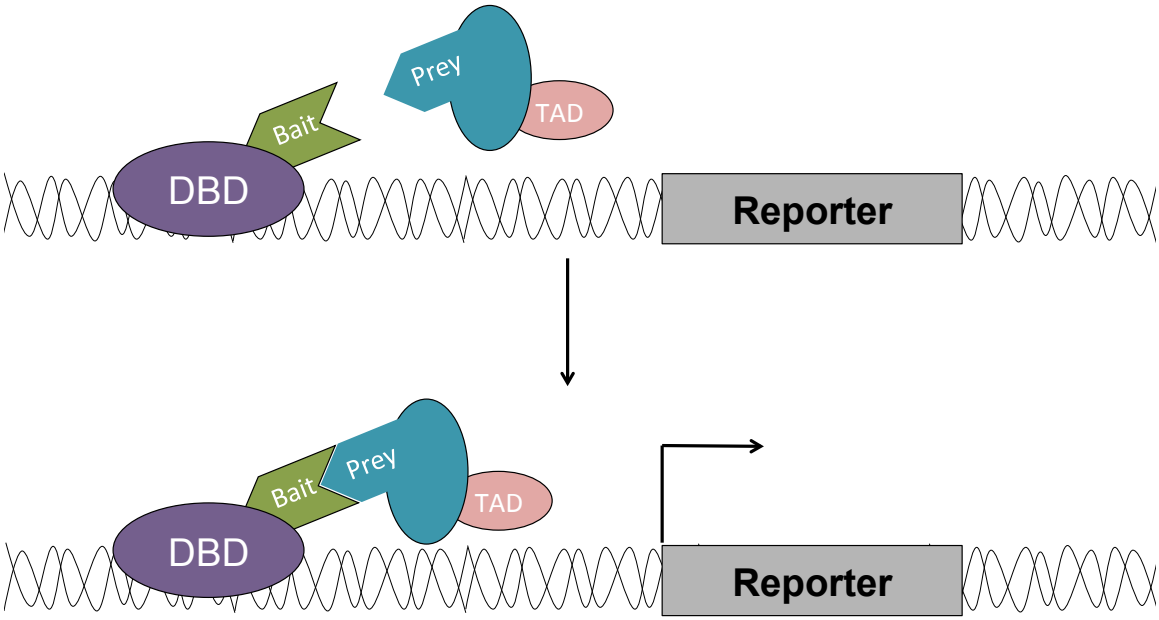

Figure 5. Schematic of the yeast-2-hybrid assay

The yeast-2-hybrid assay is a high-throughput method that is able to identify protein-protein interactions in the nucleus. Activation of a reporter gene occurs when the bait protein; which is fused to the DNA binding domain (DBD) of either GAL4 or LEXA, binds to the prey protein; which is fused to the transcriptional activation domain (TAD) of either GAL4 or B42. (Figure adapted from: Wodak, S.J., J. Vlasblom, A.L. Turinsky, and S. Pu, *Protein-protein interaction networks: the puzzling riches.* Curr Opin Struct Biol, 2013. **23**(6): p. 941-53.

reported when some proteins are fused to the DNA-binding domain without activation by the interaction partner [130]. Moreover, protein interactions dependent on post-translational modifications not present in the yeast system will be left unidentified [127].

The split ubiquitin system (SUS) is similar to the Y2H assay in that upon protein interaction the reporter protein becomes functional (Table 4) [131]. The SUS, as the name implies, utilizes a rationally bisected ubiquitin molecule: the N-terminal half (Nub) and the C-terminal half (Cub) [122, 131]. Nub is fused to protein 'X' and Cub is fused to protein 'Y' along with a transcription factor, PLV. Upon interaction of protein 'X' and protein 'Y' the ubiquitin molecule is reconstituted and ubiquitin-specific proteases cleave the fused proteins from the ubiquitin molecule thereby liberating PLV to act on target genes in the nucleus (Figure 6) [122, 131]. Recently, SUS was used to identify NCX1; a novel interacting protein of Anoctamin6 (Ano6), required for bone calcification [132]. The major advantage to SUS is that it is able to identify interactions between membrane proteins. However, cloning membrane protein ORFs in *E. Coli* often results in toxicity, but can be circumvented by cloning directly into yeast or using specialized *E. Coli* expression vectors [122]. Additionally, SUS is susceptible to false positive readouts due to cleavage of Cub by unknown proteases [122].

A fluorescence-based technique to identify protein-protein interactions is fluorescence resonance energy transfer (FRET) [122]. Essentially, protein 'X' and protein 'Y' are fused to a fluorescent donor and fluorescent acceptor, respectively, and excitation of the donor results in energy transfer to the acceptor leading to dipole-dipole

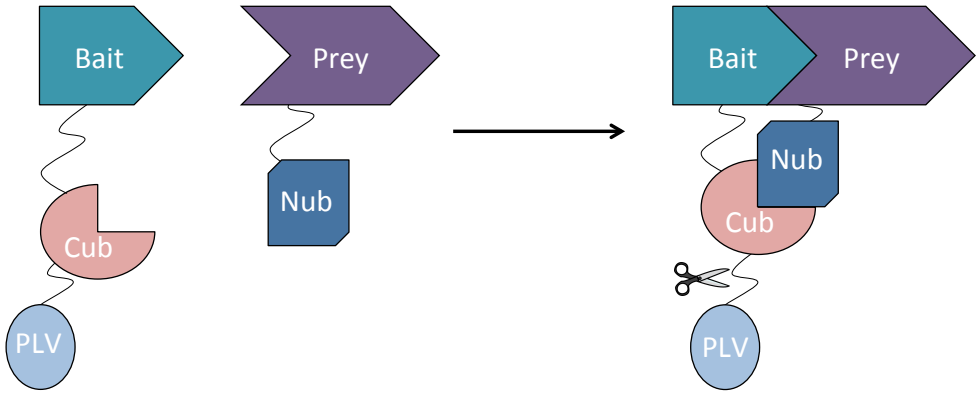

Figure 6. Schematic of the split ubiquitin system

The split ubiquitin system relies on the reconstitution of a rationally bisected ubiquitin molecule, when reconstituted, activates transcription through the cleavage and liberation of PLV into the nucleus. Specifically, the bait protein is fused to C-terminal half of ubiquitin (Cub) and the prey protein is fused to the N-terminal half of ubiquitin (Nub). Upon interaction of bait and prey proteins, Nub and Cub bind and reconstitute a full ubiquitin molecule and liberates transcription factor PLV to act on target genes in the nucleus. (Figure adapted from: Wodak, S.J., J. Vlasblom, A.L. Turinsky, and S. Pu, *Protein-protein interaction networks: the puzzling riches.* Curr Opin Struct Biol, 2013. **23**(6): p. 941-53.

coupling [133]. FRET was recently used to identify a sumoylation modification by SUMO2 on p35; which alters CDK5 activity under oxidative stress conditions in neurons [134]. The energy transfer, E, can be quantitatively measured by the energy absorbed by the donor and energy emitted by the acceptor. FRET is often described as a molecular measuring tool due to a measuring range of 1-10nm, the distance of molecules during protein complex formation, however, FRET is insufficient on its own to prove protein-protein interactions and other methods are required [122]. Additionally, FRET is subject to false signals due to autofluorescence of cells or re-absorption of emission by donor [122].

Bimolecular fluorescence (BiF), also known as protein complementation assay (PCA), is another method used to identify protein-protein interactions, but is also used mainly to validate protein-protein interactions [122]. Similar to previous methods described, a fluorescent reporter protein, such as GFP, is split into N- and C- terminal fragments and each half is fused to one of the proteins of interest [135]. The interaction between the proteins brings the two halves of the reporter protein together reconstituting fluorescence [135]. This method is a powerful tool to analyze localization of protein complexes and visualize protein-protein interactions *in vivo,* but similar to AP-MS, the size and localization of the reporter protein may result in binding interference [122].

2.1b Retrovirus-based protein complementation assay

The Retrovirus-based protein complementation assay (RePCA) is a high throughput method first utilized to identify protein-binding partners of Akt [125]. Using

RePCA, Ding et al. and colleagues found 24 protein-binding partners of Akt; one of which is ACTN4. ACTN4 was found to mediate Akt translocation to the membrane and effect cellular proliferation [125].

RePCA combines PCA strategies with enhanced retroviral mutagenesis. The enhanced retroviral mutagen (ERM) vector is superior to other retroviral-mutagenesis techniques due to its high efficiency rate, ability to infect all mammalian cell types and its available three ORFs to achieve in-frame integration sequences [136]. Furthermore, the ERM vector provides efficient and regulated mutagenesis through several engineered sequences. These sequences include a splice donor (SD) site and a mutagenesis tag controlled by a tetracycline-responsive promoter, enabling the system to be completely inducible [125].

As shown in Figure 7 the RePCA vector contains a tetracycline-responsive promoter controlling the C-terminal half of intensely fluorescent protein (IFPC) adjacent to the splice donor site. This SD site uses the endogenous splice acceptor (SA) site within the host to generate in-frame fusions. For our purposes, since Tet-on MCF7 cells were already established [137], we fused LMW-E(T1)- N-terminal half to intensely fluorescent protein (IFPN) and transduced with the RePCA vector. Upon induction with doxycycline, fluorescent cells, indicative of an LMW-E(T1)/Protein 'X' interaction, are sorted by fluorescence-activated cell sorting (FACS) to obtain clones from a single cell. Fluorescent cells are expanded and Protein 'X' is identified by RNA extraction followed by rt-PCR using primers specific to the RePCA vector. Finally, the PCR product is gel purified, sequenced, and identified using a Genbank blast.

RePCA has a numerous advantages and are summarized in Table 5. First,

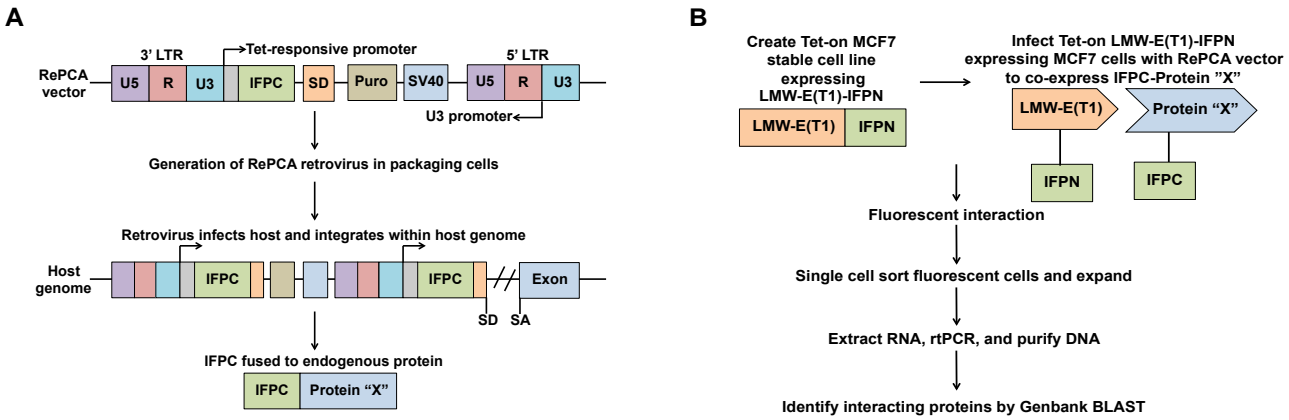

Figure 7. Schematic of RePCA screen.

(A) Creation of RePCA retrovirus. The RePCA vector contains a tetracycline responsive promoter, IFPC sequence and splice donor site. RePCA retrovirus is made in packaging cells. Inside the host genome, the RePCA vector integrates to generate IFPC-tagged "Protein X" fusions. (B) Flow chart of the RePCA screen. Stable LMW E(T1)-IFPN expressing cells were generated in a tet-on MCF7 background and infected with RePCA retrovirus. LMW-E(T1)/ "Protein X" interaction reconstitute fluorescence. Single fluorescent cells were sorted by FACS and "Protein X" is identified by RNA extraction from the fluorescent clone, followed by rt-PCR, sequencing, and a Genbank blast. (Figure adapted from: Ding, Z., J. Liang, Y. Lu, Q. Yu, Z. Songyang, S.Y. Lin, and G.B. Mills, *A retrovirus-based protein complementation assay screen reveals functional AKT1-binding partners.* Proc Natl Acad Sci U S A, 2006. **103**(41): p. 15014-9).

Table 5: Advantages and disadvantages of RePCA

Advantages	Disadvantages
• *In vivo* high-throughput screening allows for native protein folding and post-translational modifications • Does not require generation of cell-line specific cDNA libraries • Context-dependent interactions such as drug treatment or genetic manipulation • Employs endogenous splicing machinery generating full length or near-full length transcripts • Inducible system • Limited background fluorescence when the PCA fusion proteins interact • Visualization of localization of the protein complex • The reconstituted IFP molecule is highly stable and enables transient interactions to be identified • Membrane interactions are easily identified • Molecular interactions are directly identified	• The ERM vector cannot capture intron-less genes • Genes only available for virus integration are targeted • Fluorescent tag may interfere with some protein interactions • False positive interactions may be identified

Adapted from:
Ding, Z., J. Liang, Y. Lu, Q. Yu, Z. Songyang, S.Y. Lin, and G.B. Mills, *A retrovirus-based protein complementation assay screen reveals functional AKT1-binding partners.* Proc Natl Acad Sci U S A, 2006. **103**(41): p. 15014-9.

RePCA is performed in an endogenous environment and permits native protein folding and post-translational modifications [125]. RePCA is amendable to any cell type and generates full length or near-full length fusion transcripts due to the retroviral vector integrating at or near transcriptional start sites [125]. Furthermore, the RePCA vector contains a Tet-responsive promoter, which allows for inducible expression of target genes. Finally, RePCA is powerful enough to identify transient interactions such as enzyme-substrate interactions and can stabilize low affinity interactions [125].

Although RePCA has many advantages, there are disadvantages to RePCA technology as well. Similar to AP-MS, the fluorescent tag may inhibit the formation of select protein-protein interactions (Table 5). Also, intron-less genes, which comprise 3% of the human genome, are unable to be discovered by RePCA [125, 138]. Nevertheless, RePCA is a suitable method to identify a wide range of protein-protein interactions in any subcellular compartment that might otherwise go undiscovered by classical methods.

2.1c Hypothesis and specific aims

LMW-E are void of an NLS and thus, LMW-E/Cdk2 complexes accumulate in the cytoplasm with reduced levels in the nucleus [119]. Therefore, we ***hypothesize*** that ***aberrant localization of tumor-specific LMW-E in the cytoplasm leads to oncogenic protein interactions ultimately contributing to LMW-E tumorigenicity in breast cancer.***

The hypothesis will be addressed with the following specific aims:

- Generate Tet-on MCF7 cells stably expressing EL-IFPN, T1-IFPN and T2-IFPN fusion proteins.

- Identify LMW-E(T1) binding proteins in the cytoplasm utilizing the retrovirus-based protein complementation assay.
- Validate novel interactions by co-immunoprecipitation.

Briefly, results presented in this chapter reveal 11 novel interacting proteins of LMW-E(T1). The proteins identified range in function from metabolism and signal transduction to protein trafficking and stress response. Among the novel LMW-E(T1) interacting proteins identified, ATP-citrate lyase, the primary enzyme responsible for synthesis of acetyl-CoA and oxaloacetate in the cytoplasm, is a novel discovery implicating a possible link between the cell cycle machinery and cellular metabolism in breast cancer.

2.2 MATERIALS AND METHODS

2.2a. Cell culture and constructs

Breast cancer MCF7 Tet-On cells were purchased from BD Clontech (Palo Alto, CA) and cultured as previously described [139]. The LMW-E (T1) intensely fluorescent protein N terminus (IFPN) construct was generated previously [119] and used to create MCF7 Tet-On cells stably expressing the LMW-E (T1)-IFPN construct by selection with 80 µg/mL zeocin (Invitrogen, Grand Island, NY). Embryonic kidney HEK 293T/17 cells from ATCC (Manassas, VA) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS and used to produce retrovirus. Retroviral RePCA plasmids IC1, IC2 and IC3 and packaging plasmids pcGP and pVSVG were a kind gift from Gordon Mills (The University of Texas MD Anderson Cancer Center).

2.2b. Retrovirus-based protein complementation assay

RePCA was performed as previously described [125]. Briefly, IC1, IC2 or IC3 retrovirus was produced in HEK 293T/17 cells and subsequently used to transduce a total of 10 x 10^6 MCF7 Tet-On cells stably expressing the LMW-E (T1)-IFPN construct on 6-well p150 plates. Infected cells were grown for 1 week with 2 μg/mL puromycin (Invitrogen). Puromycin-resistant cells were subjected to fluorescence-activated cell sorting for green fluorescent protein (GFP) expression using a BD FACSAria II cell sorter (BD Biosciences, San Jose, CA). Single cells were grown at a very low density in minimal essential medium α containing 2 μg/mL puromycin to form clones. The clones were expanded, and RNA was extracted using RNAeasy mini kits (Qiagen, Valencia, CA). Reverse transcription was performed with a random primer (RT-1) as previously described using a Transcriptor First Strand cDNA synthesis kit (Roche, Indianapolis, IN). A T7 primer sequence is embedded within the 5' end of the RT-1 primer. The cDNA was amplified by PCR with an intensely fluorescent protein C terminus (IFPC)-specific primer and a T7 primer using HotStarTaq DNA polymerase (Qiagen). PCR products were gel purified using a QIAEX II gel extraction kit (Qiagen) and sequenced at the Sequencing and Microarray Facility at the MD Anderson Cancer Center. Sequences were identified using GenBank BLAST.

2.2c Immunocytochemical analysis

RePCA clones were cultured on coverslips in the presence of 2 μg/mL doxycycline for 48 hours. The cells were then fixed with 4% paraformaldehyde, and nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO).

2.2d Cell lysis, immunoblotting, and co-immunoprecipitation

Cell lysates were prepared and subjected to Western blot analysis as previously described [139]. Western blots were incubated with primary antibodies against Flag M2, Flag produced in rabbit (Sigma), myc-Tag 9B11, glutathione *S*-transferase (GST), ACLY (Cell Signaling, Danvers, MA), cyclin E HE12, Cdk2 (Santa Cruz Biotechnology, Dallas, TX). All antibodies were used according to the manufacturer's instructions. The blots were washed and probed with horseradish peroxidase-labeled secondary antibody and detected using enhanced chemiluminescence. Co-immunoprecipitation was performed with anti-Flag M2 antibody in lysis buffer (50 mM Tris [pH7.5], 250 mM NaCl, 0.1% NP40, and a protease inhibitor cocktail). The protein/antibody mixture was incubated with Protein G Sepharose 4 Fast Flow (GE Healthcare, Pittsburg, PA) at 4°C overnight. Beads were washed four times with lysis buffer without protease inhibitors and subjected to SDS-PAGE.

2.2e GST pull-down, GST-ACLY protein truncations and kinase assay

GST-ACLY fusion protein and GST-ACLY protein truncations were constructed using the Gateway cloning method from Invitrogen. Recombinant GST-ACLY fusion protein was expressed in *Escherichia coli* BL-21 cells and induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were lysed in NETN buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris [pH8.0], and 0.5% NP-40) plus 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM DL-dithiothreitol (DTT), 50 µg/mL lysozyme, and protease inhibitors. GST-ACLY fusion protein was purified using Glutathione Sepharose 4B beads from GE Healthcare. *In vitro* transcription and translation of cyclin E isoforms and Cdk2 were performed using the TNT-coupled

reticulocyte lysate system (Promega, Madison, WI), and these proteins were added to the mixture of GST-ACLY and glutathione beads. Purified SFB-cyclin E isoforms were expressed in 293T cells and isolated using Flag M2 antibody (Sigma) and subsequently eluted using Flag M2 peptide from Sigma. For the GST-ACLY kinase assay, lysates from Sf9 cells expressing either EL, T1 or in combination with CDK2 were immunoprecipitated using CDK2 antibody. GST-ACLY aa426-486 were incubated with Sf9 immunoprecipiates or purified AKT1 (Millipore, Billerica, MA) in kinase buffer containing 60 μM cold ATP and 5 μCi [^{32}P] ATP to a final volume of 30 μl at 37°C for 30 min. The products of the reactions were then analyzed on a 7% SDS-PAGE gel. The gel was then stained, destained, dried, and exposed to X-ray film.

2.3 RESULTS

2.3a. RePCA reveals novel LMW-E(T1) binding proteins

To identify LMW-E(T1) binding proteins in the cytoplasm we utilized RePCA technology. We first created Tet-on MCF7 cells that stably express EL-, T1- or T2-IFPN vectors and picked clones for expression. We obtained correct expression from both T1- and T2-IFPN clones, however, EL-IFPN clones showed expression at a reduced molecular weight and were not used as part of the screen (Figure 8A). The second step of RePCA is infection of the host cell line with the RePCA retrovirus. We chose to infect LMW-E(T1)-IFPN clone 5, due to its high expression, with all three ORFs of the RePCA retrovirus. After one week of 2 μg/mL puromycin selection, the infected cell population was subjected to fluorescence activated cell sorting (FACS) to isolate fluorescent cells that are indicative of an LMW-E(T1)/Protein 'X' interaction. For

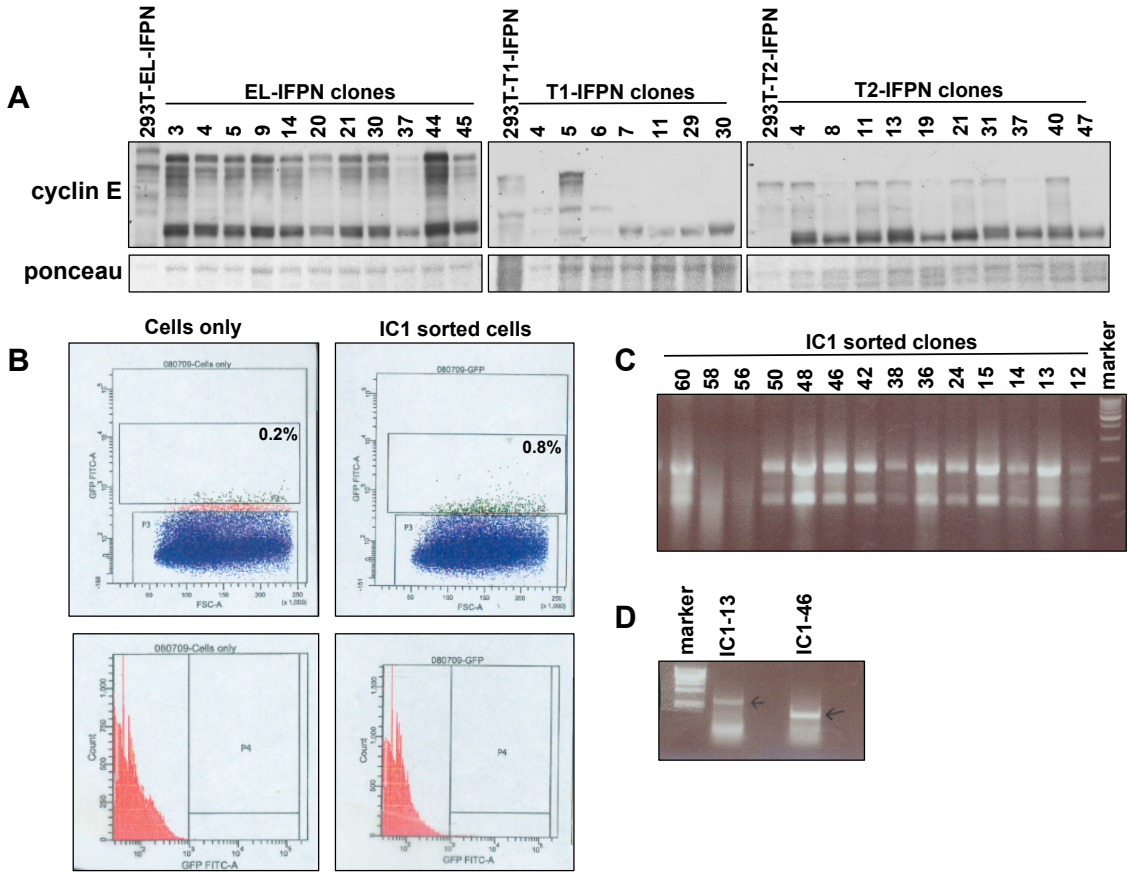

Figure 8. RePCA screening process for LMW-E(T1) interacting proteins

(A) Identification of IFPN clones. EL-IFPN, T1-IFPN and T2-IFPN clone lysates were subjected to western blot analysis for cyclin E expression using cyclin E antibodies. (B) IC1 RePCA infected cells were sorted and fluorescent cells were isolated. (C) RNA from IC1 sorted clones were analyzed on an agarose gel. (D) PCR products were analyzed on an agarose gel to examine size of LMW-E(T1) interacting partners.

example, from 10 x 10^6 cells infected with IC1 RePCA retrovirus, we isolated 0.8% (approximately 80,000 single cells) fluorescent cells (Figure 8B). The 80,000 cells were plated at a very low density and we obtained 204 colonies that were further isolated and transferred to a 12-well plate. Upon induction of doxycycline, 61 of the 204 (29.9%) colonies retained fluorescence and were further expanded. Next, we extracted RNA from the expanded clones (Figure 8C) and analyzed this for purity or degradation. Lastly, we reverse-transcribed the RNA and amplified the RePCA region of interest by PCR using primers located within the RePCA vector. The PCR products were gel purified (Figure 8D) and sequenced.

We identified 11 independent LMW-E (T1)–binding proteins in MCF7 cells (Table 6). The IFPC fusion transcript integrated at or near the transcriptional start site in 6 of the 11 transcripts, indicating that full-length or near-full-length proteins were generated. We found that the majority of the interacting proteins identified (8 of 11, 73%) were known to be localized to the cytoplasm (Table 6). YWHAQ (14-3-3β) mediates protein signaling by binding to phosphoserine-containing proteins [140]. HSP27 (heat shock protein 27) is a chaperone protein induced upon environmental stress [141]. HSP90 (heatshock protein 90), another chaperone protein, facilitates proper protein folding and protects against promiscuous protein-protein interactions [142]. VAMP8 (vesicle-associated membrane protein 8) is a component of a complex of proteins involved in the docking of synaptic vesicles with the presynaptic membrane [143]. COG4 (component of oligomeric golgi complex 4) is involved with protein trafficking at the Golgi apparatus [144]. RPL41 (ribosomal protein L41) is a component of the 60S subunit of the ribosome and important for mitosis and centrosome integrity

Table 6: Interacting proteins of LMW-E (T1) identified from RePCA

Protein name	Protein symbol	Function	Retroviral reading frame	Amino acid insertion site	Known localization
trans-2,3-enoyl-CoA reductase	TECR	Fatty acid synthesis	IC1	+4	Endoplasmic reticulum
Aldolase-A	ALDO-A	Glycolysis	IC1	-7	Cytoplasm
ATP-citrate lyase	ACLY	Fatty acid synthesis	IC1	-8	Cytoplasm; nucleus
Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, beta polypeptide	YWHAB	Signaling	IC1	-4	Cytoplasm
Heat shock protein 27	HSP27	Stress Response	IC1	-37	Cytoplasm; nucleus
Heat shock protein 90	HSP90	Stabilization	IC1	+1	Cytoplasm
Vesicle-associated membrane protein 8	VAMP8	Protein docking	IC1	+57	Plasma membrane
Component of oligomeric complex 4	COG4	Transport	IC2	+649	Golgi apparatus
RAN binding protein 1	RANBP1	Transport	IC1	+5	Cytoplasm; nucleus
Ribosomal protein L41	RPL41	Protein synthesis	IC2	+11	Cytoplasm
Eukaryotic translation initiation factor, subunit J	eIF3J	Protein synthesis	IC1	+49	Cytoplasm

[145]. RANBP1 is a GTPase that facilitates protein transport across the nuclear membrane [146]. Eukaryotic translation initiation factor 3J (eIF3J) is the 13th subunit of the eIF3 complex and aids in the initiation of transcription [147]. The three remaining novel interacting proteins of LMW-E (T1) identified constituted the largest functional group (3 of 11, 27%) of proteins discovered from the screen; these proteins are involved in metabolism. *trans*-2,3-enoyl-CoA reductase (TECR) catalyzes the final step in synthesizing long and very long chain fatty acids [148]. Aldolase A (ALDO-A), also known as fructose-bisphosphate, plays a key role in glycolysis and gluconeogenesis [149]. ACLY, catalyzes the first step of the *de novo* lipogenesis pathway and converts cytoplasmic citrate to acetyl-CoA and oxaloacetate [150]. The end products of this lipogenesis pathway are fatty acids and other metabolic intermediates needed by proliferating cells [151]. Elevated ACLY protein and activity levels correlate with tumor growth and progression in breast carcinoma, lung adenocarcinoma, and glioblastoma [152-154], and ACLY inhibition by genetic or pharmacologic methods suppresses tumor growth [155]. On the basis of its role in promoting tumor growth and its novel interaction with cyclin E, we selected ACLY for further investigation.

2.3b. Cyclin E and ACLY are interacting proteins

To examine the localization of the LMW-E (T1) protein complexes, we subjected several RePCA clones to fluorescence microscopy. The LMW-E(T1)/ACLY interaction was identified in clones #18 and #46 (Figure 9A) and the LMW-E(T1)/ACLY complex localized primarily to the cytoplasm. In another clone, #16, we identified a interaction between LMW-E(T1) and VAMP8 (Figure 9A). This interaction localizes to the plasma

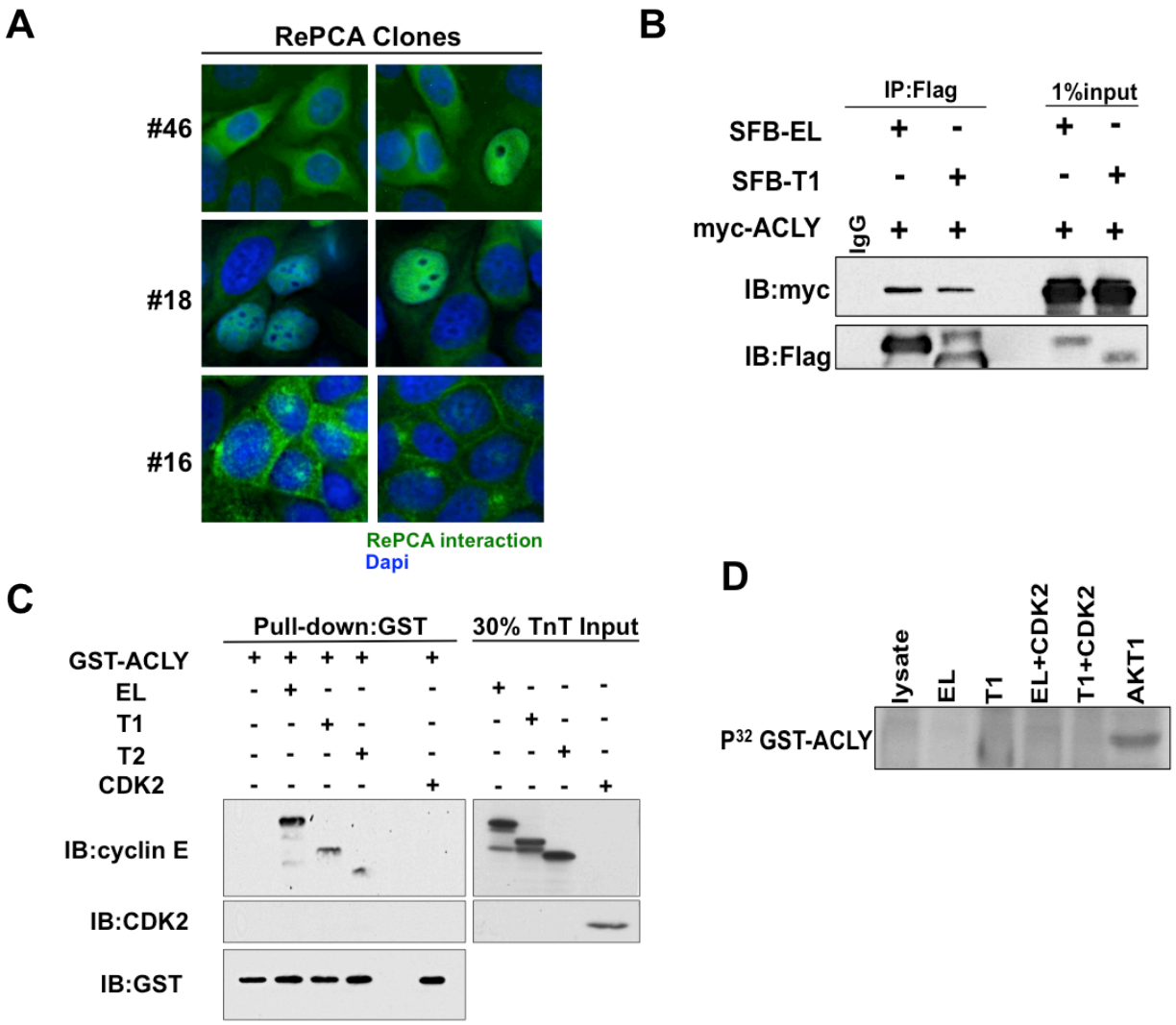

Figure 9. Validation of RePCA clones.

(A) RePCA clone #46, #18 and #16 were fixed and counterstained with Dapi. Fluorescence shows localization of the LMW-E (T1)/ Protein 'X' interaction. (B) 293T cell lysates expressing SFB-tagged cyclin E isoforms and myc-ACLY were immunoprecipitated using an anti-Flag antibody. (C) Recombinant GST-ACLY protein was incubated with *in vitro transcribed and translated (TnT)* cyclin E isoforms or Cdk2 and isolated using Glutathione sepharose beads. (D) *In vitro* kinase assay using recombinant GST-ACLY aa 426-486 and recombinant Cyclin E isoforms alone or in complex with CDK2 were isolated by Cdk2 immunoprecipitation and incubated with GST-ACLY in the presence of ^{32}P-γ-ATP. The samples were run on an SDS-PAGE gel and the autoradiography film is shown. Akt1 was used as a positive control.

membrane. The LMW-E (T1)- ACLY interaction was confirmed by co-immunoprecipitation. Lysates expressing myc-ACLY and either EL- or T1- N-terminally tagged with SFB (S-tag, Flag epitope tag, and streptavidin-binding peptide tag) showed that both full-length EL and LMW-E (T1) were able to bind ACLY (Figure 9B).

Since Cdk2 is the primary binding partner of cyclin E, we examined whether cyclin E or Cdk2 directly binds ACLY. A GST pull-down assay revealed cyclin E as the direct binding partner of ACLY and the interaction is not mediated by other proteins including CDK2 (Figure 9C). Furthermore, ACLY is phosphorylated post-translationally by multiple proteins, including cAMP-dependent protein kinase, GSK3β, nucleoside diphosphate kinase (NDPK) and protein kinase B (Akt) [156-158]. Therefore, we examined whether ACLY is a substrate of the cyclin E/Cdk2 complex. An *in vitro* kinase assay revealed that ACLY is not a substrate of EL/Cdk2 or T1/Cdk2. Akt1, known to phosphorylate was used as a positive control [153]. Together, these results indicate that ACLY directly binds cyclin E and protein binding does not result in phosphorylation of ACLY.

2.3c. Identification of the ACLY domain that binds to cyclin E

ACLY, a member of the acyl-CoA synthetase (NDP-forming) superfamily [159], is similar in structure to succinyl-CoA synthetase (SCS) and consists of five domains shared by all members of this superfamily [160]. The domains are numbered corresponding to their order in *Escherichia coli* SCS [161]. As shown in figure 10A domain 1 binds CoA and domain 2 binds the catalytic phosphohistidine residue. Together, domains 3 and 4 form an ATP grasp fold and bind nucleotide. Domain 5 binds domain 2, creating one of two “power helices”. The region of ACLY between 5

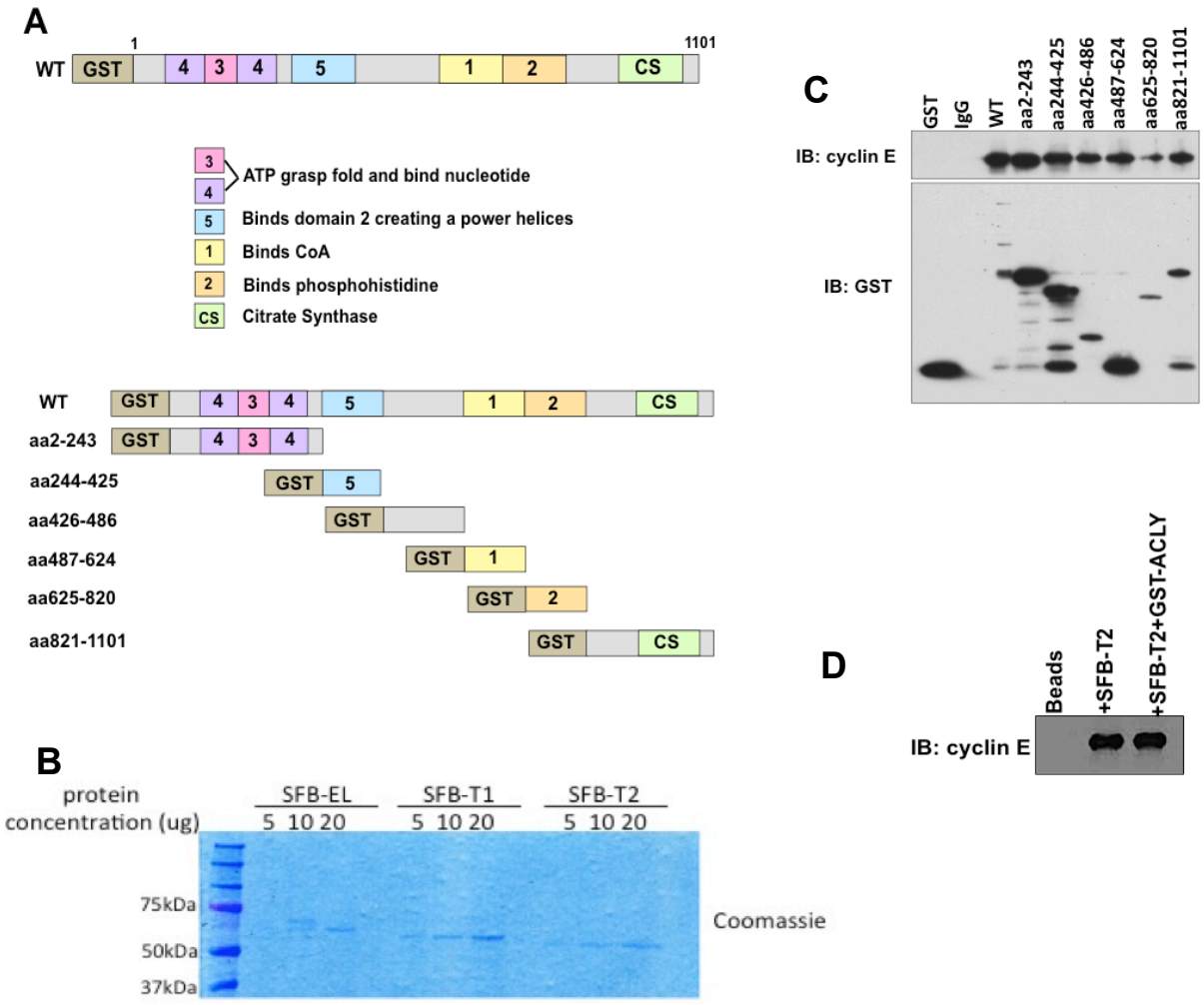

Figure 10. Analysis of interaction between cyclin E and ACLY

(A) Schematic of ACLY protein. An N-terminal GST tag was added to ACLY protein. ACLY consists of five domains followed by a citrate synthase homology domain (*adapted from [160]*). Six truncations of GST-ACLY were generated to study the interaction between cyclin E and ACLY. (B) Coomassie stained gel showing purification of SFB-tagged cyclin E isoforms from HEK 293T cells. (C) Recombinant GST-ACLY protein truncations were incubated with purified SFB-LMW-E(T2) and complex formation was isolated using Glutathione sepharose beads. (D) SFB-LMW-E(T2) alone or SFB-LMW-E(T2) + GST-ACLY WT were incubated in the presence of glutathione beads and complex formation was assessed.

and 1 is the highly phosphorylated region of the protein [160]. The C-terminal end of ACLY displays high sequence homology to the large domain of citrate synthase [160]. To identify which region of ACLY interacts with cyclin E we generated six GST-ACLY fusion protein truncations utilizing the Gateway cloning system and expressed these recombinant proteins in *E coli* (Figure 10A). Additionally, we overexpressed SFB-tagged isoforms of cyclin E in HEK 293T cells and purified the cyclin E complexes by Flag pull-down and elution with Flag peptide (Figure 10B). Purification resulted in isolation of all three isoforms of cyclin E.

We performed a GST pull-down assay using our purified cyclin E isoforms and GST-ACLY protein truncations. Expression of GST-ACLY truncations was variable, with aa 2-243, aa 244-425 and aa 426-624 showing very high expression, while GST-ACLY WT was the least expressed, probably due to the size of the fusion protein (over 150kDa). Surprisingly, pull-down analysis showed that all truncations of ACLY bound very effectively to LMW-E(T2) (Figure 10C). This result was also seen in the other isoforms of cyclin E (EL and LMW-E(T1)) (data not shown) and was suspicious because every truncation created should not be able to bind to cyclin E. Therefore, we suspected non-specific binding could be due to something that each of the truncations has in common, the SFB-tag or the glutathione beads. To examine non-specific binding to the beads, we incubated SFB-T2 alone or in combination with the glutathione beads and as suspected, SFB-T2 was able to bind without the requirement of the GST tag (Figure 10D). In our hands, the use of GST and SFB tags on ACLY and LMW-E(T2), respectively, did not result in identification of the region of binding and future

studies will require creation of a different cyclin E fusion protein that minimizes non-specific binding.

2.4 CONCLUSIONS

In the age of proteomics, methods to identify protein-protein interactions are essential to understanding protein function. The methods to identify protein-protein interactions have become increasingly more reliable by reducing false positive rates and enhancing specificity. In this chapter we have shown that RePCA is an effective, high throughput method to identify protein-protein interactions in an endogenous environment. RePCA was first utilized to identify protein binding partners of Akt1 and we confirm that RePCA is a useful tool to identify protein-protein interactions [125]. We created a host cell line, MCF7, containing IFPN-tagged forms of cyclin E and subsequently transduced LMW-E(T1) with the RePCA vector. From 10×10^6 cells originally infected, we obtained 61 colonies from which to extract RNA. Of the 61 colonies, we identified 11 novel interacting proteins of LMW-E(T1) and most proteins identified localized to the cytoplasm (Table 6). Ding and colleagues found 24 independent binding partners of Akt from all three RePCA ORFs [125]. Specifically, from the IC2 ORF, 2×10^7 cells were infected and subsequently formed 384 colonies. Furthermore, 14% of colonies retained fluorescence after doxycycline induction. These results are similar to what we found using RePCA screening methodology in that RePCA yields tens of candidates, not hundreds like AP-MS.

To validate our results from the RePCA screen, we first subjected several clones to fluorescence microscopy to investigate subcellular localization of the LMW-E(T1)

protein complex. The localization of the LMW-E(T1)/ACLY protein complex from RePCA clones #46 and #18 were localized primarily to the cytoplasm with a small percentage of cells showing nuclear localization (Figure 9A). Moreover, we confirmed the interaction between LMW-E(T1) and ACLY by co-immunoprecipitation and identified the interaction is direct and not mediated by other proteins (Figure 9B,C)

Regulation of ACLY, in part, occurs through multiple phosphorylation events [156-158]. For example, an antibody raised against an Akt substrate consensus sequence proved ACLY to be a substrate of Akt in rat primary adipocytes in an insulin-dependent manner [158]. Therefore, we investigated whether ACLY could be a substrate of cyclin E/Cdk2 and our results revealed that cyclin E/Cdk2 does not phosphorylate ACLY (Figure 9D).

To gain insight to function of the cyclin E/ACLY protein complex, we investigated which domain of ACLY bound to cyclin E. We created six truncations of a GST-ACLY fusion protein and created purified isoforms of cyclin E to examine binding (Figure 10A,B). However, our binding analysis revealed that our SFB-cyclin E fusion protein was non-specifically binding to the glutathione beads, thus preventing the identification of the region that binds to cyclin E (Figure 10D). Future experimental designs will avoid SFB-tagged proteins and glutathione beads and will utilize other tags such as MBP (maltose binding protein) for purification. Nevertheless, the use of RePCA technology to identify LMW-E(T1) binding protein in the cytoplasm was successful and the identification of ACLY as a cyclin E binding protein sheds new light on how the cell cycle and cellular metabolism are coupled in breast cancer.

CHAPTER 3: **ATP-CITRATE LYASE LINKS LOW MOLECULAR WEIGHT CYCLIN E TO CELLULAR METABOLISM IN BREAST CANCER**

3.1 INTRODUCTION

3.1a. Overview of cancer cell metabolism

In 2000, Douglas Hanahan and Robert Weinberg published the seminal paper, "The Hallmarks of Cancer" describing six characteristics of human cancers that allow cancer cells to proliferate, survive and metastasize throughout the body [72]. These characteristics include: growth signal self-sufficiency, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, maintained angiogenesis, and tissue invasion and metastasis [72]. A decade later, they revised the hallmarks to include emerging hallmarks of cancer such as deregulated cellular energetics and evasion from immune destruction [162].

The idea that cancer cells display defects in metabolic regulation was first described almost 100 years earlier by Otto Warburg [163]. Under normal conditions, non-proliferating cells undergo aerobic respiration; a process in which glucose is converted to pyruvate via glycolysis, then to carbon dioxide following oxidative phosphorylation (OXPHOS) in the mitochondria yielding a net total of 36 ATP from one glucose molecule [162, 164]. On the other hand, proliferating cells undergo anaerobic respiration either when oxygen levels are limited or to meet the demands of macromolecular synthesis (DNA, membrane or protein synthesis) and primarily utilizes glycolysis for energy yielding a total of 2 ATP molecules [162]. However, many cancer cells, even under conditions when oxygen is present, primarily exploit glycolysis for

energy production; an event coined the ‘glycolytic switch’, ‘aerobic glycolysis’ or ‘Warburg effect’ [163, 165].

The Warburg effect, on its own, is a highly inefficient method to produce the energy required to fuel a proliferating cell population and therefore, to keep up with demand, the cancer cell increases glucose uptake which in turn results in lactate excretion [165, 166]. The elevated level of glucose uptake by cancer cells is exploited clinically through the use of [^{18}F] fluorodeoxyglucose positron emission tomography (FDG-PET) imaging [163, 165-167]. Specifically, FDG-PET uses a radiolabeled glucose analogue to expose regions of high glucose consumption and has been shown to be efficacious in diagnosing and monitoring tumors [165].

Ultimately, the metabolic reprogramming of cancer cells to aerobic glycolysis is advantageous to fuel growth since aerobic glycolysis consumes glucose; one of the most plentiful extracellular nutrients, and uses it for ATP production [165]. Additionally, elevated influx of glucose provides the substrates required for macromolecular biosynthetic pathways [165, 166]. For example, ribose sugars are used during nucleotide synthesis, and citrate, from a truncated tricarboxylic acid cycle (TCA), provides substrates required for fatty acids production, and nonessential amino acids for protein production [166, 167].

3.1b. Deregulated metabolic pathways in cancer

The PI3K/Akt signaling pathway serves as a hub for multiple downstream pathways required for cell survival, proliferation and protein synthesis (Figure 11) [168]. Consequently, this pathway is also one of the most frequently mutated pathways in

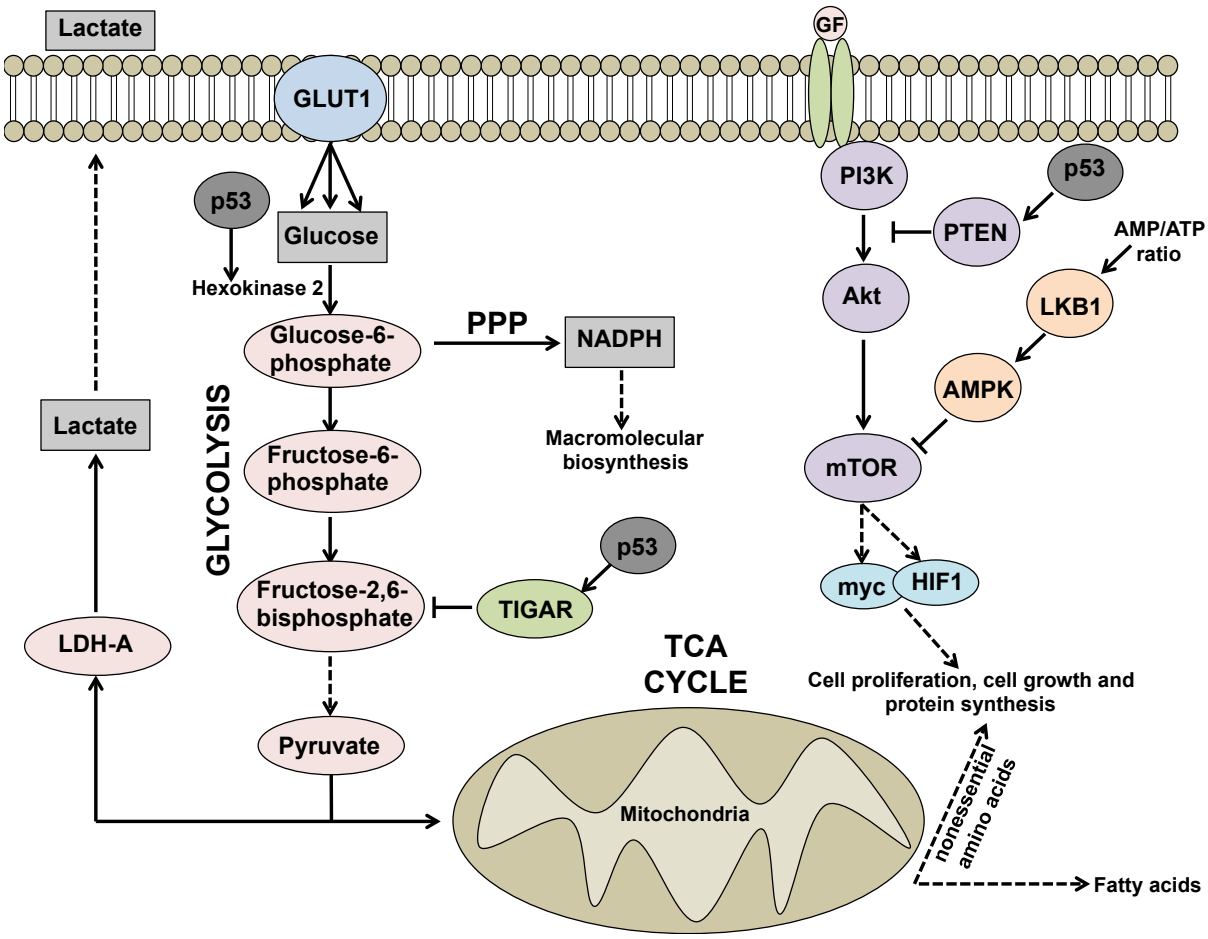

Figure 11. Molecular pathways that induce the Warburg effect

The Warburg effect can be induced by oncogenic pathways to confer a glycolytic phenotype. The PI3K/Akt/mTOR pathway enhances the glycolytic phenotype by regulating enzymes directly involved in glycolysis or by activating key transcription factors and can be negatively regulated by the AMPK pathway. p53 also plays an important role in regulating the expression of key metabolic enzymes including HK2 and TIGAR as well as influencing PI3K pathway through inhibition of tumor suppressor, PTEN. *(Figure adapted from Cairns, R.A., I.S. Harris, and T.W. Mak, Regulation of cancer cell metabolism. Nat Rev Cancer, 2011. **11**(2): p. 85-95.)*

human cancer (Table 7) [165]. Mutations in the tumor suppressor, phosphatase and tensin homolog (PTEN), mutations in the PI3K complex or inappropriate signaling from receptor tyrosine kinases (RTKs) all confer a growth and survival phenotype in cancer cells through constitutively activating Akt and promoting the Warburg effect through multiple mechanisms [165]. First, Akt can facilitate glucose transporters such as GLUT1, to translocate to the membrane to increase glucose uptake [165, 166, 169]. Additionally, Akt influences transcriptional changes that result in elevated glycolytic function through inhibition of the forkhead box subfamily O (FOXO) family of transcription factors [165, 170]. Lastly, Akt is responsible for promoting signaling through mammalian target of rapamycin (mTOR) by inhibition of its negative regulator, tuberous sclerosis 2 (TSC2) [165]. The mTOR pathway acts as a sensor of nutrient availability and if growth conditions are sufficient, can promote protein synthesis, specifically, mRNA translation and ribosome biogenesis [165, 171]. Furthermore, the mTOR pathway in tumor cells can promote transcriptional changes through activation and stabilization of hypoxia-inducible factor 1 alpha (HIF1α) even when oxygen conditions are normal [165].

Another pathway often deregulated in cancer cells is the AMPK pathway (Figure 11). AMPK (AMP-activated protein kinase) acts a checkpoint for energy stress [165]. Under conditions of energy stress such as a low AMP/ATP ratio, AMPK becomes activated and signals the cell to switch from glycolytic metabolism to oxidative metabolism; thus, abrogating cellular proliferation [165, 172, 173]. Tumor cells are able to bypass this checkpoint pathway by mutating the tumor suppressor, LKB1 (liver kinase B1) (Table 7). LKB1 mutations are responsible for Peutz-Jeghers syndrome; a

Table 7: Molecular alterations that affect metabolic circuits

Molecule	Activity in cancer	Consequence in cancer	Refs
PI3K	Mutation; gain-of-function	Activation of Akt and facilitates upregulation of glucose transporters, transcription factors and mTOR	165, 166, 169, 170
PTEN	Mutation; loss-of-function	Activation of Akt and facilitates upregulation of glucose transporters, transcription factors and mTOR	165
LKB1	Mutation; loss-of-function	Increased glycolytic flux; activation of mTOR and HIF1α	165, 173-175
HIF1α	Constitutive expression; gain-of-function	Increases expression of genes encoding glycolytic enzymes, VEGF and other proteins involved in the hypoxic response	165, 166, 176
Myc	Constitutive expression; gain-of-function	Increases expression of LDH-A; increases glycolysis and lactate production	165
Ras	Mutation; gain-of function	Increases glycolysis and VEGF expression	165
p53	Mutation; loss-of-function	Stabilized by HIF1α; activates HK2; induces apoptosis under hypoxic and acidic conditions through TIGAR expression	165, 179, 180
VHL	Mutation; loss-of-function	Destabilizes hypoxia induced transcripts; VEGF and GLUT1	165, 177, 178
PKM2	Alternative splice variant; lower activity	Guides carbon sources away from	165, 182-

		OXPHOS	185
IDH1/2	Mutation; loss-of-function; gain-of-alternative function	Creates onco-metabolite	165, 190, 191
SDH	Mutation; loss-of-function	Elevated levels of succinate; impairment of PHD3; elevated levels of HIF	166, 192
FH	Mutation; loss of function	Elevated levels of fumarate; modification of cysteine residues leading to negative regulation of Nrf2; upregulation of anti-oxidant response genes	166, 192

disease which starts out as benign lesions of the gastrointestinal tract and oral cavity, but shifts to increase the risk of a wide range of other malignancies [165]. Moreover, LKB1 is mutated in 15-35% of non-small cell lung cancer (NSCLC) cases and 20% of cervical carcinomas [173-175]. Ultimately, loss of AMPK signaling can lead to activation of mTOR and HIF1, both leading to an increased glycolytic phenotype [165].

HIF1α, as previously mentioned, can be induced through transcriptional activation downstream of PI3K, Akt and mTOR (Figure 11) [165]. Under normal oxygen conditions, HIF1α is posttranslationally modified by prolyl hydroxylation and this modification signals von Hippel-Lindau (VHL) tumor suppressor, a ubiquitin ligase, ultimately leading to HIF1α degradation [165, 166]. However, under hypoxic conditions, HIF1α is stabilized and transcriptionally activated to increase expression of glucose transporters and glycolytic enzymes [166, 176]. Of note, HIF1α can be activated under normoxic conditions in tumor cells by the inactivation of VHL or mutations in the PI3K pathway (Table 7) [165]. For example, in renal-cell carcinoma, mutations in VHL lead to constitutively active HIF1α and re-expression of VHL is able to rescue HIF1α function and decrease the aerobic glycolysis phenotype [177, 178].

Lastly, although primarily known for its essential role in regulating DNA damage and the apoptosis response, p53 is also a player in regulating key metabolic genes (Figure 11) [165]. First, p53 can induce the expression of HK2 (hexokinase 2), the first enzyme in glycolysis and produces glucose-6-phosphate (Figure 11) (Table 7) [165, 179]. Glucose-6-phosphate is an important molecule and is present at the crossroads of two metabolic pathways: glycolysis and the pentose phosphate pathway (PPP). Glycolysis produces ATP and synthesizes pyruvate for the tricarboxylic acid cycle

(TCA) and the PPP produces NADPH and ribose sugars, both important for nucleotide synthesis [165]. Furthermore, p53 regulates expression of TIGAR (TP-53 induced glycolysis and apoptosis regulator), an enzyme that controls the levels of a key glycolysis enzyme, fructose-2,6-bisphosphate [165, 180]. Lastly, it is well known that p53 is a regulator of PTEN, a key inhibitor of the PI3K pathway [165, 181].

3.1c. Onco-enzymes and onco-metabolites in metabolic pathways

Pyruvate kinase (PK) functions in the final irreversible step of glycolysis converting phosphoenolpyruvate (PEP) to pyruvate (Figure 11) [165, 182]. In mammals, there are multiple isoforms of PK including type L, type R, type M1 and type M2 [165, 183]. Type L is expressed in the liver and kidney; type R is found in erythrocytes; type M1 is expressed in the muscle cells and brain; and type M2 is found in embryonic stem cells, adult stem cells and tumor cells [165, 183]. PK is found either as an inactive monomer, low activity dimers or as active tetramers [182]. The low activity dimer is what is most often found in many cancers and although counterintuitive, its low enzymatic activity decreases the rate of glycolysis and thus, increases metabolic flux required for macromolecular biosynthesis through PPP for nucleotide synthesis and other pathways for phospholipid and amino acid synthesis [165, 167, 183-185]. Indeed, expression of PKM2 in lung cancer cells is more conducive to tumor growth than the PKM1 isoform [184].

The expression of PKM2 over PKM1 in cancer cells is due to preferential expression by myc coupled with unique splicing events [165]. Specifically, myc induces the expression of heterogeneous nuclear ribonucleoproteins (hnRNPs) that bind and inhibit exon 9, which encodes PKM1 and liberates exon 10, which encodes PKM2 [165,

186]. At the enzymatic level, negative regulation of PKM2 occurs by growth factor-derived phosphotyrosine signaling by FGFR1, Bcr-Abl and JAK2 [187].

PKM2 is primarily a cytoplasmic enzyme, but has recently been shown to localize to the nucleus as well [188]. This translocation is due to apoptotic signals, response to IL-3, acetylation on Lys 433 or by phosphorylation by extracellular signal-related kinase 2 (ERK2) [182]. Specifically, upon EGFR stimulation, ERK2 interacts with and phosphorylates PKM2 at serine 37 [188]. This phosphorylation recruits PIN1 for cis-trans isomerization leading to exposure of its NLS and interaction with importin 5α [188, 189]. Nuclear PKM2 in concert with β-catenin induces myc expression, eventually resulting in GLUT1 and LDH-A expression forming a positive feedback loop [188, 189]. Ultimately, PKM2 expression in tumor cells has the ability to guide carbon precursors away from pyruvate production and into pathways responsible for macromolecular biosynthesis required for growth and proliferation.

Additional onco-enzymes are isocitrate dehydrogenases (IDH1 and IDH2) that function in the TCA cycle to produce α-ketoglutarate (αKG) from isocitrate [165]. In 2008, Parsons et al. and colleagues sequenced 20,661 protein-coding genes in glioblastoma multiforme (GBM) and found a number of genetic aberrations not previously known to be present in GBM [190]. Of particular interest was a mutation in the active site of IDH1 [190]. Studies have now shown that either IDH1 or IDH2 is mutated in almost 80% of grade II and III glioblastomas and acute myeloid leukemias (AML) [165]. Mutations in IDH1 are heterozygous; an indication that this mutation is a gain-of-function event [165]. A year later, in 2009, Dang et al. and colleagues discovered a single mutation at arginine 132 and instead of synthesizing αKG, mutated

IDH1 is responsible for production of a completely new metabolite, R (-)-2-hydroxyglutarate (2HG) [191]. Moreover, this onco-metabolite, in excess, results in a higher risk of formation and progression of glioblastoma and AML [165, 191]. Thus, the presence of a tumor-specific metabolic alterations such as a mutations in IDH1 yields a valuable screening protocol for patients as well as a providing a plausible treatment strategy, inhibiting 2HG production.

Lastly, although rare, mutations in succinate dehydrogenase (SDH) and fumarate hydratase (FH) occur in pheochromocytoma, paraganglioma, leiomyoma, and renal carcinoma [166, 192]. Elevated levels of succinate and fumarate are the results of SDH and FH mutations and succinate specifically deregulates PHD3, an αKG-dependent enzyme responsible for regulating HIF1α stability [192]. Similarly, fumarate can deregulate PH2, but is also responsible for negative regulation of the transcription factor, Nrf2, leading to anti-oxidant gene upregulation [192].

3.1d. Therapeutic targets in metabolic pathways

Eradicating tumor cells and leaving normal cells unharmed is the challenge of modern cancer therapy. Indeed, tumor cells undergo a 'metabolic transformation' that aids in proliferation and survival; and consequently, renders altered metabolic pathways susceptible to inhibition (Figure 12) [193]. About 20 years after Otto Warburg described aberrant glycolysis in cancer cells, the first antimetabolites were used in cancer therapy [193, 194]. Farber et al. and colleagues used a folic acid antagonist and observed temporary remission of acute leukemia in children [194]. Since then a number of inhibitors of either metabolic enzymes or inhibitors of metabolic pathways have been generated to target these 'metabolically transformed' cells (Table 8) [193].

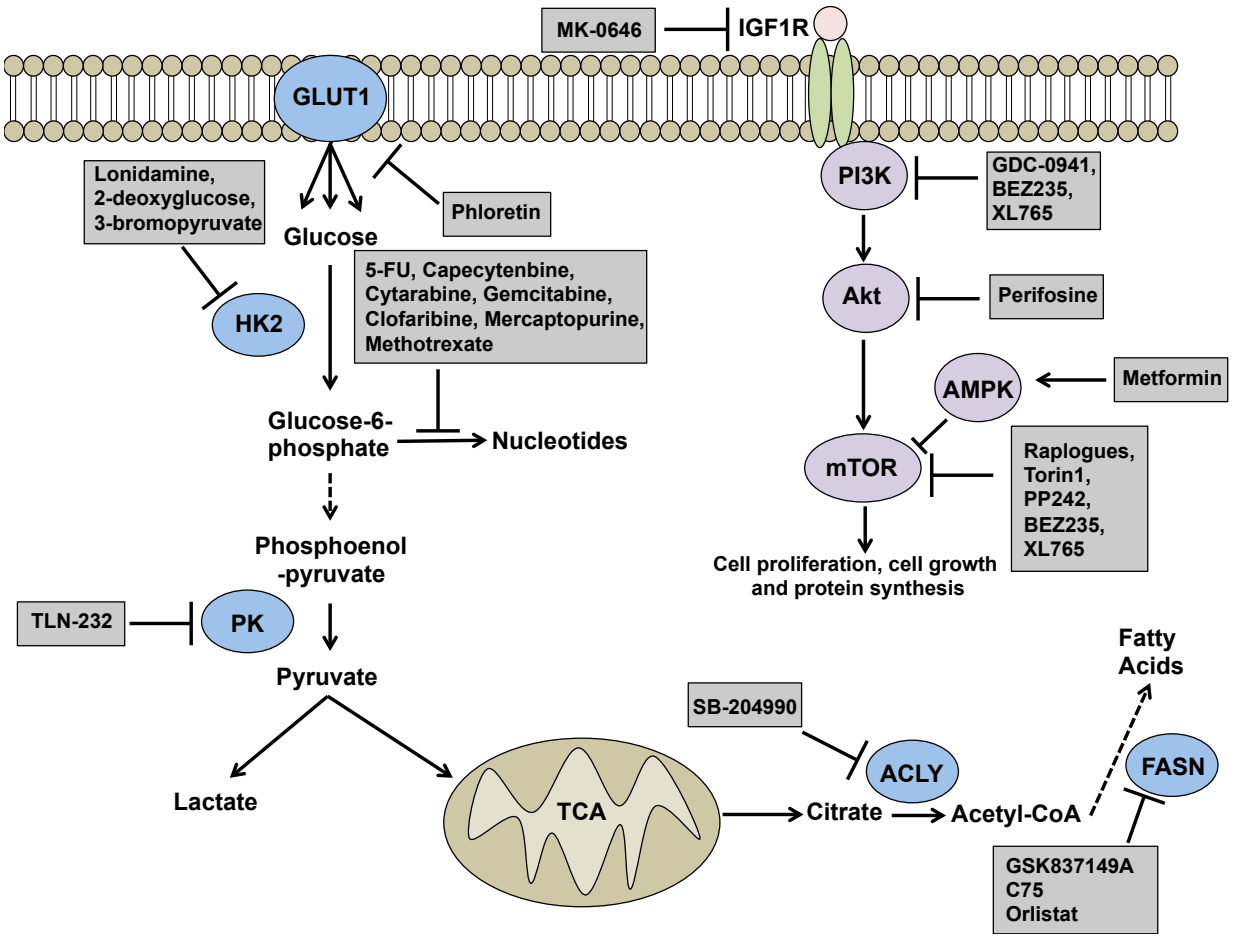

Figure 12. Therapeutic targets in metabolic pathways

Metabolic pathways that control growth and proliferation or provide macromolecular intermediates are attractive targets for intervention. PI3K/Akt/mTOR inhibitors indirectly impact metabolic pathways by inhibiting cell growth, proliferation and survival. Direct targets of metabolic enzymes reduce the yield of pathway output leading to a reduction in macromolecular intermediates required for cell proliferation. (Adapted from: Tennant, D.A., R.V. Duran, and E. Gottlieb, *Targeting metabolic transformation for cancer therapy.* Nat Rev Cancer, 2010. **10**(4): p. 267-77.)

Table 8: Inhibitors of metabolic enzymes or effectors of common pathways

Compound	Target	Effect	Stage of approval	Types of cancers	Study number(s)
2-deoxyglucose	Hexokinase	Inhibits glycolysis	Phase I&II	Lung, breast, prostate, gastic, head and neck	• NCT00633087 • NCT00096707 • NCT00247403
Lonidamine	Hexokinase	Inhibits glycolysis	Phase III	Benign prostatic hyper-plasia	• NCT00435448 • NCT00237536
3-bromopyruvate	Hexokinase	Inhibits glycolysis	Pre-clinical	N/A	N/A
TLN-232	Pyruvate Kinase	Inhibits glycolysis	Phase II	Metastatic melanoma and renal cell carcinoma	• NCT00735332
Anti-metabolites (i.e. 5-FU, cytarabine, methotrexate)	Nucleotide biosynthetic pathway	Inhibits cell proliferation	FDA approved	Multiple cancer types	>100
MK-0646	IGF1R	Blocks IGF signaling	Phase I&II	Lung, pancreas, liver, breast	• NCT00799240
BIIB022	IGF1R	Blocks IGF signaling	Phase I&II	Lung, pancreas, liver, breast	• NCT00555724
AVE1642	IGF1R	Blocks IGF signaling	Phase I&II	Lung, pancreas, liver, breast	• NCT00791544
GDC-0941	PI3K	Inhibits PI3K signaling	Phase I&II	Breast, lymphoma	• NCT00876109
PX866	PI3K	Inhibits PI3K	Phase I&II	Breast, lymphoma	• NCT00726583

		signaling			
BEZ235	PI3K/mTOR	Inhibits signaling from PI3K and mTORC1 and mTORC2	Phase I&II	Advanced solid tumors (i.e. lung, brain)	• NCT00485719
XL765	PI3K/mTOR	Inhibits signaling from PI3K and mTORC1 and mTORC2	Phase I&II	Advanced solid tumors (i.e. lung, brain	• NCT00777699
SF1126	PI3K/mTOR	Inhibits signaling from PI3K and mTORC1 and mTORC2	Phase I&II	Advanced solid tumors (i.e. lung, brain	• NCT00704080 • NCT00907205
BGT226	PI3K/mTOR	Inhibits signaling from PI3K and mTORC1 and mTORC2	Phase I&II	Advanced solid tumors (i.e. lung, brain	• NCT00600275
Torin1	mTORC1 and mTORC2	Inhibits mTORC1 and mTORC2	Pre-clinical	N/A	N/A
PP242	mTORC1 and mTORC2	Inhibits mTORC1 and mTORC2	Pre-clinical	N/A	N/A
Periforsine	Akt	Inhibits Akt	Phase I&II	Renal cancer, lung, lymphoma	• NCT00399789 • NCT00399152
GSK690693	Akt	Inhibits Akt	Phase I&II	Renal cancer, lung, lymphoma	• NCT00493818
PX-478	HIF1α	Inhibits HIF	Phase I	Advanced	• NCT00522652

		signaling		solid tumors and lymphoma	
Acriflavine	HIF1α	Inhibits HIF signaling	Pre-clinical	N/A	N/A
Metformin	AMPK complex and Complex I of mitochondria	Activates AMPK	Phase I&II	Solid tumors and lymphoma	• NCT00659568 • NCT00881725 • NCT00984490 • NCT00909506
SB-204990	ATP-citrate lyase	Inhibits fatty acid synthesis	Pre-clinical	N/A	N/A
Orlistat	FASN	Inhibits fatty acid synthesis	Pre-clinical	N/A	N/A
GSK837149A	FASN	Inhibits fatty acid synthesis	Pre-clinical	N/A	N/A
C75	FASN	Inhibits fatty acid synthesis	Pre-clinical	N/A	N/A
Adapted from: Tennant, D.A., R.V. Duran, and E. Gottlieb, *Targeting metabolic transformation for cancer therapy*. Nat Rev Cancer, 2010. **10**(4): p. 267-77.					

Growth signaling pathway inhibitors such as those targeting IGF1R, PI3K and Akt are used as an indirect method to target tumor cell metabolism and have had success in decreasing cancer cell progression [195]. One of the most well known metabolic drugs identified that is clinically used is metformin, an AMPK activator. Metformin was first used in patients with type 2 diabetes; however, a study found that diabetes patients that were treated with metformin had a higher probability of being cancer-free after an 8-year follow-up compared to patients treated with other regimens [193, 196]. These results were corroborated in preclinical mouse models as well as in diabetes patients with breast cancer and found that metformin has a profound anti-tumor effect [193, 197, 198].

Another attractive target for intervention is glycolysis. As previously stated, many tumor cells display elevated levels of glycolysis and greatly uptake glucose regardless of bioenergetic requirement [165]. However, many inhibitors of glycolysis used as a monotherapy, such as 2-deoxyglucose (a glucose mimetic which targets hexokinase 2), are unsuccessful in osteosarcoma and NSCLC and require combination treatment with either chemotherapy and/or radiotherapy [193, 199, 200]. For example, 2-deoxyglucose is synergistic when combined with adriamycin and paclitaxel in both osteosarcoma and non-small cell lung cancer [199].

Moreover, the conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase is an important rate-limiting step of glycolysis and the PK inhibitor, TLN-232, is currently in Phase II clinical trial [193]. However, TLN-232 is a PKM1 specific inhibitor and as previously mentioned in chapter 3.1c, PKM2 is the predominant isoform of PK in cancer cells. Recently, a PKM2 knockdown in a mouse model showed that PKM2 is

not required for tumor growth *in vivo* and that other growth pathways may become activated following its removal [201].

Another mode of intervention includes agents developed to reduce HIF1 levels either by targeting effector molecules or by inhibiting HIF1 dimerization (Table 8) [193]. For example, acriflavine prevents dimerization between HIF1α and HIF1β subunits and has been shown to be effective at inhibiting xenograft prostate cancer growth [193, 202]. Similarly, PX-478 has been shown to decrease HIF1α levels leading to a profound inhibition of tumor growth both *in vitro* and *in vivo* in multiple cancer types [193, 203].

Inhibition of the mTOR pathway is also an attractive target due to its overactivity in multiple types of cancers [193]. Unfortunately, the mTOR inhibitor, rapamycin, and its many rapalogues have had limited success as a monotherapy due to additional activation of PI3K/Akt [193, 204]. Therefore, the creation of compounds targeting the combination of mTORC1, mTORC2 and PI3K such as BEZ235 and XL765 have been developed for lung and brain cancers and are currently in Phase I and II clinical trial [193].

Lastly, cancer cells are largely dependent on *de novo* fatty acid synthesis for their supply of fatty acids rather than from exogenous accumulation and as a consequence, upregulate the *de novo* lipogenesis pathway [205]. The *de novo* lipogenesis pathway will be described in more detail in Chapter 3.1e, however, agents have been developed that target enzymes in this pathway; mainly inhibitors of fatty acid synthase (FASN) and ATP-citrate lyase (ACLY) [193, 205]. FASN is overexpressed in a variety of cancers, including cancers of the breast, colon and endometrium and the

inhibition of FASN using C75, orlistat and GSK837149A, are very effective *in vitro* and *in vivo* [206-208]. Additionally, FASN inhibitors have been used in combination with 5-FU and traztuzumab to synergistically induce cell death [209, 210]. Likewise, ACLY is overexpressed at the mRNA, protein and enzymatic level in lung adenocarcinoma [153]. Inhibition of ACLY by either genetic or pharmacologic means, reduces glucose-dependent lipid synthesis, proliferation and survival *in vitro* and *in vivo* [211]. ACLY will be discussed in further detail in Chapter 3.1f and 3.1g. Ultimately, targeting metabolic pathways and enzymes as a monotherapy or in combination in transformed cells provides an effective avenue to target tumor-specific alterations and prevent toxicity to normal tissues.

3.1e. The *de novo* lipogenesis pathway

In order to promote growth and proliferation, tumor cells are required to synthesize macromolecular building blocks such as nucleotides, amino acids and fatty acids. Fatty acids are derived from two sources: exogenously via dietary means and endogenously via *de novo* synthesis [212]. The *de novo* lipogenesis pathway synthesizes complex fatty acids such as phospholipids, triglycerides and cholesterylesters that are important for synthesis of cell membranes and lipid-based post-translational modifications on proteins [212]. In contrast to normal cells, tumor cells obtain almost 93% of triacylglycerols from *de novo* synthesis [212]. Therefore, enzymes of this pathway are commonly upregulated in a variety of cancers including cancers of the ovary, breast, stomach, colorectum, lung, bladder and prostate among others [212].

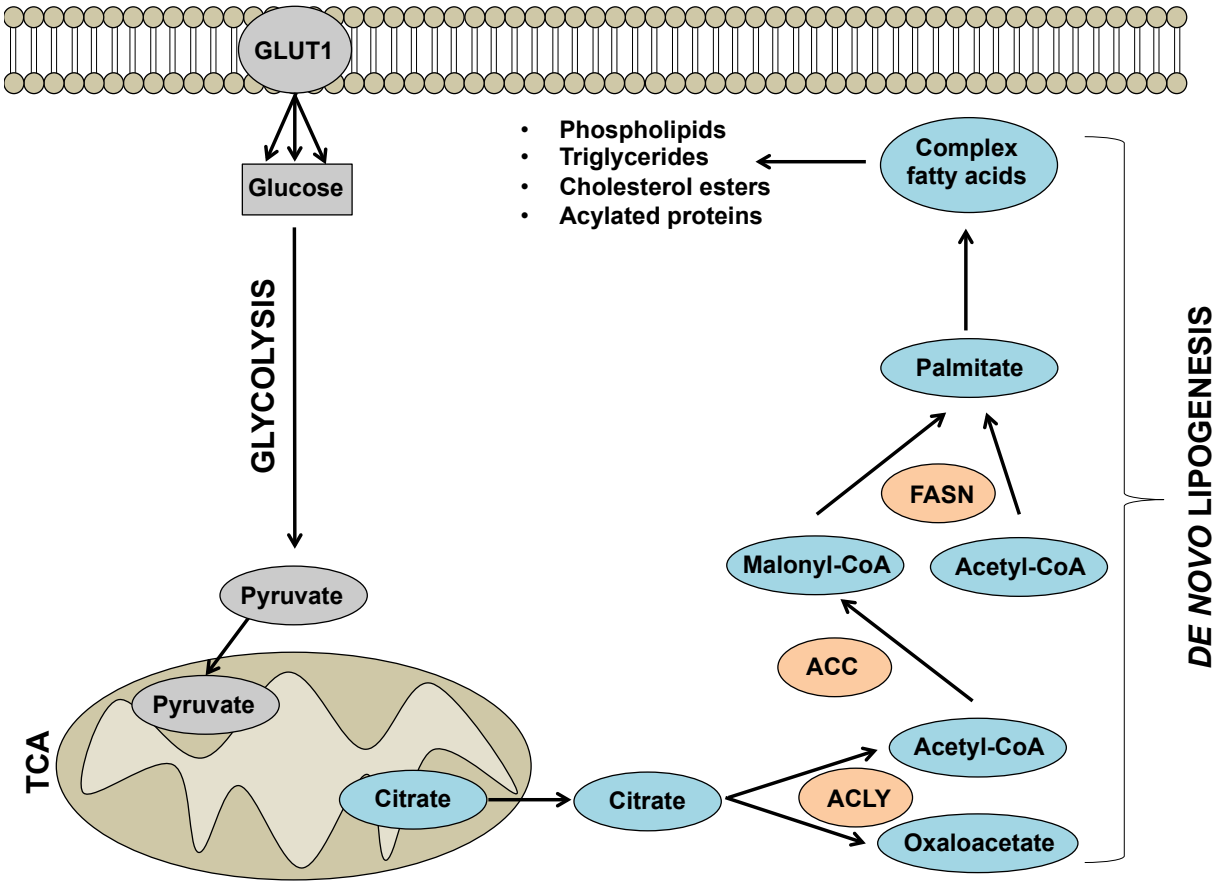

Figure 13. The de novo lipogenesis pathway

The de novo lipogenesis pathway is downstream of glycolysis and a truncated citric acid cycle. Citrate is exported from the mitochondria into the cytoplasm and converted to acetyl-CoA and oxaloacetate by ATP-citrate lyase (ACLY). Acetyl-CoA is used as a substrate for Acetyl-CoA carboxylase (ACC) and synthesizes malonyl-CoA. Fatty acid synthase (FASN) condenses malonyl-CoA and acetyl-CoA to form palmitate and other complex fatty acids such as phospholipids, triglycerides, cholesterol esters. (Adapted from: Menendez, J.A. and R. Lupu, *Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis.* Nat Rev Cancer, 2007. **7**(10): p. 763-77.)

The first enzyme of the lipogenesis pathway is ATP-citrate lyase (ACLY). ACLY catalyzes the conversion of cytoplasmic citrate to acetyl-CoA and oxaloacetate downstream of a truncated citric acid cycle (Figure 13) [205, 212]. Next, acetyl-CoA carboxylase (ACC) synthesizes malonyl-CoA in a rate-limiting fashion [212]. In breast carcinomas, ACC is overexpressed at the mRNA and protein level with its inhibition resulting in decreased cell proliferation and cell viability [213-215]. The last reaction of the pathway is the condensation of malonyl-CoA and acetyl-CoA by FASN to palmitate and other complex fatty acids (Figure 13) [212]. Elevated levels of FASN are found in over 20 types of cancer including breast, colorectal, bladder, ovary and lung [212]. Moreover, high FASN expression is also correlated with increased risk of disease recurrence and death in breast cancer patients [208, 216].

3.1f. ATP-citrate lyase

As previously stated, ATP-citrate lyase (ACLY) catalyzes cytoplasmic citrate into acetyl-CoA and oxaloacetate in the *de novo* lipogenesis pathway. In 2004, Beigneux et al. and colleagues attempted to generate an ACLY knockout mouse by crossing ACLY heterozygous mice [217]. However, no ACLY -/- mice were obtained from at least 60 litters and therefore, ACLY is required for embryonic development. Interestingly, heterozygous mice were completely normal in spite of displaying only 50% of ACLY mRNA and protein expression [217].

In mammals, ACLY is highly expressed in white adipose tissue and liver cells, whereas brain, heart, small intestine and muscle cells display low levels of ACLY [218, 219]. Moreover, ACLY is localized primarily in the cytoplasm of cells to synthesize acetyl-CoA in the *de novo* lipogenesis pathway, but recently has been found to be in

the nucleus in murine embryonic fibroblasts, murine pro-B-cell lymphoid cells, glioblastomas and in colorectal carcinomas [220]. In the nucleus, it has been proposed that ACLY is responsible for acetyl-CoA production required for histone acetylation [220].

Sterol regulatory element binding protein-1 (SREBP-1) is a lipogenic transcription factor suggested to regulate the expression of lipogenic enzymes, including ACLY, however, it found that ACLY mRNA levels do not correlate with SREBP-1 levels [153]. Therefore, it was suggested that ACLY might be regulated not at the transcriptional level, but post-translationally by phosphorylation and that the phosphorylation may lead to enzyme activity and protein stability [153]. Indeed, ACLY is phosphorylated by Akt on Ser454 in primary adipocytes as well as Thr 446 and Ser 450 by GSK3β in an insulin dependent manner [158]. However, Migita et al. and collegues noted that ACLY phosphorylation and enzymatic activity were not completely abolished and only exhibited a modest reduction when lung adenocarcinoma cells were treated with the PI3K inhibitor, LY294002 [153]. Therefore, these data indicate that ACLY activity may be regulated in part by another pathway.

3.1g. ATP-citrate lyase and cancer

ACLY has shown to be upregulated either at the protein level or the enzymatic level in multiple cancer types including cancers of the lung, prostate, bladder, breast, liver, stomach and colon [218]. Specifically in breast cancer, ACLY activity is 160 fold higher in breast carcinoma compared to normal breast tissue [152]. Moreover, in lung adenocarcinoma, ACLY is elevated at both mRNA and protein levels with high levels of activated ACLY correlating with stage, grade and poor survival. In contrast, inhibition

of ACLY at both genetic and pharmacologic levels has been effective at suppressing proliferation and tumor growth in both *in vitro* and *in vivo* [211, 221]. These data support the notion that increased ACLY expression and activity provide tumor cells with selective growth advantages in order to support the tumorigenesis program.

3.1f. Hypothesis and specific aims

Metabolic transformation is a common feature of many tumor cells. Upregulation of lipid synthesis as well as upregulation of lipogenic enzymes provide tumor cells with essential building blocks required to maintain growth and survival in unfavorable conditions. In the previous chapter, we found ACLY, a lipogenic enzyme, to be a novel interacting protein of LMW-E in the cytoplasm. Therefore, we ***hypothesize*** that ***LMW-E mediated tumorigenesis requires ACLY***. The following specific aims will address this hypothesis:

- Examine LMW-E mediated changes of ACLY enzymatic activity.
- Analyze lipid synthesis in an LMW-E inducible model system.
- Determine the affects of ACLY knockdown on anchorage-independent growth, migration and invasion of LMW-E expressing cells.
- Examine the requirement of ACLY in xenograft tumor growth in LMW-E expressing breast cancer cells.

Briefly, results presented in this chapter reveal that ACLY is regulated at the enzymatic level by LMW-E. In addition to total ACLY activity increases, ACLY enzymatic activity in the cytoplasm is elevated upon the induction of LMW-E and correlates with lipid droplet formation; which is indicative of activation of the *de novo* lipogenesis pathway. Moreover, ACLY is required for anchorage-independent growth, migration and invasion

in LMW-E expressing cells. Lastly, ACLY is required for xenograft tumor growth of MCF7 cells expressing LMW-E. Taken together, these data provide a unique insight of how metabolic pathways and the cell cycle are intrinsically linked and will help delineate treatment strategies for LMW-E expressing breast cancer patients.

3.2 MATERIALS AND METHODS

3.2a. Cell culture and plasmids

Human breast cancer MCF cells stably overexpressing cyclin E isoforms were cultured as previously described [113]. The immortalized non-tumorigenic human mammary epithelial cell line, 76NE6, which ectopically express cyclin E isoforms under a doxycycline-inducible promoter were cultured as previously described [111, 112]. 76NE6-tumor derived cells (TDCs) were generated and cultured as previously described [111]. The shRNA constructs were purchased from the shRNA and ORFeome core at the University of Texas MD Anderson Cancer Center (Houston, TX) and were generated in a lentiviral packaging system as previously described [117]. Briefly, HEK293T cells were co-transfected with packaging vectors, pCMVΔR8.2 and pMD2.G, along with either pGIPZ scrambled shRNA sequence or shACLY using LipoD293T transfection reagent. Lentiviral supernatant was filtered through a 0.45μm filter and transduced target cells for 48 hrs. Infected cells were selected with 0.5 μg/ml (76NE6) or 2 μg/ml (MCF7) puromycin.

3.2b. Cell lysis, immunoblotting and kinase assays

Cell lysates were prepared and subjected to western blot analysis as previously described in Chapter 2.2d. Western blots were incubated with primary antibodies

against ACLY, Parp and β-tubulin (Cell signaling, Danvers, MA), cyclin E HE12 and CDK2 were obtained from Santa Cruz Biotechnology (Dallas, TX), and β-actin was obtained from EMB Millipore (Billerica, MA). For the kinase assay, lysates from MCF7 cells expressing the cyclin E isoforms and shRNA toward ACLY were immunoprecipitated using a CDK2 antibody. Lysates were incubated with Histone H1 in kinase buffer containing 60 µM cold ATP and 5 µCi of [^{32}P] ATP to a final volume of 30 µl at 37°C for 30 min. The products of the reactions were then analyzed on a 7% SDS-PAGE gel. The gel was then stained, destained, dried, and exposed to X-ray film.

3.2c. In vitro transcription and translation (TnT)

Cyclin E isoforms and CDK2 proteins were generated according to the manufacturers instructions using the TnT T7 Quick Coupled Transcription/Translation System from Promega (Madison, WI).

3.2d. ACLY activity

ACLY activity was measured by the malate dehydrogenase-coupled method as previously described [153]. Briefly, cell lysates were extracted from MCF7 and 76NE6 cell lines and incubated with the reaction mixture (200mM Tris Hcl pH 8.7, 20mM $MgCL_2$, 20mM potassium citrate, 1mM DTT, 0.2mM NADH, 1U/mL MDH, 0.5mM CoA) with and without ATP and subjected to the assay. ACLY activity was measured every 3 minutes for 30 minutes using a NanoDrop 2000c spectrophotometer from Thermo Scientific (Wilmington, DE). ACLY specific activity was calculated as the change in absorbance with ATP minus the change in absorbance without ATP normalized to protein concentration, however, 25ng was used in *in vitro* assays from utilizing purified recombinant ACLY enzyme (US Biological, Salem, MA).

3.2e. Cell fractionation

2 x 10^6 cells were plated on p150 tissue culture plates for 48 hours before harvesting. To harvest cell lysates, cells were washed twice with PBS and removed from the plate using a cell scraper in ice cold PBS. Cells were centrifuged at 2500rpm for 5 minutes at 4°C. Cells were resuspended in RSB buffer (10mM Tris-HCl, pH 7.4, 10mM NaCl, 3mM $MgCl_2$) and centrifuged at 2500rpm for 5 minutes at 4°C. Cells were again resuspended in RSB buffer and homogenized using a loose fitting glass homogenizer then passed through a 25-gauge syringe to break the cell membrane. Lysate was incubated on ice for 10 minutes and centrifuged at 2500rpm for 5 minutes at 4°C. The pellet is the nuclear fraction and the supernatant is the cytosol. The supernatant was removed and placed in a clean tube. The pellet was washed with RSB buffer and centrifuged again. The pellet was resuspended in 50μl Nuclear Extraction Buffer (Cell Biolabs, San Diego, CA) along with 0.5μl of DL-dithiothreitol (DTT) and 5μl protease inhibitors. The pellet was kept on ice for 30 minutes and vortexed at the highest setting every 10 minutes. The pellet was centrifuged for 30 minutes at 14,000 x g at 4°C with the supernatant being the soluble nuclear fraction.

3.2f. Lipid staining

Lipid staining was conducted using the LipidTox reagent according to the manufacturers instructions (Invitrogen, Grand Island, NY). Briefly, cells were fixed using 4% paraformaldehyde and permeabilized with 0.1% saponin for 60 minutes at room temperature. LipidTox reagent was diluted 1:200 in PBS and incubated with cells for 45-60 minutes and imaged on an Olympus Epi-fluorescence microscope with a 60X objective and Hamamatsu Orca camera.

3.2g. Anchorage-independent growth assay

Anchorage-independent growth assays were performed as previously described with the following modifications [222]. Briefly, MCF7 cells were plated at a density of 2,500 cells/well on 12-well plates in 0.35% Seaplaque Agarose (Lonza, Rockland, ME). Cells were grown for 10 to 30 days in their recommended media and fed every 3-4 days. Colonies were measured by using the GelCount system (Oxford Optronix, Abingdon, UK) with colonies greater than 50μm in diameter scored. Three biological replicates were conducted in duplicate, with results reported as average number ± SD.

3.2h. Migration assay

A total of 300,000 cells were plated on a 6-well tissue culture plate and allowed to grow until confluent. The plate was then scratched with a pipet tip. Pictures were taken at 0 and 24 hours using an EVOS XL Core light microscope with a 10X objective (Life Technologies, Grand Island, NY) and scratch opening was measured using Adobe Photoshop (San Jose, CA).

3.2i. Transwell invasion assay

The transwell invasion assay was performed as previously described [111]. Briefly, for each sample, 100 μl of 1 mg/ml Matrigel in serum-free cold MEM media was placed into the upper chamber of 24-well transwell plate (Corning, Corning, NY) and incubated at 37°C for 4–5 hours. Trypsinized cells were washed and suspended in serum-free medium at a concentration of 1×10^6 cells/ml. 100μl of cell suspension was transferred onto the upper chamber, while the lower chamber contains 600 μl of complete media containing 10μg/ml fibronectin. After 24 hours, the cells were fixed

and permeabilized with 4% formaldehyde for 15 minutes, rinsed with PBS three times, incubated with 100% methanol for 20 minutes and washed again. The wells were stained with 0.2% crystal violet for 15 minutes. The crystal violet was removed and the invaded cells were photographed with an EVOS XL Core light microscope. Cells were quantified by trypsinizing the cells and counted using a BioRad TC20 cell counter (Hercules, CA). Each sample was repeated 3 times independently.

3.2j. Cell proliferation assay

Cells were seeded at a density of 1000 cells/well in a 96-well plate and harvested every 24 hours for 96 hours. On day of harvest, 50µl per well of 2.5 mg/mL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphentltetrazolium bromide) (Sigma) was added to serum-free media and allowed to incubate at 37°C for 3 hours. After incubation, media was removed and 100µl solubilization solution (0.04M HCl, 1%SDS, in isopropanol) was added to each well. Plates were incubated with shaking for 1 hour before reading on a plate reader (Epoch Microplate Spectrophotometer Gen 5 software, BioTek, Winooski, VT) at 590nm. Doubling time was calculated in Graphpad Prism software (La Jolla, CA).

3.2k. Real-time PCR

Total RNA was isolated from cell culture with RNAeasy kit with DNase treatment according to the manufacture's protocol (Qiagen). 2 µg of the RNA samples was reverse-transcribed using cDNA synthesis kit (Applied Biosystems). Realtime PCR was done with aliquots of the cDNA samples mixed with SYBR Green Master Mix (Sigma). Reactions were carried out in triplicate. The fold difference in transcripts was calculated by the ΔΔCT method using GAPDH as a control. E-cadherin forward 5'-

TGCCCAGAAAATGAAAAA GG, reverse 3'-GTGTATGTGGCAATGCGTTC Twist forward 5'-GGAGTCCGCAGTCTTA CGAG, reverse 3'-TCTGGAGGACCTGGTAGAG G; Slug forward 5'- GGGGAGAAGCCTTT TTCTTG, reverse 3'-TCCTCATGTTTGTGCAGG AG; and GAPDH forward 5'- ACCCAGAA GACTGTGGATGG-3', reverse 5'-CTGGACTGG ACGGCAGATCT-3'

3.2l. *In vivo* xenograft model

Nude mice were obtained from the department of Experimental Radiation Oncology at The University of Texas MD Anderson Cancer Center (Houston, TX). MCF7 cells were injected in a 50:50 ratio of cells:Matrigel at a density of 2.5 x 10^6 cells/mL in the mammary fat pad. Tumors were measured twice per week with caliper starting at 3 weeks for 12 weeks.

3.2m. Statistical analysis

Experiments were performed in at least duplicate with at least three technical replicates per experiment. All error bars are representative of standard deviation from the mean Statistical analyses were performed using the Student's *t*-test (two sample equal variance; two-tailed distribution) using Graphpad Software, La Jolla, CA).

3.3 RESULTS

3.3a. LMW-E isoforms affects ACLY enzymatic activity

ACLY enzymatic activity has shown to be elevated in breast carcinoma and lung adenocarcinoma compared to normal tissue [152, 153]. Therefore, we examined the effect of cyclin E expression on ACLY enzymatic activity using the malate dehydrogenase in human breast cancer cells that ectopically express the cyclin E

isoforms. Expression of LMW-E(T1) and LMW-E(T2) significantly increased ACLY enzymatic activity by 25-60% , while EL expression resulted in no change (Figure 14A). Moreover, increased ACLY enzymatic activity was only detected upon doxycycline induction of the LMW-E isoforms using the non-tumorigenic human mammary epithelial cell line (hMEC), 76NE6; that contains inducible expression of cyclin E (Figure 14C). The phosphorylation of ACLY on Ser454 by Akt is thought to regulate ACLY enzymatic activity, however, the increase in ACLY enzymatic activity observed upon expression of the LMW-E isoforms did not result in changes of total or phosphorylated ACLY protein levels, indicating the increase in activity is independent of Akt (Figure 14B,D) [153]. Furthermore, examination of the LMW-E isoforms revealed that LMW-E(T1) and LMW-E(T2) directly upregulate ACLY enzymatic activity 35-50%, while addition of EL or CDK2 did not result in a significant increase of ACLY enzymatic activity (Figure 14E).

3.3b. Cytoplasmic ACLY activity results in lipid droplet accumulation

ACLY is primarily found in the cytoplasm, but has also been found to localize to the nucleus in murine embryonic fibroblasts, murine pro-B-cell lymphoid cells, glioblastomas and in colorectal carcinomas [220]. To examine whether the LMW-E isoforms specifically upregulate cytoplasmic ACLY activity, 76NE6 cells with inducible expression of cyclin E were fractionated and enzymatic activity was measured by the malate dehydrogenase coupled method. ACLY enzymatic activity was higher in the cytoplasm compared to the nucleus of all conditions examined, however, ACLY enzymatic activity was increased by 40% in the cytoplasmic fraction of cells containing LMW-E(T1) and LMW-E(T2) expression compared to uninduced conditions (Figure

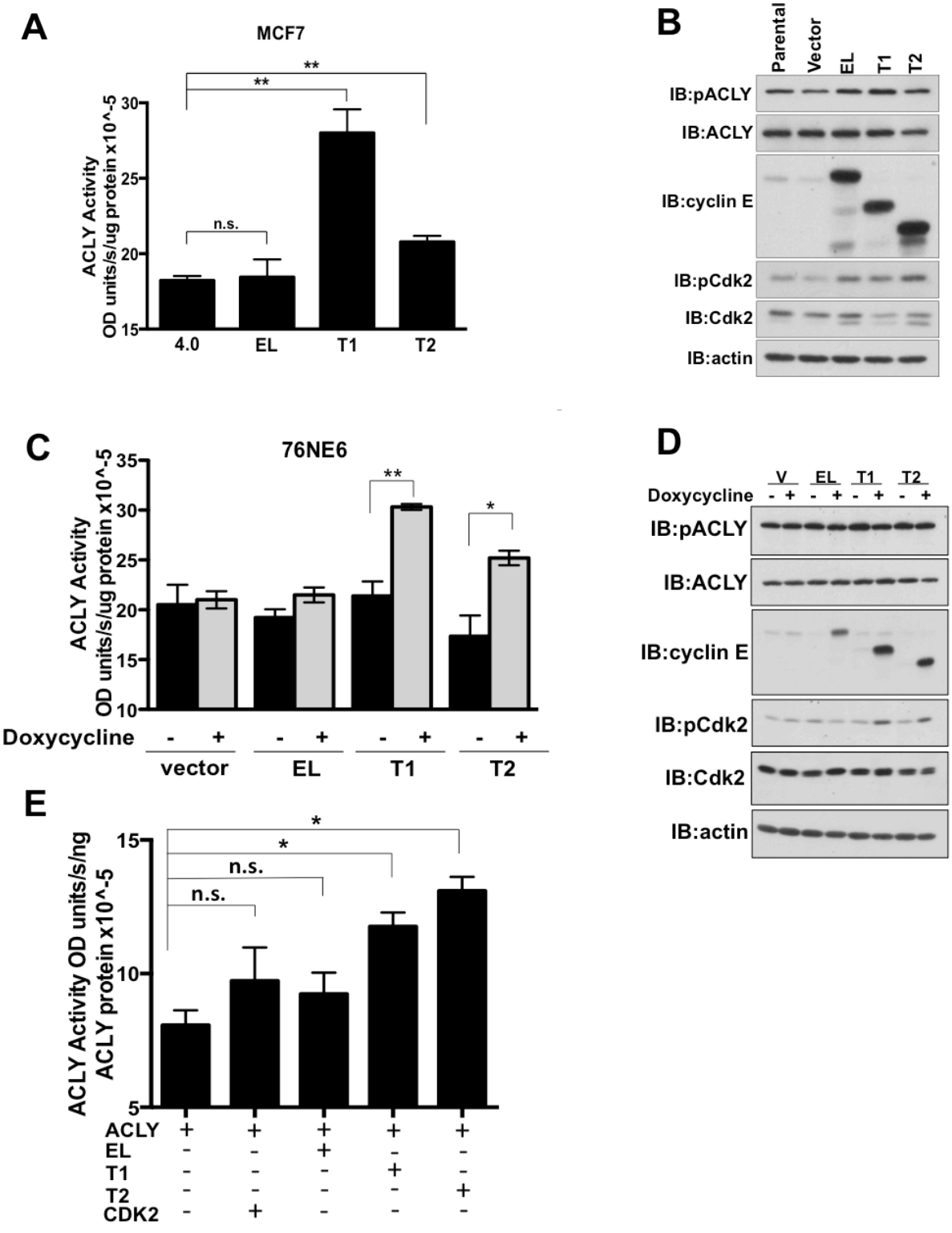
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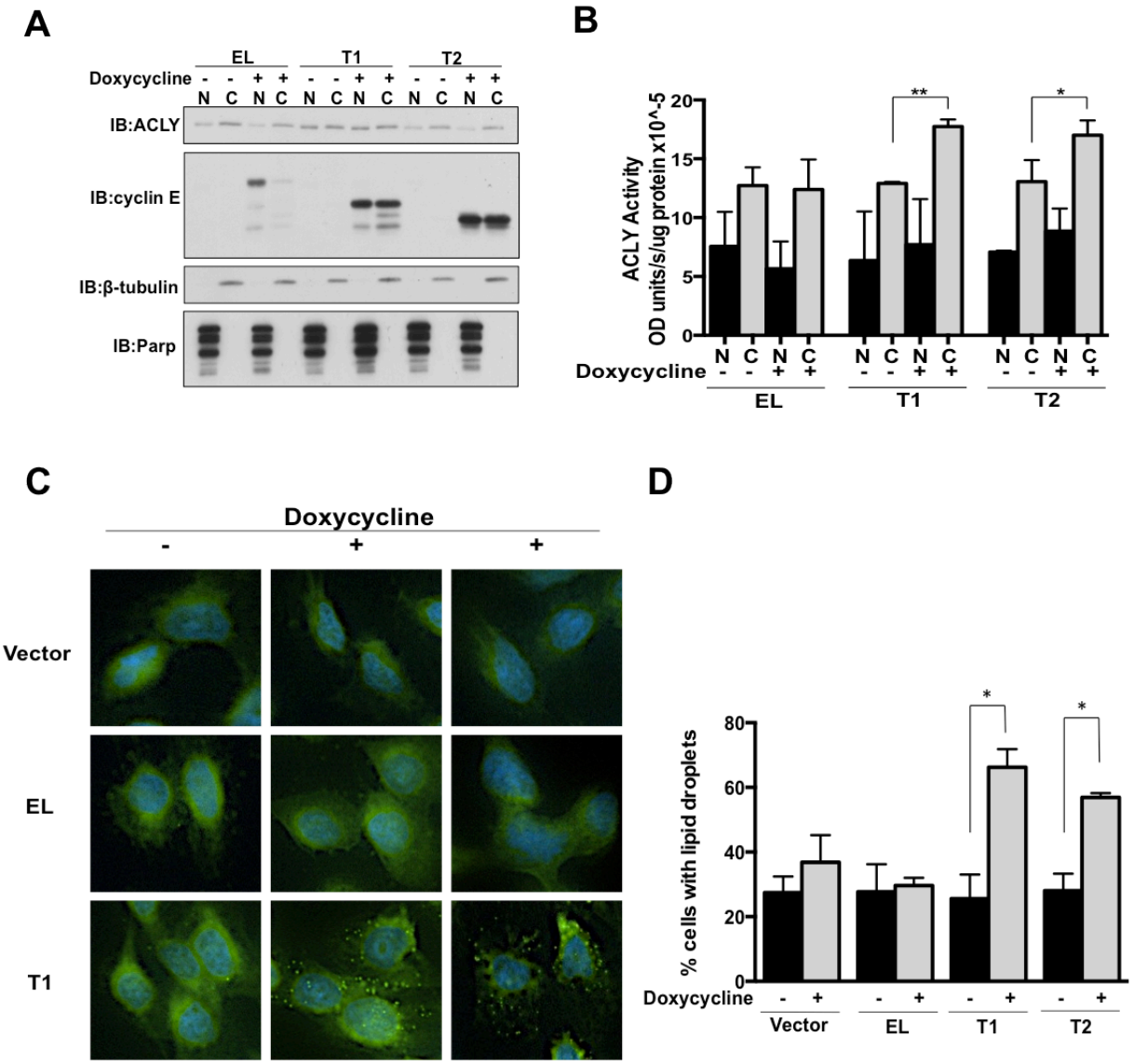
Figure 14. LMW-E isoforms affects ACLY enzymatic activity

(A) Stable expression of LMW-E isoforms increases ACLY enzymatic activity. ACLY activity was determined by the malate dehydrogenase coupled method for 3 independent replicates. Error bars= SEM (Student's t-test,**p<0.007). (B) Western blot analysis showing protein expression. (C) Inducible expression of the LMW-E isoforms increases ACLY enzymatic activity. ACLY activity was determined by the malate dehydrogenase coupled method for 3 independent replicates. Error bars= SEM (Student's t-test,**p<0.004 and *p<0.03). (D) Western blot analysis showing protein expression. (E) ACLY activity is shown from 2 independent replicates generated from *in vitro transcribed and translated* proteins incubated with recombinant ACLY. Error bars= SEM (Student's t-test,**p<0.03).

15B). Importantly, EL had no effect on activity, while nuclear LMW-E isoforms only increase ACLY activity about 10% (Figure15B).

End products of the *de novo* lipogenesis pathway include phospholipids and complex fatty acids resulting in lipid droplet formation [212]. Lipid droplets (LDs) are intracellular structures comprised of a phospholipid and sterol outer layer and hydrophobic core containing neutral lipids such as triacylglycerides and cholesterylesters [223, 224]. Thus, lipid droplet stores provide building blocks for membrane production, protein degradation, substrates for lipid-based post-translational modifications and viral replication [224]. Therefore, to investigate lipid accumulation, 76NE6 cells containing inducible expression of the cyclin E isoforms were stained with a neutral lipid dye. Neutral lipid staining under uninduced conditions showed a diffuse pattern in all cyclin E isoforms, however, upon doxycycline induction, only LMW-E expressing cells revealed a punctate pattern of neutral lipid staining indicating lipid droplet formation (Figure 15C,D). LMW-E expression increased LD formation from 20% to 60% in LMW-E(T1) and from 20% to 45% in LMW-E(T2) expressing cells (Figure 15 D).

Moreover, lipid droplet accumulation in LMW-E expressing cells requires ACLY. Specifically, lipid droplets were present in only 20-30% of cells when ACLY is knocked down, compared to 40-60% in control shRNA and indicates that expression of LMW-E(T2) does not independently induce lipid droplet formation (Figure 15G,H). Together, these data show that ACLY is highly active in the cytoplasm due to the presence of LMW-E and results in lipid droplet formation in human mammary epithelial cells.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EL
T1
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OD units/s/ug protein x10^-5
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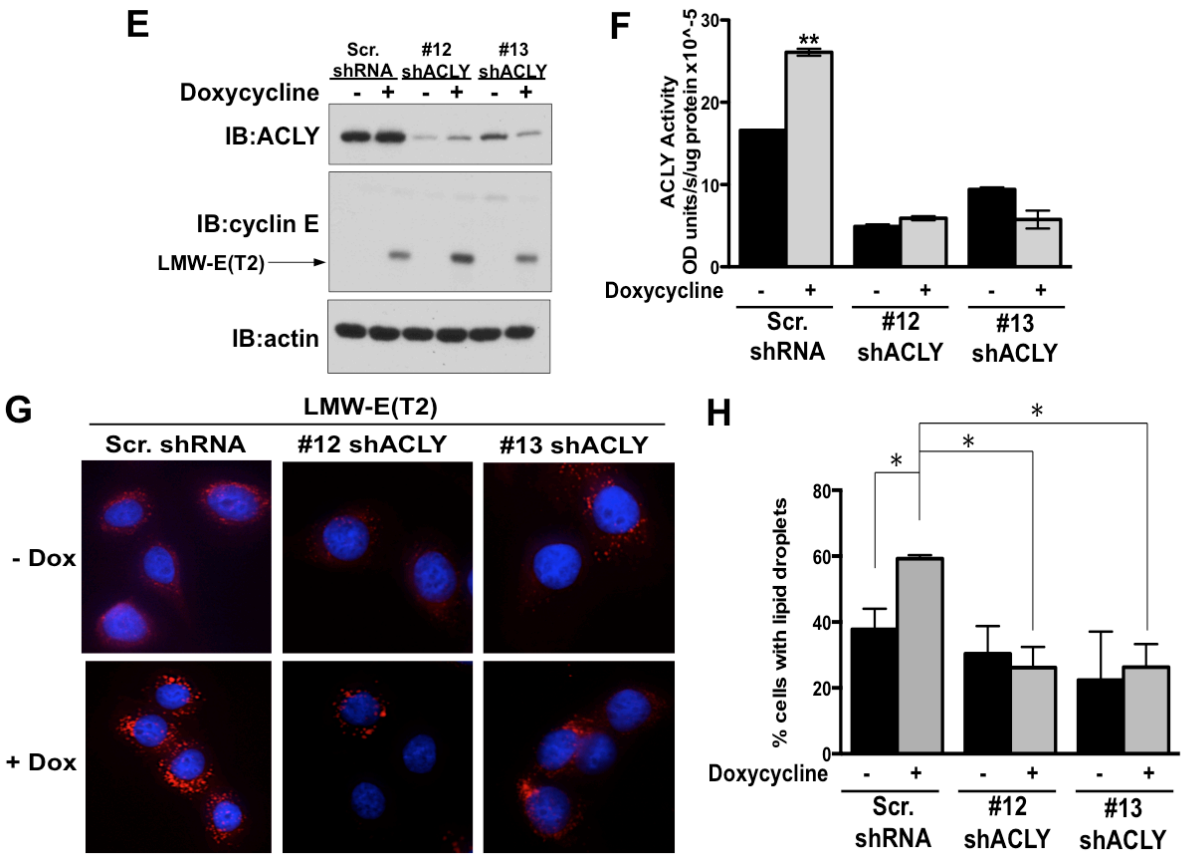


Figure 15. Cytoplasmic ACLY activity results in lipid droplet accumulation

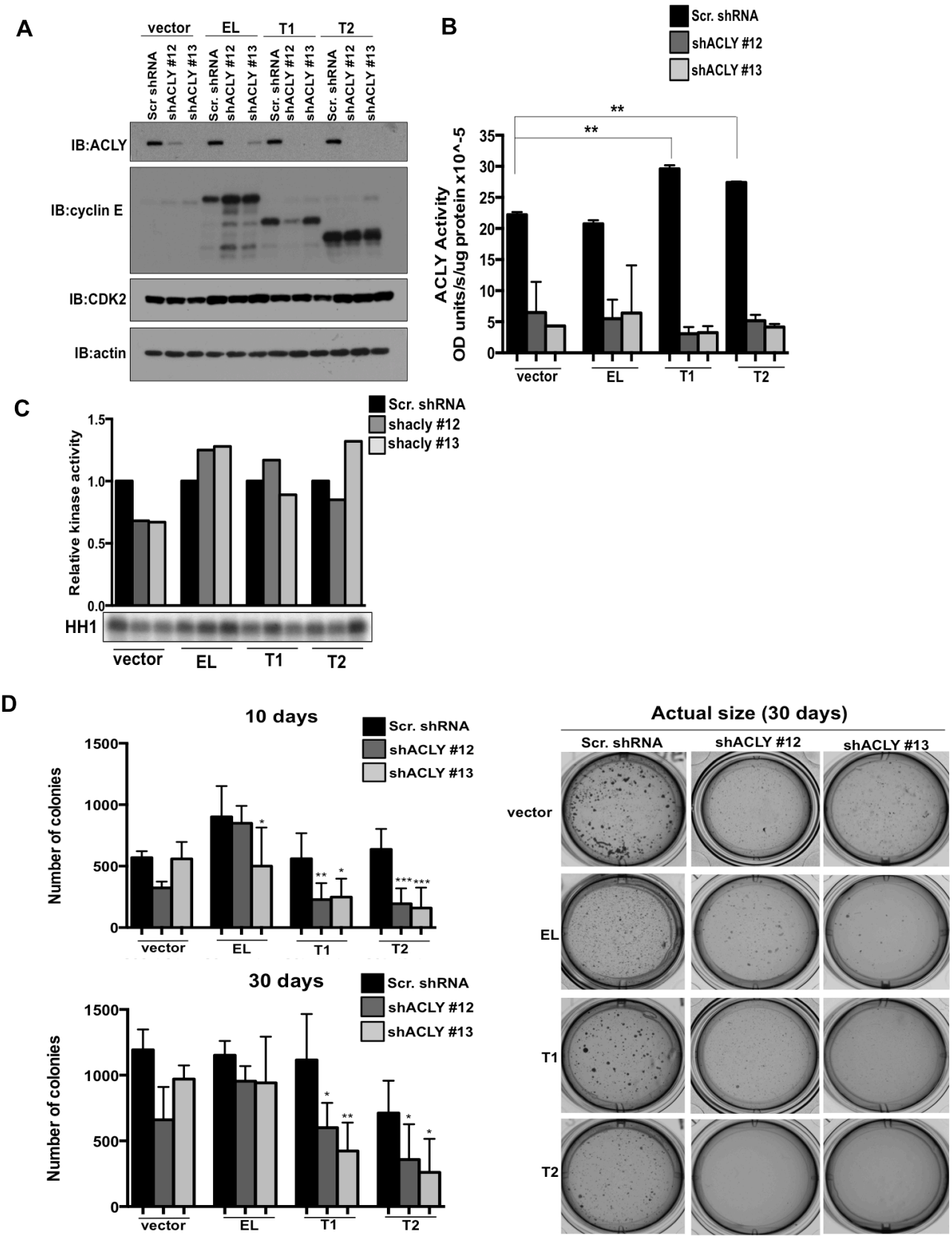
(A) Western blot showing protein expression from fractionated cells. (B) ACLY activity is shown for 3 independent replicates from fractionated 76NE6 cells. Statistical analysis was conducted using the student's t-test. Error bars= SEM (**p<0.008 and *p<0.04). (C) Intracellular lipid accumulation was measured using fluorescent dyes. (D) Cells were counted for the formation of lipid droplets before and after addition of doxycycline (n=100). Statistical analysis was performed using the student's t-test (*p<0.05). (E) Western blot showing protein expression. (F) ACLY activity is shown for 3 independent replicates. Error bars=SEM (**p<0.002). (G) Intracellular lipid accumulation was measured using fluorescent dyes. (H) Cells were counted for the formation of lipid droplets before and after addition of doxycycline (n=100). Statistical analysis was performed using the student's t-test (*p<0.05).

3.3c. ACLY is required for LMW-E mediated transformation

The LMW-E isoforms are biologically hyperactive and display increased tumorigenic potential compared to EL [113]. Therefore, to examine the requirement of ACLY in LMW-E mediated tumorigenesis, an anchorage-independent growth assay was performed in MCF7 cells containing knockdown of ACLY. Knockdown of ACLY had no effect on vector cells and a slight reduction was observed in EL cells at 10 days, but was rescued by 30 days (Figure 16D). Knockdown of ACLY in LMW-E expressing cells resulted in colony formation being reduced up to 50% (Figure 16D). Moreover, the average colony diameter was reduced by 20-40% in LMW-E expressing cells with knockdown of ACLY (Figure 16E). Importantly, knockdown of ACLY did not affect CDK2 associated kinase activity in cyclin E expressing cells, indicating that reduced growth is not due to reduced proliferation in LMW-E expressing cells (Figure 16C). Together, these data suggest that ACLY contributes to LMW-E mediated transformation in the context of anchorage-independent growth.

3.3d. Inhibition of ACLY reduces migratory and invasive capabilities of HMECs

ACLY has been implicated in playing a role during migration in glioblastoma [225]. In addition, it has been shown that LMW-E expression in non-tumorigenic human mammary epithelial cells (76NE6-HMECs) that have been serially passaged through mice; called tumor derived cells (TDCs), have an increased propensity to invade using a transwell invasion assay [111]. Therefore, to examine whether ACLY is required for LMW-E mediated migration and invasion in TDCs, ACLY was knocked-down using shRNA (Figure 17A,B). The wound healing assay showed that migration of LMW-E expressing TDCs was significantly inhibited within 24 hours in the ACLY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Scr. shRNA
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shACLY #13
ACLY Activity
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EL
T1
T2
C
Scr. shRNA
shacly #12
shacly #13
Relative kinase activity
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vector
EL
T1
T2
D
10 days
Scr. shRNA
shACLY #12
shACLY #13
Number of colonies
30 days
Actual size (30 days)
Scr. shRNA
shACLY #12
shACLY #13
vector
EL
T1
T2

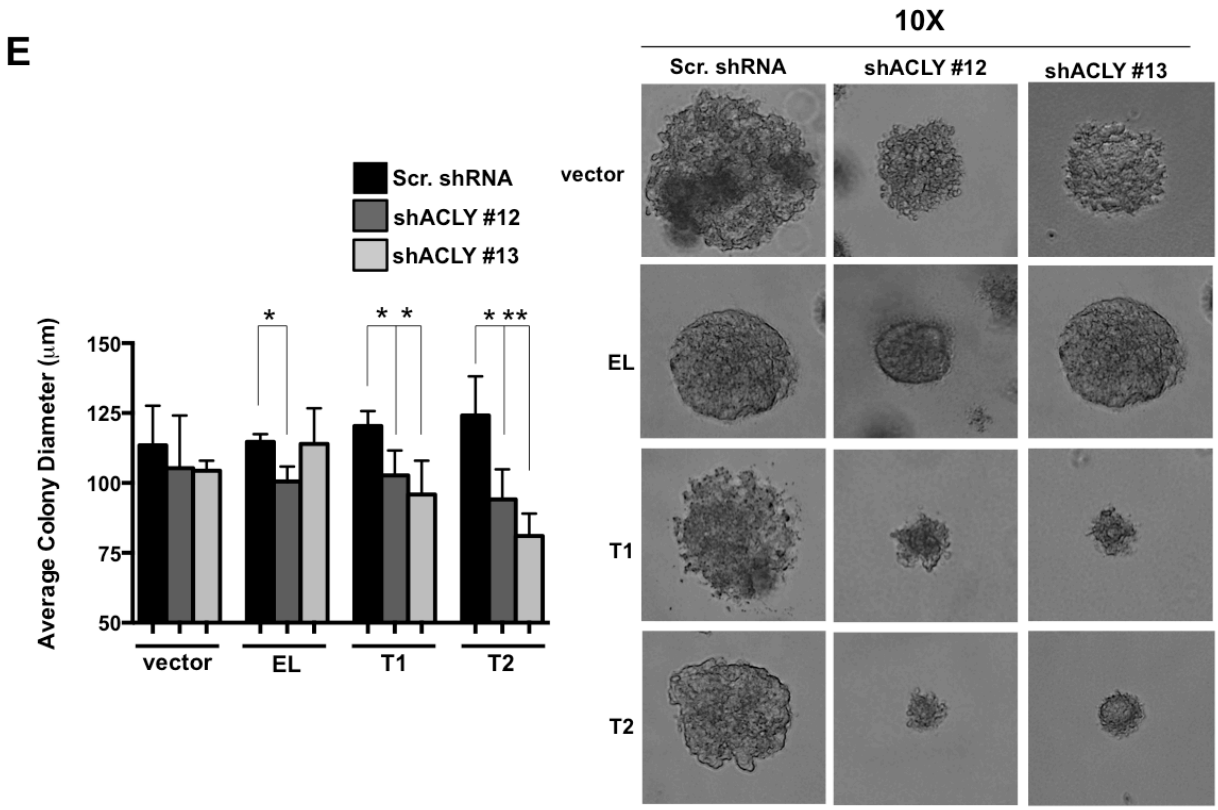
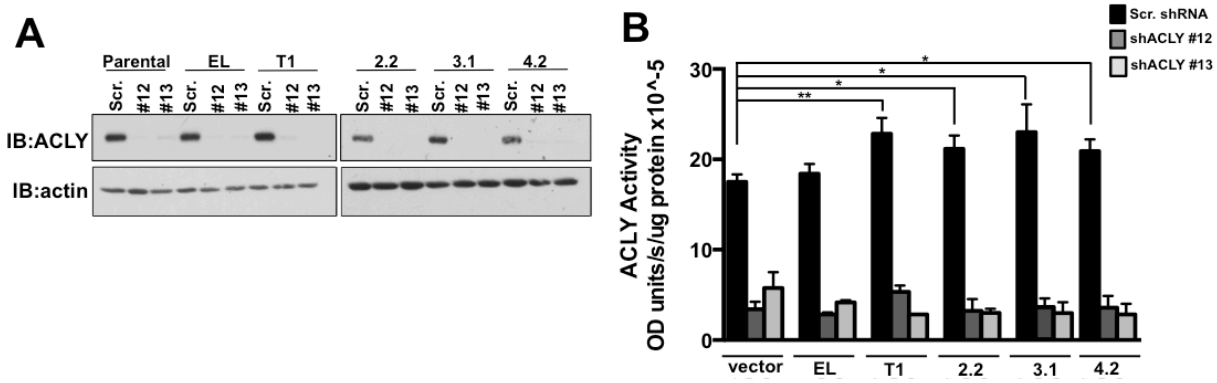
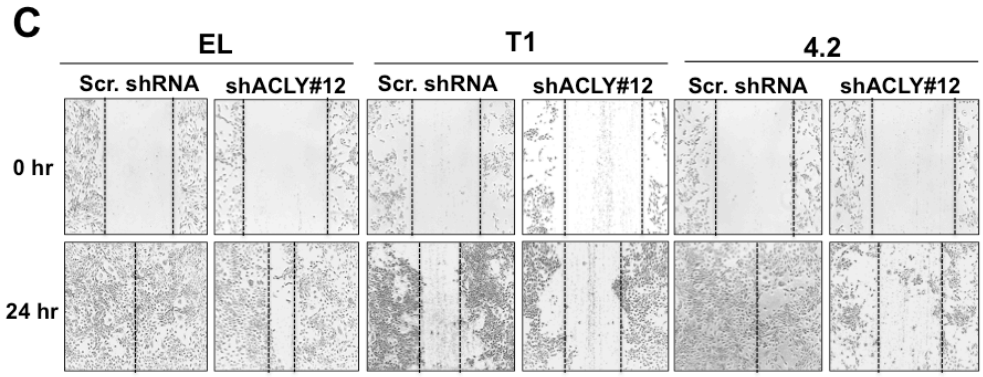
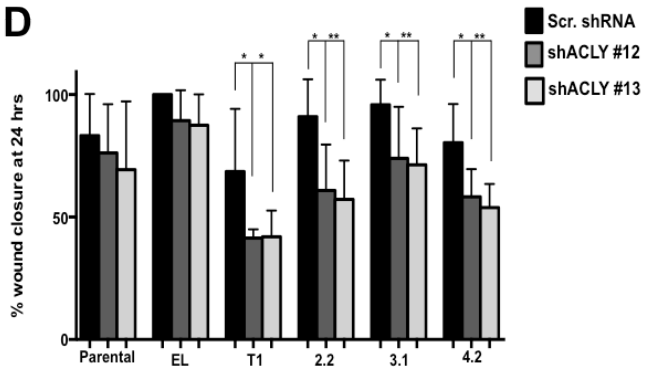


Figure 16. ACLY is required for LMW-E mediated anchorage-independent growth

(A) Western blot showing protein expression. (B) ACLY enzymatic activity in MCF7 cells containing shACLY. Error bars=SEM, **p<0.005. (C) CDK2 associated kinase activity in knockdown cells. (D) Quantitation of colonies formed after 10 days and 30 days in anchorage-independent growth conditions. Statistical analysis was performed using the student's t-test. For 10 days *p<0.04,**p<0.009, ***p,0.0002. For 30 days *p<0.04 and **p<0.0025. The assay was performed in triplicate with images from whole wells from one representative experiment are shown. (E) Average colony diameter. Statistical analysis was performed using the student's t-test with **p<0.0085 and performed in triplicate with images from representative colonies are shown.







E

Cells	shRNA	Doubling time (hrs.)
Parental	Scrambled	40.24 +/- 0.670
	#12shACLY	34.42 +/- 6.790
	#13shACLY	37.25 +/- 3.495
EL	Scrambled	29.10 +/- 2.010
	#12shACLY	29.31 +/- 0.245
	#13shACLY	33.61 +/- 1.160
T1	Scrambled	24.16 +/- 2.145
	#12shACLY	24.37 +/- 1.535
	#13shACLY	24.44 +/- 1.975
2.2	Scrambled	29.34 +/- 3.230
	#12shACLY	28.96 +/-2.355
	#13shACLY	29.76 +/- 2.800
3.1	Scrambled	36.27 +/- 0.370
	#12shACLY	27.89 +/- 8.756
	#13shACLY	34.92 +/- 4.195
4.2	Scrambled	30.48 +/- 3.170
	#12shACLY	29.52 +/- 4.305
	#13shACLY	30.41+/- 5.760

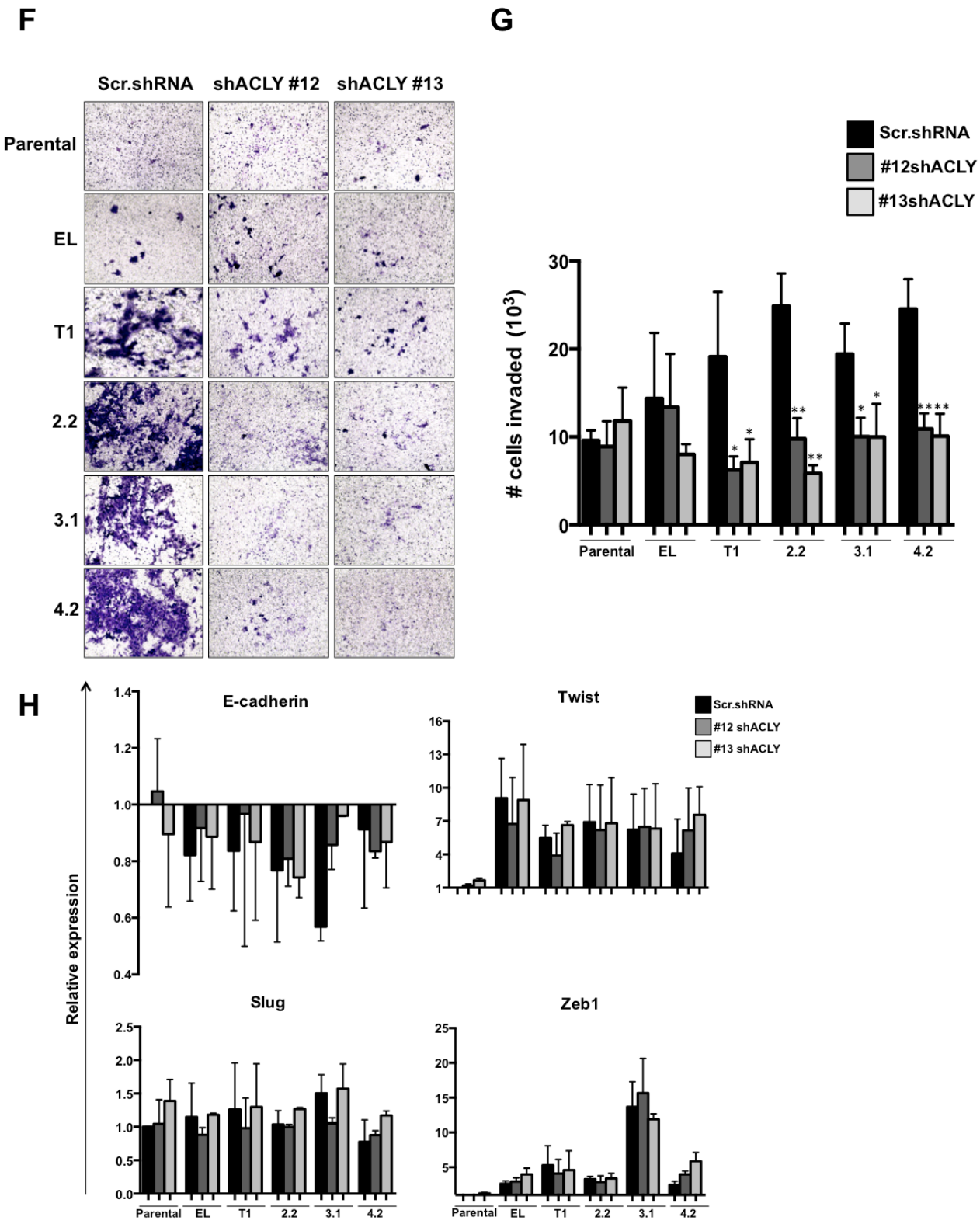

F
Scr.shRNA
shACLY #12
shACLY #13
Parental
EL
T1
2.2
3.1
4.2
G
Scr.shRNA
#12shACLY
#13shACLY
cells invaded (10^3)
30
20
10
0
Parental
EL
T1
2.2
3.1
4.2
H
Relative expression
E-cadherin
Twist
Slug
Zeb1
Scr.shRNA
#12 shACLY
#13 shACLY

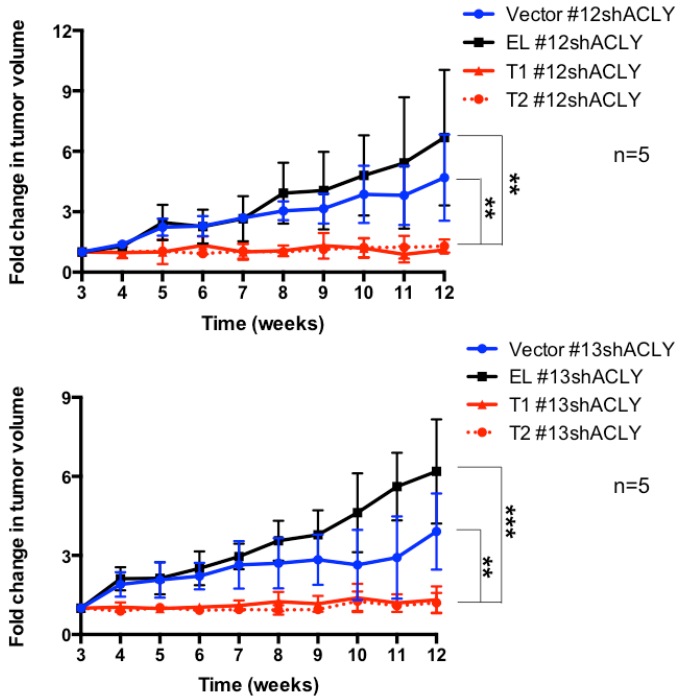
Figure 17. Inhibition of ACLY reduces migration and invasion in HMECs
(A) Western blot analysis showing protein expression. (B) ACLY enzymatic activity in 76NE6 and TDCs containing shACLY. Error bars=SEM, *p<0.05, **p<0.009. (C) Proliferation was assessed by MTT assay and read at 590nm. Doubling time was calculated in GraphPad Prism software. (D) 76NE6 and TDCs containing either scrambled shRNA or shRNA toward ACLY were allowed to reach confluency and scratched. Images were taken at 0 and 24 hours post-scratch. (E) Quantitation of migration assay. Statistical analysis was conducted using the student's t-test from 3 independent replicates for each condition. Error bars=SEM; *p<0.05, **p<0.008. (F) Cells were plated on a transwell chamber containing Matrigel and incubated on top of fibronectin-containing media for 24 hours and invaded cells were stained with crystal violet. Images of 20X magnification were taken with a light microscope. (G) Quantitation of transwell invasion assay. Cells on the bottom of the transwell were collected at 24 hours and counted. Statistical analysis was conducted using the student's t-test from 3 independent replicates for each condition. Error bars=SEM, p<0.05,**p<0.005. (H) qRT-PCR for selected EMT related genes. Reactions were carried out in triplicate with the fold difference in transcripts was calculated by the ΔΔCT method using GAPDH as a control.

knockdown cells, while vector control and EL had no effect (Figure 17C,D). Specifically, migration was reduced in 76NE6-T1 shACLY cells by 50% and up to 30% in ACLY knockdown TDCs. Importantly, reduced migration was not due to changes in the proliferative capacity of the ACLY knockdown cells, as no changes were observed in doubling time (Figure 17E). Moreover, using the transwell invasion method, indeed, ACLY is required for LMW-E TDC mediated invasion. The invasive capacity of LMW-E TDCs was reduced up to 60%, with minimal or no effect in vector or EL cells containing knockdown ACLY (Figure 17F,G). LMW-E TDCs have also been reported to undergo EMT, however, ACLY is not required for this process. Examination of EMT related genes such as E-cadherin, Twist, Slug and Zeb1 displayed no change when ACLY was inhibited (Figure 17H). Together, these data demonstrate that ACLY is required for migration and invasion of LMW-E expressing TDCs and that proliferation and the EMT process are unaffected.

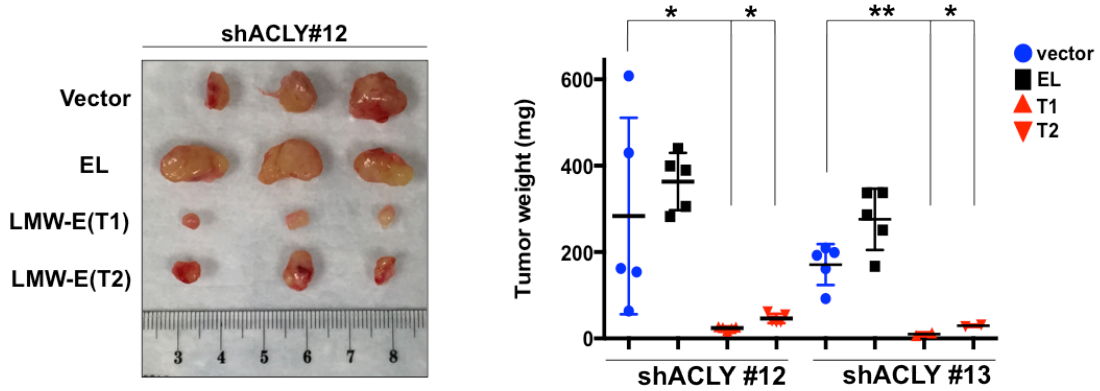
3.3e. ACLY is required for LMW-E mediated tumor growth

To further examine the requirement of ACLY in LMW-E mediated tumorigenesis, we performed an *in vivo* xenograft tumor assay and injected MCF7 cells containing both stable expression of the cyclin E isoforms and stable knockdown of ACLY into the mammary fat pad of nude mice. Knockdown in MCF7 vector cells displayed a modest reduction in tumor growth, while knockdown of ACLY in EL expressing cells were uninhibited (Figure 18A). Knockdown of ACLY in LMW-E expressing cells, however, displayed substantial inhibition of tumor growth (Figure 18A). Specifically, on average, vector expressing cells increased in volume about 3-fold and EL expressing cells increased in volume about 6-fold, however, LMW-E expressing cells containing

A



B



C

Cyclin E expression	shRNA sequence	# of tumors 3 weeks	# of tumors 12 weeks
Vector	Scr.shRNA	100% (5/5)	100% (5/5)
	#12shACLY	100% (5/5)	100% (5/5)
	#13shACLY	100% (5/5)	100% (5/5)
EL	Scr.shRNA	100% (5/5)	100% (5/5)
	#12shACLY	100% (5/5)	100% (5/5)
	#13shACLY	100% (5/5)	100% (5/5)
LMW-(T1)	Scr.shRNA	100% (5/5)	100% (5/5)
	#12shACLY	80% (4/5)	100% (5/5)
	#13shACLY	40% (2/5)	40% (2/5)
LMW-E(T2)	Scr.shRNA	100% (5/5)	100% (5/5)
	#12shACLY	0% (0/5)	100% (5/5)
	#13shACLY	20% (1/5)	40% (2/5)

Figure 18. ACLY is required for LMW-E mediated tumor growth
(A) Inhibition of tumor growth in MCF7 cells containing stable overexpression of the cyclin E isoforms and either scrambled shRNA or shRNA targeted to ACLY. Cells were injected at a concentration of $2.5x10^6$cells/100μL into the mammary fat pad of nude mice. Tumors were measured for 12 weeks starting at 3 weeks. Statistical analysis was conducted using the student's t-test. N=5; error bars=SEM; **p<0.008 and ***p<0.0007. (B) Representative pictures of #12shACLY tumors and quantitation of tumor weights. . Statistical analysis was conducted using the student's t-test. Error bars=SEM; *p<0.05, ***<0.0002 and ****p<0.0001. (C) Propensity of tumor growth. LMW-E expressing cells containing knockdown of ACLY have delayed tumor latency.

knockdown of ACLY exhibited virtually no change in tumor volume over the 12 week period (Figure18A). These changes were corroborated with examination of tumor weight, in that LMW-E expressing cells with knockdown of ACLY were extremely small with weights ranging in size from 6mg-65mg (Figure 18B). Moreover, knockdown of ACLY in LMW-E expressing cells delays tumor latency. Specifically, transcript #12 delayed tumor formation to 6 weeks in LMW-E(T1) and up to 9 weeks in LMW-E(T2) expressing cells. Similarly, tumor formation was delayed in transcript #13, with only 40% of tumors forming after 12 weeks (Figure 18C). Taken together, these data show that ACLY is required for LMW-E mediated tumor formation and tumor growth.

3.4 CONCLUSIONS

Energy deregulation is now recognized as a hallmark of cancer cells and a characteristic of the transformed phenotype [162]. Indeed, metabolic enzymes have been shown to be deregulated in cancer cells including deregulation of enzymes in the *de novo* lipogenesis pathway [223]. Specifically, lipogenesis enzymes, ACC and FASN, have shown to have elevated expression in breast and prostate cancers [223]. Moreover, ACLY has shown to be upregulated either at the protein level or the enzymatic level in multiple cancer types including cancers of the lung, prostate, bladder, breast, liver, stomach and colon [218].

This study demonstrates, for the first time, a direct link between the metabolic enzyme, ACLY, with cell cycle protein, cyclin E. Specifically, the LMW-E isoforms significantly increased ACLY enzymatic activity in human breast cancer, MCF7 cells, and non-tumorigenic human mammary epithelial cells. Other studies have shown that

ACLY enzymatic activity is mediated by phosphorylation by Akt [153]. However, in lung adenocarcinoma A549 cells treated with the PI3K inhibitor, LY294002, showed only partial inactivation of ACLY enzymatic activity, suggesting additional mechanisms of ACLY activation [153]. We propose that ACLY is also enzymatically regulated by the LMW-E isoforms.

Moreover, LMW-E mediated activation of ACLY is specific to cytoplasmic ACLY and results in lipid droplet formation. Lipid droplets are energy reservoirs that provide cells with the substrates required to support cell growth, proliferation, energy homeostasis, resistance to oxidative stress and function in signaling [223]. Therefore, these data indicate that LMW-E activation of ACLY and subsequent formation of lipid droplets provides LMW-E expressing cells the necessary building blocks to sustain growth.

Additionally, ACLY has been implicated in playing a role during migration in glioblastoma [225]. In 76NE6-TDCs with LMW-E expression, knockdown of ACLY significantly reduced migration and invasion with migration reduced up to 30% and invasion reduced up to 60%. Changes in EMT related gene expression where not detected, therefore, the reduced migratory and invasive phenotype may be due to changes in lipid-based signals. Indeed, eicosanoids, phosphoinositides and sphingolipids are signaling lipids shown to mediate multiple cellular processes such as cell proliferation and migration [226]. It would be interesting to determine whether changes in migratory behavior of LMW-E TDCs with ACLY expression was due to depletion of these key lipid signaling molecules.

Lastly, ACLY is required for LMW-E mediated tumor growth *in vitro* and *in vivo.* Knockdown of ACLY in MCF7 cells expressing the LMW-E isoforms significantly reduced anchorage-independent growth as early as 10 days with colony diameter after 30 days being reduced up to 40%. Moreover, reduced colony formation was irrespective of CDK2-associated kinase activity, as kinase activity was only moderately inhibited in vector cells containing knockdown of ACLY. Moreover, inhibition of ACLY in LMW-E expressing cells significantly reduced tumor formation *in vitro*. Nude mice bearing xenograft tumors expressing the LMW-E isoforms and containing knockdown of ACLY exhibited substantial inhibition of tumor formation and tumor growth, while knockdown of ACLY in vector cells had minimal reduction in tumor growth. No inhibition of tumor growth was observed in EL expressing cells containing knockdown of ACLY. These data indicate that LMW-E expressing tumors rely on ACLY and presumably through the *de novo* lipogenesis pathway to support tumor growth and formation. Taken together, LMW-E mediated activation of ACLY serves as a mechanism of LMW-E mediated tumorigenesis and inhibition of ACLY may serve as a viable target in breast cancers and other cancers with LMW-E expression.

CHAPTER 4: **CONCLUSIONS AND FUTURE DIRECTIONS**

4.1 CONCLUSIONS AND MAJOR FINDINGS

Deregulation of the LMW-E isoforms leads to tumorigenesis and research provided in this dissertation has addressed the primary hypothesis that ***aberrant localization the LMW-E isoforms leads to molecular interactions that ultimately contribute to LMW-E breast cancer tumorigenicity.*** The evidence delineated in this dissertation has provided answers to the key questions outlined in chapter 1.

1. Does LMW-E have protein-binding partner(s) in the cytoplasm?

- Indeed, using a retrovirus-based protein complementation assay, we found 11 novel interacting proteins of LMW-E. One of the interacting proteins identified, ATP-citrate lyase (ACLY), is capable of directly binding all cyclin E isoforms, but is not a substrate of cyclin E/CDK2.

2. Are the function(s) of the LMW-E cytoplasmic binding partner(s) enhanced and/or suppressed?

- Expression of the LMW-E isoforms elevates ACLY enzymatic activity in breast cancer cells and non-tumorigenic human mammary epithelial cells. Moreover, in cyclin E inducible cells, we found that activation of ACLY by LMW-E occurs in the cytoplasm and results in lipid droplet formation, a cellular energy reservoir important for providing lipid/energy substrates required to fuel many cellular processes.

3. Are cytoplasmic LMW-E binding partners required for LMW-E mediated transformation, invasive capabilities and tumor growth?

- Downregulation of ACLY impairs anchorage-independent growth in MCF7 cells expressing LMW-E, but not in control cells or EL expressing cells. Migration and invasion are also diminished when ACLY is downregulated, that is irrespective of the EMT program. *In vivo*, we found that downregulation of ACLY in LMW-E expressing cells is required for tumor growth and well as tumor formation.

4.2 FUTURE DIRECTIONS

Research provided in this dissertation contributes to the body of work addressing the mechanisms of LMW-E induced tumorigenesis. While data presented here advanced our knowledge by identifying cytoplasmic LMW-E binding proteins, due to the number of binding protein identified, it is clear that LMW-E mediated tumorigenesis is multifocal with many pathways and/or proteins contributing to LMW-E mediated tumorigenicity. Therefore, further investigation is required; particularly examination of the metabolic requirements of LMW-E expressing breast cancer cells.

From the RePCA screen, three metabolic proteins where identified as cytoplasmic binding partners of LMW-E (T1): trans, 2,3-enoyl-CoA reductase (TECR), Aldolase A (ALDO-A) and ATP-citrate lyase (ACLY). TECR functions in lipogenesis to reduce long chain and very long chain fatty acids [148]. Exome sequencing revealed a mutation that converts a leucine residue to a proline residue that is implicated in autosomal recessive non-syndromic mental retardation (NSMR), but no known link to cancer has been established [227]. ALDO-A, on the other hand, has been linked to human neoplasias. ALDO-A functions in glycolysis to catalyze the conversion of

fructose-1,6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate [228]. For example, in Ras transformed NIH-3T3 cells, ALDO-A inhibition by siRNA resulted in a reduction in cell proliferation upwards of 90% [229]. Moreover, malignant adrenocortical tumors exhibit high expression of ALDO-A compared to benign tumors and high expression of ALDO-A in lung squamous cell carcinoma (LSCC) correlates with poor prognosis, reduced differentiation and increased tumor grade [228, 230].

Since multiple metabolic enzymes from different pathways were identified in the RePCA screen, it is plausible that LMW-E expressing cells broadly deregulate cellular metabolism and exploit flux through glycolysis and the *de novo* lipogenesis pathway. Glucose uptake is a characteristic of a more aggressive breast cancer phenotype and in general, MCF7 cells are less aggressive [177]. However, it can be hypothesized that breast cancer cells containing LMW-E expression shift to a more glycolytic phenotype since LMW-E expression renders breast cancer cells more aggressive and have a poor prognosis compared to breast cancer cells only containing expression of full-length cyclin E (EL) [98, 109, 111, 231]. Therefore, examination of glucose uptake in LMW-E expressing cell would provide additional insight regarding the metabolic alterations in LMW-E expressing breast cancer cells.

We also found that LMW-E expression results in lipid droplet formation. Lipid droplets (LDs) are intracellular energy reservoirs composed of triacylglycerides and cholesterylesters surrounded by a phospholipid monolayer [223]. LDs and increased fatty acid synthesis have shown to be involved in many areas of cancer progression [223]. In general, fatty acids promote membrane synthesis important for cell growth

and proliferation, but also exhibit non-proliferative roles. For example, fatty acids lead to membrane saturation important for resistance to oxidative stress and survival under energy stress [223]. Additionally, metabolic intermediates such as NADPH provide redox balance and fatty acid derived lipids facilitate signaling through membrane receptors or lipid-based posttranslational modifications [223]. Since knockdown of ACLY did not affect proliferation in LMW-E expressing 76NE6 human mammary epithelial cells, LDs with respect to LMW-E may play a non-proliferative role. We showed that knockdown of ACLY resulted in reduced migration and invasion that may be due to decreased fatty acids that regulate these functions. For example, water-soluble phospholipid, lyosophosphatidic acid (LPA), has shown to promote migration through G-protein-coupled receptors and the PI3K pathway [223, 232]. Furthermore, prostaglandins derived from cyclooxygenase 2 (COX2), also promote migration and support tumor-microenvironment interactions [233]. Preliminary examination of these lipid signaling molecules may prove to be involved in LMW-E mediated migration and invasion.

Another function of LDs is resistance to oxidative stress [223]. Activation of *de novo* lipogenesis results in an intracellular increase of saturated and mono-unsaturated phospholipids that are more resistant to peroxidation while decreasing poly-unsaturated phospholipids [234]. Therefore, inhibition of *de novo* fatty acid synthesis or inhibition of lipogenic enzymes has been shown to sensitize cells to oxidative stress causing cell death [234]. Moreover, decreased membrane saturation enables diffusion of chemotherapeutic agents such as doxorubicin; thereby providing rationale for the combination of lipogenesis inhibitors with chemotherapeutic agents [234].

In addition to investigating the broad scope of metabolic deregulation in LMW-E expressing breast cancer cells, the mechanism behind the LMW-E/ACLY interaction has yet to be established. It can be hypothesized that ACLY binds cyclin E in the MRAIL motif; which facilitates recognition of RXL containing proteins [81]. ACLY contains three RXL regions of interest. The first RXL motif is located at the N-terminus of ACLY, but is unlikely the site of cyclin E binding due steric hindrance by the ATP grasp fold between domains 3 and 4 of ACLY [235]. The next RXL motif and the most likely site of cyclin E binding, is found in the highly variable region of ACLY (aa426-486). It is within this region ACLY is phosphorylated by multiple kinases including Akt and GSK3β [158]. This RXL motif lies just 27 amino acids downstream of S454, the site of phosphorylation by Akt [158]. The final RXL motif in ACLY is located within domain 1 which binds CoA [235]. Due to the power helices fold between domains 2 and 5, domain 1 is probably not easily accessible to a large protein such as cyclin E and can also be ruled out due to steric hindrance.

Since ACLY binds all isoforms of cyclin E (EL, LMW-E(T1) and LMW-E(T2)), it is logical that ACLY would bind in a common region of all cyclin E isoforms and not the N-terminus; which LMW-E are lacking. CDK2 binds cyclin E through the cyclin box motif and the last 50 amino acids [82]. It is possible that ACLY requires multiple regions on cyclin E to bind as well. Therefore, truncations of cyclin E can be generated to examine this possibility. Lastly, if ACLY binds cyclin E in the cyclin box, it is possible that ACLY has affinity to other cyclins as well since the cyclin box is a conserved region shared among all cyclins [75]. To examine this possibility, co-immunoprecipitation

assays can be performed to investigate interaction between ACLY and other cyclins involved in the cell cycle such as cyclin D, cyclin A or cyclin B.

Finally, we found that genetic downregulation of ACLY resulted in reduced anchorage-independent growth *in vitro* and xenograft tumor growth *in vivo* that was specific to LMW-E expressing breast cancer cells. Thus, in order to utilize this anti-tumor affect clinically, it would be imperative to examine whether pharmacologic inhibition was equally effective. There are several competitive and non-competitive inhibitors of ACLY [236]. Hydroxycitrate, one of the most studied competitive inhibitors, has shown modest success [225, 237, 238]. Hydroxycitrate is derived from Garcinia fruits and/or flowers and is commonly added to diet supplements, however, studies with mice have shown no differences in regards to weight [158, 236]. The anti-neoplastic effect of hydroxycitrate has been shown in bladder and colon carcinoma cell lines with growth inhibition ranging from 5-60% [238].

Butanedioic acid derivatives are another type of competitive inhibitor of ACLY [236]. Specifically, SB-204990, the pro-drug of SB-201076, has shown to be effective at reducing glucose-dependent lipid synthesis *in vitro* and tumor growth *in vivo* [211, 221]. Hatzivassiliou et al. and collegues report SB-204990 reduces lipid synthesis in a dose-dependent manner in a murine pro-B-cell lymphoid cell line as well as a significant reduction in xenograft tumor growth from mouse pancreatic ductal cell lines containing K-Ras^{G12D} mutations [221]. Finally, non-competitive inhibitors of ACLY are effective at inhibiting ACLY *in vitro*, with only a few examining the inhibitory effect *in vivo*, but their toxicity is problematic and there is no evidence relating to their anti-neoplastic properties [236].

4.3 SIGNIFICANCE

Breast cancer continues to be among the leading causes of cancer death in women in the United States and the identification of the molecular mechanisms of sporadic breast cancer remain largely elusive for this complex disease [1]. Breast cancer patients that express high levels of LMW-E have a poor prognosis and this dissertation identified ACLY, a cytoplasmic LMW-E binding protein, that mediates breast cancer tumorigenicity [98]. Genetic inhibition of ACLY in LMW-E expressing breast cancer cells attenuates tumor growth. Pharmacologic inhibition of ACLY in LMW-E positive breast cancer patients has the potential to be a viable treatment strategy to combat sporadic breast cancer. Moreover, inhibition of ACLY may prove to be beneficial in targeting other cancer types that display LMW-E expression such as ovarian, colorectal cancers and melanomas.

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VITA

Kimberly Marie Szymanski was born March 19, 1983 to Alan and Peggy Szymanski in Cleveland, Ohio. After graduating from Kingwood High School in 2001, she attended Baylor University in Waco, Texas and graduated in 2005 with a Bachelor of Arts degree in Biochemistry. Unsure as to whether she wanted to pursue a career in research, she worked as a research assistant at UT Southwestern in Dallas, Texas under Joel M. Goodman, Ph.D. from 2005-2007 and this experience initiated her passion for medical research. In 2008, she married Charles Lucenay and began studies at The University of Texas Health Science Center Graduate School of Biomedical Sciences to pursue a doctor of philosophy degree in cancer biology. In 2011, she and her husband welcomed their first son, Connor, and three years later welcomed their second son, Camden. Both boys fill their lives with endless happiness and joy.