THE ROLE OF THE CDP-CHOLINE PATHWAY IN THE ANOIKIS RESISTANCE OF RAS-TRANSFORMED INTESTINAL EPITHELIAL CELLS

by

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DEDICATION PAGE

This work is dedicated to the many family and friends who, without want of recognition or repayment, presented me with the opportunity to pursue an education. To my parents: Susan, Raymond, my brother and sister, Samuel and Margaret for their candid enthusiasm and interest in my work. To my aunts Barbara and Brenda for practical guidance in all things separate from academia. And to my grandparents, Lois and Samuel F. Hale for acting as exemplars of success attained through perseverance.

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ABSTRACT

Phosphatidylcholine (PC) is an essential component of biological membranes and is synthesized by the CDP-choline pathway under the control of the rate-limiting enzyme CTP:phosphocholine cytidylyltransferase-alpha (CCTα). Ras transformed cells have increased lipid synthesis; the aim of this study was to determine if upregulation of CCTα was part of this transformed phenotype. Rat intestinal epithelial cell lines (IEC) and three oncogenic H-ras expressing IEC (IEC-Ras) were used to investigate the role of CCTα and phosphatidylcholine (PC) synthesis in resistance to detachment dependant apoptosis, termed anoikis. IEC-Ras have increased CCTα expression within the nucleus. Reduction of CCTα expression with lentiviral short hairpin RNA sensitized IEC-Ras to anoikis and decreased PC degradation, but did not change PC synthesis. Thus, in addition to CCTα being involved in anoikis-resistance in IEC-Ras these data indicate the possibility that it may also have nuclear-specific functions.

LIST OF ABBREVIATIONS USED

Apaf-1	Apoptosis protease activating protein-1
APC	Adenomatous polyposis coli
BSA	Bovine serum albumin
C/EPT	Choline/ethanolamine phosphotransferase
CCT	CTP:phosphocholine cytidylyltransferase
CDP	Cytidine 5'-diphosphate
CHO	Chinese hamster ovary
CHT	High affinity choline transporter
CL	Cardiolipin
ConA	Concanavalin A
CTP	Choline phosphotransferase
CytC	Cytochrome C
DAG	Diacylglycerol
DED	Death effector domain
D-MEM	Dulbecco's modified Eagle's media
Dpm	Disintegration per minute
ECM	Extra cellular matrix
EGF	Epidermal growth factor
EMT	Epithelial to messenchymal transition
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
ERK	Extracellular signal regulated kinase
FADD	Fas-associated death domain
FAK	Focal adhesion kinase
FASN	Fatty acid synthase
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GLUT	Glucose transporter
GPC	Glycerophosphocholine
GPCR	G-protein coupled receptor
HC-3	Hemicholinium-3
HEK	Human embryonic kidney
IAP	Inhibitor of apoptosis
IEC	Intestinal epithelial cell
IEC-Ras	Ras-transformed intestinal epithelial cell
IF	Immunofluorescence
KO	Knockout
LSC	Liquid scintillation counter
MAPK	Mitogen activated protein kinase
mRNA	Messenger RNA
MW	Molecular weight

NLS	Nuclear localization signal
NPC	Nuclear pore complex
NT	Non-targeting
PA	Phosphatidic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
pChol	Phosphorylcholine
PC-PL	Phosphatidylcholine-specific Phospholipase
PDGF	Platelet derived growth factor
PE	Phosphatidylethanolamine
PEI	Polyethyleneimine
PEMT	Phosphatidylethanolamine N-methyltransferase
PH	Pleckstrin homology
PI	Phosphatidylinositol
PIP	Phosphatidylinositol phosphate
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PM	Plasma membrane
PS	Phosphatidylserine
RTK	Receptor tyrosine kinase
SDS	sodium dodecyl sulfate
SEM	Standard error of mean
shNT	Non targeting short hairpin RNA
shRNA	Short hairpin RNA
SP-agarose	Sea Plaque agarose
Sp1	Specificity protein 1
SRE	Sterol response element
SREBP	Sterol response element binding protein
TBS	Tris-buffered saline
TLC	Thin layer chromatography
XBP1	Xbox-binding protein 1

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CHAPTER 1 INTRODUCTION

1.1 Phosphatidylcholine Synthesis And Regulation

1.1.1 Phospholipid Structure and Function

Biological membranes are fluid, dynamic structures that separate cellular compartments from each other and form a barrier to the external environment [1]. Membranes are composed of phospholipids, proteins, sphingolipids, cholesterol, and glycolipids/proteins, all existing as a fluid mosaic [2]. In eukaryotes, the lipid portion of the membrane is primarily comprised of phospholipids such as phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), cardiolipin (CL) and phosphatidylinositol (PI) [3]. Phospholipids are not only involved in membrane structure, but also serve many other essential functions within the cell. For example, phosphorylated PI (PIP) serve as organelle-specific binding sites for various lipid-binding domains such as pleckstrin homology (PH) domains, or as secondary messengers by release of phosphorylated inositol. PE makes up 20-30% of membrane content and forms a cone-like shape arising from its smaller headgroup and therefore can induce negative curvature into lipid bilayers [4]. The plasma membrane has an asymmetrical distribution of PS between the bilayer leaflets [5]. This asymmetry is maintained by flippases that can transfer lipids from one leaflet of the bilayer to the other. This process occurs during apoptosis when flippases are inactive and PS can be exposed on the cell surface, which signals engulfment of the cell by macrophages [5]. CL is present in the inner mitochondrial membrane and is required for oxidative phosphorylation [3]. When CL is oxidized by CL-specific

oxygenases during initiation of apoptosis, cytochrome C is released from the mitochondria triggering additional apoptotic events [6].

PC is the most abundant phospholipid in the eukaryotic cell, comprising 40-60% (mol%) of membrane lipids [4, 7]. PC is essential for membrane integrity due to its cylindrical-like shape that stabilizes the lipid bilayer and is a precursor for signaling molecules and required for secretory products like lipoproteins [4]. Duplication of all cellular components such as proteins, and membranes is required during cell division [8]. The synthesis of PC is increased during mitosis thereby supplying sufficient PC to allow the formation of a stable lipid bilayer in both daughter cells [8]. Without PC the cell cannot create sufficient membranes and becomes growth arrested, eventually undergoing apoptosis [8]. As cancerous cells are characterized by constant proliferation and division, they require rapid duplication of all cellular components including membranes. This implicates the synthesis and regulation of PC as an important factor in cell cycle progression, programmed cell death and cancer (discussed further in Section 1.3.3). It is this function of PC that is the subject of research in this thesis.

1.1.2 Phosphatidylcholine Structure and Function

PC is composed of 2 long-chain fatty acyl groups esterified to the *sn-1* and the *sn-2* positions and phosphate and choline at the *sn-3* position of glycerol [2]. PC can be esterified with a variety of fatty acids with different acyl chain length and saturation, resulting in hundreds of different molecular species in the lipid bilayer [2, 9]. Aside from being a structural component of the lipid bilayer, PC is involved in signal transduction through hydrolysis by various phospholipases. The cleavage of the phosphocholine

headgroup from PC by phospholipase C (PLC) results in the formation of diacylglycerol (DAG), an activator of protein kinase C (PKC) [10]. As well, PC hydrolysis by phospholipase A_2 (PLA_2) releases arachidonic acid, a precursor of leukotrienes and prostaglandins [11]. Phospholipase D (PLD) releases phosphatidic acid (PA) from PC in response to growth factors such as insulin, platelet derived growth factor (PDGF) and epidermal growth factor (EGF) [12]. The production of PA in turn activates signaling mediators such as Raf and Sos, both of which promote cell growth. PC serves to sequester these secondary messengers until activation by hydrolysis by a signaling event.

PC is synthesized by the PE *N*-methyltransferase (PEMT) and CDP-choline pathways [13]. Hepatocytes utilize the PEMT pathway to produce 30-40% of PC by successive methylations of the ethanolamine head group using S-adenosyl-methionine [14, 15]. All other tissues have insignificant PEMT activity and do not make significant amounts of PC via this pathway. The CDP-choline pathway is present in all tissues, including the liver. Although both pathways produce PC, each is distinct in terms of the molecular species produced [14]. The CDP-choline pathway produces medium-chain, saturated fatty acid-rich PC, while the PEMT pathway produces long-chain, unsaturated fatty acids because the substrate PE is composed primarily of these fatty acids [14]. *PEMT* -/- mice cannot synthesize PC through the hepatic PEMT pathway but are still viable [15-17]. However, feeding a choline-deficient diet to these mice results in hepatic steatosis and death in 4-5 days because this is the only pathway for the *de novo* synthesis of choline in mammals [15-17]. *PEMT* -/- mice fed choline-supplemented diets have reduced PC in the bile, lipoprotein secretion from the liver as well as mild hepatic steatosis, indicating that hepatic PEMT supplies PC to these critical secretory processes [15].

The CDP-choline pathway, identified by Eugene Kennedy in 1956, is unique in that phosphocholine combines with CTP to form the activated CDP-choline intermediate as the rate-limiting step [18]. The CDP-choline pathway begins with uptake of exogenous choline, which is then phosphorylated, activated with the addition of CDP, and phosphocholine is subsequently transferred to DAG to form PC (**Fig. 1.1**) [19]. The enzymes of the CDP-choline pathway are localized throughout the cell (**Fig. 1.1**). Several studies have been undertaken to understand the requirements of PC derived from these two pathways. MT-58 CHO cells express a temperature-sensitive CCTα that disrupts the CDP-choline pathway at the non-permissive temperature of 40°C [20, 21]. When cultured under relatively fatty acid deficient conditions at the non-permissive temperature, ectopic expression of PEMT in these cells cannot complement the lack of CCTα activity [20, 21]. The lack of complementation is likely due to depletion of PE by PEMT, which is not normally expressed in CHO cells [20]. Liver-specific CCTα knockout mice have fatty liver but are viable, therefore the PEMT pathway is sufficient for viability of this organ. These studies show that the CDP-choline pathway is the essential for the synthesis of PC in most tissues whereas PEMT has a significant role only in the liver.

1.2 Enzymes of the CDP-Choline Pathway

1.2.1 Choline Transport

Choline is an essential nutrient required for PC synthesis by the CDP-choline pathway [22]. The majority of choline used in the CDP-choline pathway is from the diet [13]. The uptake of choline into the cell is by facilitated diffusion and high- and

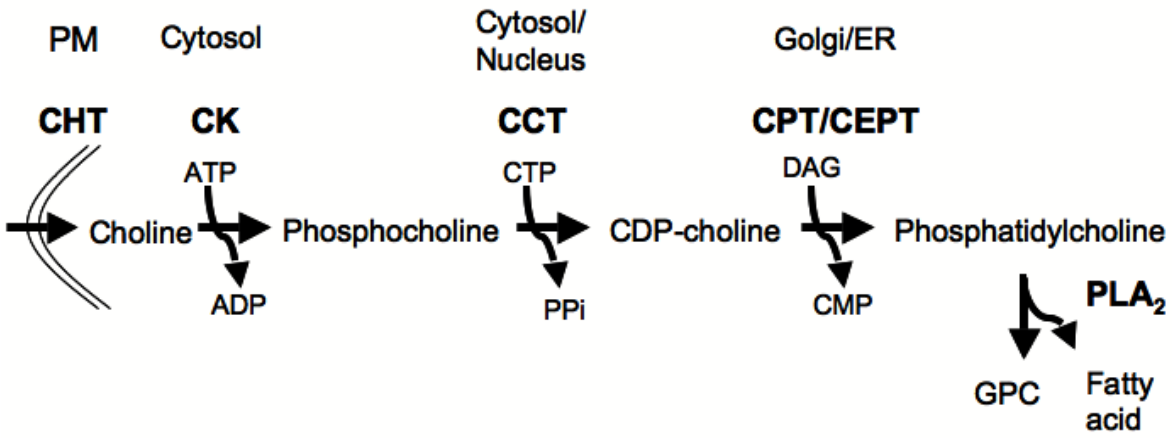

Figure 1.1: Enzymes of the CDP-choline pathway and localization within the cell. Choline kinase is cytosolic, CCTα/β are nuclear/cytoplasmic enzyme, and CPT/CEPT is ER and ER/Golgi localized, respectively. Abbreviations are: High affinity choline transporter (CHT), choline kinase (CK), CTP:phosphocholine cytidylyltransferase (CCT), choline phosphotransferase (CPT), glycerophosphocholine (GPC), phospholipase-A_2 (PLA_2), choline/ethanolamine phosphotransferase (CEPT) and plasma membrane (PM).

intermediate-affinity Na^+-dependent transporters (CHT) at the plasma membrane [23]. Once inside the cell, choline is rapidly converted to phosphocholine by choline kinase for use in the CDP-choline pathway. Choline also contributes to PC synthesis through oxidation to betaine that is required for formation of S-adenosyl methionine, which is used in the PEMT pathway [23].

Aside from being used in PC synthesis, choline is also required in cholinergic neurons for the synthesis of the neurotransmitter acetylcholine [13]. These neurons contain especially high amount of high-affinity choline transporters to maintain synaptic activity. As such, dysfunctional choline transport results in impaired neuronal function leading to Parkinson's and Alzheimer's disease [22].

1.2.2 Choline Kinase

The first step of the CDP-choline pathway commits exogenous or recycled choline to this pathway by the ATP-dependent phosphorylation of choline to produce phosphocholine [19, 24]. This step is catalyzed by the cytosolic enzyme choline kinase, which has bifunctional activity toward choline and ethanolamine [25, 26]. Choline kinase has two isoforms, choline kinase α (choline kinase α1 and choline kinase α2 splice variants) and choline kinase β that share 60% amino acid identity [26]. Both isoforms of choline kinase are expressed ubiquitously in mammalian tissues but choline kinase α is expressed most highly in testis and liver, and choline kinase β is expressed highly in the heart and liver [25-27]. Choline kinase exists as an active heterodimer (α/β) and less active homodimers (α/α or β/β) in a 60/40 distribution [26].

While CCT catalyzes the rate-limiting step in most instances, choline kinase can be limiting for flux through the pathway if its expression is reduced [28]. Several types of cancer have elevated expression and activity of choline kinase which contributes to the transformed phenotype (Discussed further in Section 1.3.2) [29-31].

Heterozygous gene deletion of choline kinase α in mice does not effect PC levels, however homozygous gene deletion of choline kinase α is embryonic lethal at day 3.5-7.5 [32, 33]. Choline kinase β -/- mice are viable but have muscular dystrophy in the hind limbs as well as neonatal bone deformities [32]. Muscular dystrophy does not occur in the forelimbs as choline kinase α expression is high enough to compensate for the loss of choline kinase β [34]. While choline kinase is not rate-limiting, it clearly has significant influence on PC synthesis in rapidly dividing cells and during development.

1.2.3 Choline/Ethanolamine Phosphotransferase

The last step in the CDP-choline pathway is catalyzed by choline/ethanolamine phosphotransferase (CEPT) and/or choline phosphotransferase (CPT). The genes for these two enzymes share 60% amino acid sequence similarity, including nearly identical catalytic domains [35]. CEPT and CPT have a similar seven membrane spanning structure with the catalytic region in a soluble loop at the C-terminus [35]. CEPT is localized primarily to the endoplasmic reticulum (ER) while CPT is localized to the Golgi apparatus [36].

CPT is specific for CDP-choline pathway, and thus PC synthesis, and is highly expressed in the testis and small intestine [37]. In contrast, CEPT can utilize either CDP-choline or CDP-ethanolamine for the formation of PE or PC [37]. Transcript levels of CEPT are ubiquitously and uniformly expressed in all human tissues [37].

Upregulation of CPT results in the expansion of the ER during ER-associated protein degradation (ERAD) [38]. Increased expression of CPT occurs following activation of X-box-binding protein 1 (XBP1) [39]. In XBP1-transduced NIH-3T3 fibroblasts, choline kinase activity was unchanged, CCT activity was increased ~30% and CPT activity was increased 5-fold as determined by [^{14}C]choline incorporation [39]. This results in increased PC synthesis during ERAD, resulting in ER expansion [39]. In transformed breast epithelial cells (MCF-12A), CPT activity is upregulated 2.4-fold compared to wild-type cells [40]. In both cases, increased CPT results in proliferation of the ER, which increases the surface area for the rough ER and potentially protein synthesis. These studies suggest that increased CPT expression and/or activity may facilitate the transformed phenotype through increasing ER size and functionality.

1.2.4 CTP:phosphocholine Cytidylyltransferase α (CCT)

The rate-limiting middle step of the CDP-choline pathway is catalyzed by CCT. Two isoforms of CCT, CCTα and CCTβ, are encoded by the genes *pcyt1a* and *pcyt1b*, respectively [41]. CCTα has an N-terminal nuclear localization signal (NLS), a catalytic domain, an α-helical membrane binding domain and a C-terminal phosphorylation domain [42] (**Fig 1.2**). The main structural difference between the two isoforms is the absence of the NLS in CCTβ [41]. There are 3 different isoforms of CCTβ resulting from alternate splicing [41, 43, 44]. CCTβ1 does not contain a C-terminal domain present in CCTα, and CCTβ2 contains a unique N-terminal sequence [41, 44]. The final isoform CCTβ3 is identical to the CCTβ2 isoform but lacks the amino terminal 28 amino acids; a difference that does not apparently give rise to any functional variation [43] (**Fig 1.2**).

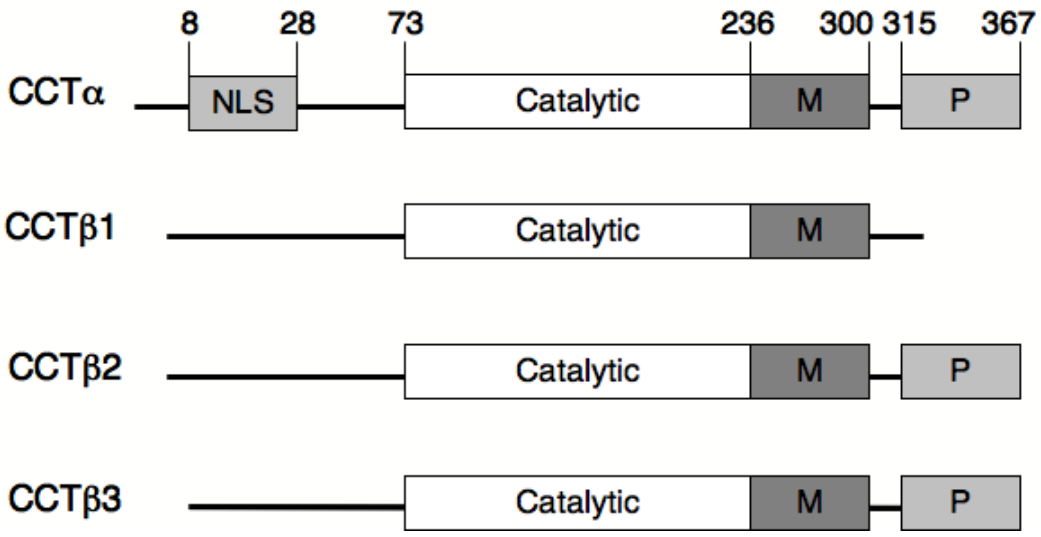

Figure 1.2: Domain structure of CCT isoforms. The main difference between the two isoforms of CCT is the absence of the nuclear localization signal (NLS) in the β isoforms. Membrane binding domain and phosphorlyation domains are abbreviated as M and P, respectively. Amino acid positions are noted at the top.

Although CCTα is ubiquitously expressed in all mammalian tissues, CCTβ 2/3 expression is limited to the brain and gonads and is developmentally regulated [43]. During differentiation of the neuronal cell line PC12 by nerve growth factor, the expression and activity of CCTα remained constant while CCTβ was increased, suggesting that the β isoform contributes to increased PC production during differentiation [45]. Reduction of CCTβ2 during PC12 differentiation resulted in decreased cell division, growth and impaired neurite growth [46]. CCTβ2 expression in the gonads is responsible for 30% of PC synthesis, and CCTβ2 -/- mice displayed reduced fertility [47]. Heterozygous CCTα knockout mice have increased *pcty1b* transcription, suggesting that CCTβ may partially compensate for the loss of CCTα at the transcript level [48]. Thus, the role of CCT isoforms in PC production seems to be divided, with CCTβ providing PC during tissue-specific development, and CCTα required for bulk PC production.

CCTα has tissue-specific functions in the lung and liver [49]. In pulmonary epithelial cells, CCTα is localized to the cytoplasm and contributes to the production of lung surfactant, specifically dipalmitoyl-PC [49]. Infection of the lung by *P. aeruginosa* results in caspase cleavage and reduced transcription of CCTα causing reduced PC synthesis, less surfactant secretion and respiratory distress [50]. Inducable knockout of CCTα in lung epithelial cells results in respiratory distress, but does not affect lung devleopment [50]. This suggests that CCTα is responsible for increased PC synthesis required for secretion of lung surfactant possibly due to its cytoplasmic localization [51].

CCTα expression and the CDP-choline pathway are required in the liver for the production of PC in order to secrete lipoproteins, such as VLDL and HDL [52, 53]. Mice

with a liver-specific knockdown of CCTα, using the Cre/loxP system, are viable but have deficient VLDL formation, resulting in reduced plasma triacylgylerol and PC [52, 53]. This suggests that CCTα activity in the liver is required for VLDL-dependent delivery of TG and cholesterol to peripheral tissues.

1.2.4.1 The N-Terminal Nuclear Localization Signal (NLS)

CCTα is localized in the cytoplasm and nucleus, however nuclear locailzation is seen in the majority of cultured cells and tissues [54]. Nuclear localization is due to an N-terminal nuclear localization signal (NLS) that is rich in basic amino acids (residues 8-28) [54]. The NLS of CCTα is sufficient to promote nuclear import in a heterologous system, for example, NLS fusion to β-galactosidase [54]. CCTβ does not have an NLS and is localized to the cytoplasm and ER [41]. Growth of CCTα-deficient cells can be complemented by CCTα lacking an NLS indicating that catalytic activity is not dependent on the NLS or cellular localization [44, 54].

The localization of CCTα changes after induction of apoptosis. The NLS contains a caspase cleavage site at D28 within the TEED motif that is cleaved during farnesol-, UV- and chelerythrine-induced apoptosis [55]. This allows CCTα to be localized to the cytoplasm as import into the nucleus is abolished by this cleavage event. However, relocalization to the cytosol does not affect the activity of CCTα, suggesting that nuclear export of CCTα during apoptosis could prevent interaction with nuclear factors, and not specifically affect PC synthesis.

1.2.4.2 The Catalytic Domain

The catalytic domain, henceforth referred to as Domain C, spans amino acids 73-236 and was first identified by its sequence identity to other members of the cytidylyltransferase superfamily [56]. Domain C is conserved at the amino acid level between rat and human CCTα and almost identical to mouse and hamster [57]. CHO58 (MT-58) cells express a temperature sensitive form of CCTα that has 100-fold reduced activity at non-permissive temperatures [21, 58] due to an R140H mutation in Domain C but still has normal membrane binding activity and cellular localization [56], indicating that catalytic activity does not affect other domains [58]. CCTα is a homodimer *in vivo* as determined by cross-linking experiments [59], and the site of dimerization is amino acids 139-145 in Domain C [60, 61].

The catalytic motif in cytidylyltransferases is HXGH (HSGH in CCTα, amino acids 89-91) [62]. The two histidines in this motif are required for transition state stabilization while serine is the catalytic amino acid [62]. This motif was previously characterized in class I tRNA synthetases where it mediates nucleotide triphosphate binding [62]. Another conserved motif in CCTα is RTEGISTS (amino acids 196-203) where mutation of Arg 196 to Lys reduce substrate binding by 23-fold and the V_{max} by 3-fold [63]. This suggests that these amino acids are present in or near the binding site and are required for catalysis [63].

1.2.4.3 The Membrane Binding Domain

The membrane-binding domain, henceforth referred to as Domain M, spans amino acids 236-300 as a continuous 52 residue amphipathic α-helix [64, 65]. The polar face of Domain M is composed mainly of acidic amino acids, the non-polar face has 18 hydrophobic residues and basic amino acids are at the interface between the two [64].

CCTα exists as an active membrane-bound form and also as an inactive soluble form, and it is primarily through the reversible binding to membranes mediated by Domain M that activation is regulated [64]. A conformational change upon Domain M binding to membranes results in activation of K_{cat} by more than 80-fold [63]. A mutated form of CCTα lacking Domain M was found to be insensitive to lipid stimulation and constitutively active *in vitro* [66-68]. In this model, Domain M inhibits catalysis in soluble CCTα but when activated and bound to membranes the inhibition is relieved [69] (**Fig 1.3**). Membrane binding is proposed to induce a conformational change of dimer interface of Domain C that increases catalytic activity [68].

Domain M binds to membranes through a combination of ionic interactions with type I lipids and by insertion into membranes with packing defects induced by type II lipids [64, 70, 71]. Type I activators include anionic phospholipids and fatty acids, and type II lipid activators of CCTα (such as DAG and PE) induce membrane curvature [64, 72]. Reduced membrane packing induces curvature strain in the membrane and binding of Domain M to the membrane is favoured since it alleviates this strain through intercalation into the lipid bilayer [66, 73]. Other proteins can influence membrane binding of CCTα indirectly through remodeling membrane content [74]. Phospholipase C and A_2 (PLC and PLA) generate DAG and fatty acids, both of which are activators of membrane binding and activity of CCTα [36, 75]. Domain M is also necessary and sufficient for the export of CCTα from the nucleus in response to lipid activators such as fanresol and oleate [76]. This was found by following the export of a reporter protein, composed of 2 copies of Domain M linked to a GFP, under activating conditions [76]. Thus, Domain M is responsible for

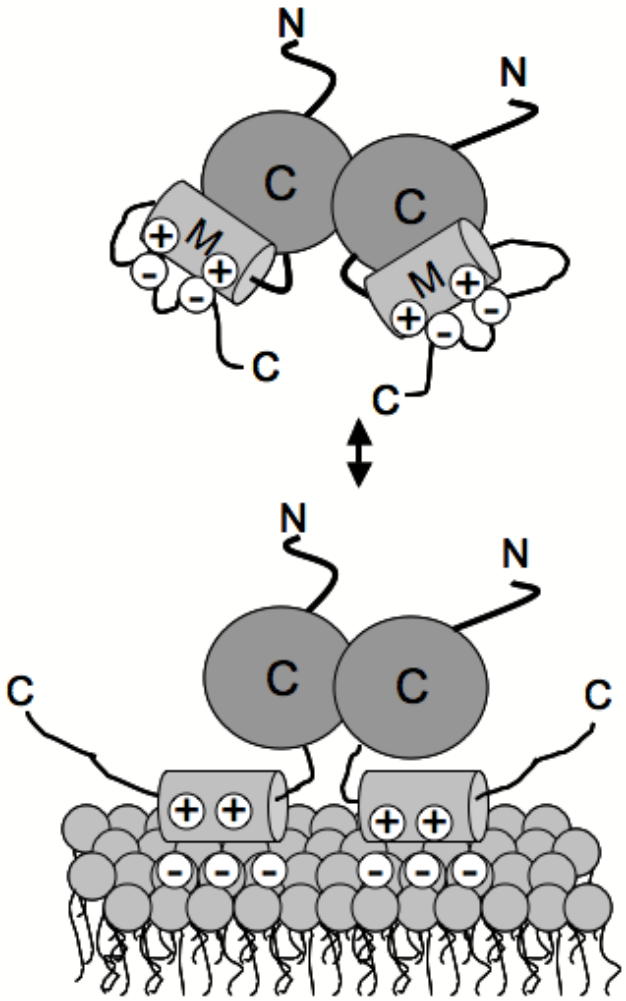

Figure 1.3: Auto-inhibition of Domain C of CCTα is relieved by membrane binding of Domain M. Inactive CCT exists in a soluble phosphorylated form where Domain M binds and inhibits CCT at Domain C. Activation by anionic phospholipids allows for binding of Domain M to membranes, relieving autoinhibition. Figure adapted from Cornell [64].

CCTα localization, translocation and change in catalytic activity through its membrane sensing properties [76, 77].

1.2.4.4 The Phosphorylation Domain

The CCTα phosphorylation domain, henceforth referred to as Domain P, is located at the C terminus (amino acids 315-367) and negatively regulates enzyme activity through attenuation of membrane binding by Domain M [67]. Increased phosphorylation of Domain P is correlated with periods of low catalytic activity, such as during G_o and M phase, while decreased phosphorylation is associated with increased activity during G1 phase [78]. There are 16 serines that are phosphorylated in Domain P but the precise kinases and phosphatases that are involved are not known [79, 80]. Consensus sequences and *in vitro* studies implicate casein kinase II, PKC, cell division control 2, glycogen synthase kinase-3 and mitogen activated protein kinase (MAPK) in CCTα phosphorylation [66, 80, 81].

Site-directed mutants of CCTα lacking the 16 serine phosphorylation sites by mutation to alanines have increased membrane localization [67]. When all serine phosphorylation sites are mutated to be phosphomimetic (serine to glutamate), CCTα is bound to membranes in the presence of oleate, indicating that the excess negative charge from Domain P when fully phosphorylated is not able to completely abolish membrane binding [82]. The deletion of Domain P activates catalytic activity of CCT, as Domain M is not longer inhibited by phosphorylation of this domain [83]. Overall, phosphorylation serves to modulate CCTα membrane binding by producing negative cooperativity when assayed with PC/oleate vesicles.

1.2.4.5 Regulation of CCT by PC Degradation

Cellular PC levels and fatty acid composition are kept constant by a balancing synthesis, degradation and remodeling [9]. The fatty acid content of PC is dependent on tissue type and cell function. For example, acyl-CoA: lysophospholipid acyltransferase is essential in neurons to replace arachidonic acid (released as a secondary messenger) with a fatty acid to restore membrane integrity [84]. Excess PC is degraded though the activity of PC-specific phospholipases (PC-PL). In Chinese hamster ovary (CHO) cells, overexpression of CCTα results in a 7-10-fold increase in PC production that is countered by an upregulation of PLA_2 [85]. Conversely, when PC levels are reduced through treatment of the cell with exogenous PLC, PLD or PLA_2, synthesis of PC is upregulated [36, 86, 87]. This balance between PC synthesis and degradation by PC-PL is crucial for the maintenance of membrane integrity during proliferation and division.

1.2.5 Transcriptional Regulation of CCTα

While cell cycle linked post-transcriptional membrane binding is the primary form of CCTα regulation, transcriptional regulation occurs under the same conditions. Since the synthesis of PC is essential during periods of cell growth, the specific transcriptional mediators temporally regulating CCTα have been extensively investigated. The expression of CCTα is influenced by transcriptional activators such as NFκB, E2F, sterol regulatory element (SRE) and members of the specificity protein-1 (Sp1) family [88, 89]. An SRE in the promoter of CCTα can be activated by sterol regulatory element binding protein (SREBP) after it is cleaved by site 1 and 2 protease in low cholesterol conditions (reviewed in [90] and [65]). SREBP is responsible for upregulation of many lipogenic genes, particularly fatty acid synthase (FASN) for fatty acid synthesis, HMG CoA reductase for

cholesterol synthesis, and CCTα [91]. Although the upregulation of CCTα by SREBP is low, the increase in fatty acids synthesis that is concurrent with SREBP activation results in the post-transcriptional activation of CCTα [92].

Sp1 is the founding member of a protein superfamily, which binds to GC-rich promoter elements using conserved zinc fingers domains, and positively and negatively regulates the expression of hundreds of genes [93]. Sp1 is an essential protein, illustrated by the multiple developmental abnormalities in Sp1 -/-mice that die by day 10 [94]. Sp1 expression requires deacetylation of histones to promote an open chromatin for transcription. The expression of histone deacetylases results in Sp1 downregulation [95]. Additionally, an inhibitor of histone deacetylases caused upregulation of CCTα expression, implicating Sp1 as its regulator [95]. Sp3 has been shown to upregulate the expression of CCTα downstream of H-Ras [94, 96]. The regulation of CCTα by Sp3 is important in this study as this links oncogenic H-Ras to increased CCTα expression and possibly PC synthesis.

During S phase there is increased Sp1-mediated upregulation of CCTα expression through activation by cyclin E and cyclin A/CDK2 [89, 97]. In CHO cells, the synthesis of PC and subsequent degradation to glycerophosphocholine (GPC) is increased early in the G_1/G_0 transition without any change in CCTα expression [98]. During this time CCTα was found to be rapidly translocated to nuclear membranes in agreement with its increased activity [98]. This increased PC synthesis and degradation provide activators for CCTα, resulting in increased PC synthesis late in G_1, which decreases almost completely approaching M phase [98]. In human macrophages treated with colony stimulating factor, the synthesis of PC decreases sharply during S phase while the expression of CCTα is

upregulated [78]. This increased expression of CCTα during S phase is thought to be required so that both daughter cells will have sufficient CCTα after division and not for additional PC synthesis. Again, the main mechanism of CCTαactivation is the rapid induction of membrane association not transcriptional activation [78]. During cell division CCTα phosphorylation gradually increases reaching a peak during M phase before being completely dephosphorylated at the start of the next G_1/G_0 transition [78]. All of these regulatory mechanisms support a system in which CCTα phosphorylation is directly coupled to cell cycle progression and PC synthesis and degradation.

1.3 The Effect of Apoptosis on PC Synthesis

1.3.1 Programmed Cell Death: Apoptosis

Apoptosis is known as programmed cell death because it results in the ordered and regulated cessation of key metabolic processes in a cell [99]. This is important for 1) proper development and function of the immune system 2) maintaining normal cell populations in tissues and 3) embryonic development [99]. Apoptosis differs from cell necrosis, which is uncontrolled and involves loss of cell viability due to acute environmental insult leading to cell swelling, rupture and inflammation [100]. Dysregulation of apoptosis is extremely detrimental to an organism. Without the induction of a coordinated apoptotic program, organ formation will be impaired due to ischemia when the growth of tissues is faster than vasculature growth. If apoptosis is not induced in T cells that target self-antigens, systemic autoimmunity reactions will result. As well, the apoptosis of unused or damaged neurons is a required process that if not maintained results in neurodegenerative diseases. Finally, the resistance of a cell to apoptosis is a common observation in cancer [99].

Apoptosis was first identified in the nematode *Caenorhabditis elegans* as its development is well described and understood [101]. Using this model organism, the exact number of cells generated during development could be determined, along with those that undergo programmed cell death [101]. Analysis of mutations that inhibited or enhanced apoptosis could then be used to identify relevant genes. An example of one such gene is *ced-9*, an analogue of mammalian *Bcl-2* which can act as an oncogene due to its ability to inhibit apoptosis thereby allowing uncontrolled cell growth [101].

1.3.1.1 Initiation and Execution of Apoptosis by Caspases

Apoptosis is executed by a series of cysteine proteases named initiator and effector caspases [102]. Initiator caspases are regulated through two prominent domains, a death effector domain (DED) and caspase recruitment domain (CARD). For example, apoptotic protease activating factor-1 (Apaf-1) and its target, caspase-9, bind by interaction of the CARD on each protein. Similarly, Fas ligand activates Fas-activating death domain (FADD) that contains a death domain to bind to an activated receptor and a DED to bind to initiator caspases [102, 103]. Caspases 2, 8, 9 and 10 are initiator caspases that act as adaptors and points of convergence/divergence in extrinsic or receptor-mediated apoptotic signals [102]. All caspases exist in an inactive procaspase form until activated by a pro-death signal, such as FADD, Apaf-1 or RIP-associated ICH-1/Ced-3 homologous protein with a death domain (RAIDD) [102]. Once activated, initiator caspases bind to and cleave effector caspases. Effector or executioner caspases then cleave cellular components required for cell growth and metabolism. Caspases are negatively regulated by inhibitors of apoptosis (IAPs), through direct binding and inhibition of cleavage [104].

Apoptosis can occur through two alternate but overlapping pathways, the intrinsic or extrinsic pathway. Intrinsic apoptosis is regulated by Bcl-2 family member proteins and is initiated by the release of cytochrome C in response to environmental/nutrient stress or developmental signals. Extrinsic apoptosis is receptor-mediated by death signals through Fas-L for example (**Fig 1.4**).

Caspase activity is also regulated by anti-apoptotic proteins such as, Bcl-2, Bid, and Bcl-x_L and pro-apoptotic Bak and Bax [105-108]. Bcl-2 family members interact by binding to Bcl-2 homology domains (BHD) [106]. When not bound by an anti-apoptotic Bcl-2 family member, Bax and Bak promote the release of mitochondrial proteins into the cytoplasm, including the potent activator of apoptosis cytochrome C (cyt C) [106].

Effector caspases 2, 3, 4, 5, 6, 7, 11, 12 and 13 kill the cell by cleaving various target proteins [109]. The targets of effector caspases have been found serendipitously through sequence identity of caspase cleavage sites, well known targets include, lamin, focal adhesion kinases, gelsolin and polyADP-ribose polymerase [103, 109]. Overall, effector caspases operate to destroy the cytoskeleton and chromatin structure, opposing anti-apoptotic proteins (such as Bcl-2) and disable proteins through cleavage of regulatory and catalytic domains [109]. During apoptosis DNA is also cleaved to completely abrogate any transcription by the cell.

Redundancy between some caspases has been identified through knockout studies in mice. For example, targeted disruption of caspases 1, 2, or 11 had no effect on apoptosis induction [110-112]. However, caspases 3, 8 and 9 are essential, and when disrupted cells are unable to execute apoptosis resulting in uncontrolled cell growth and division [113-115].

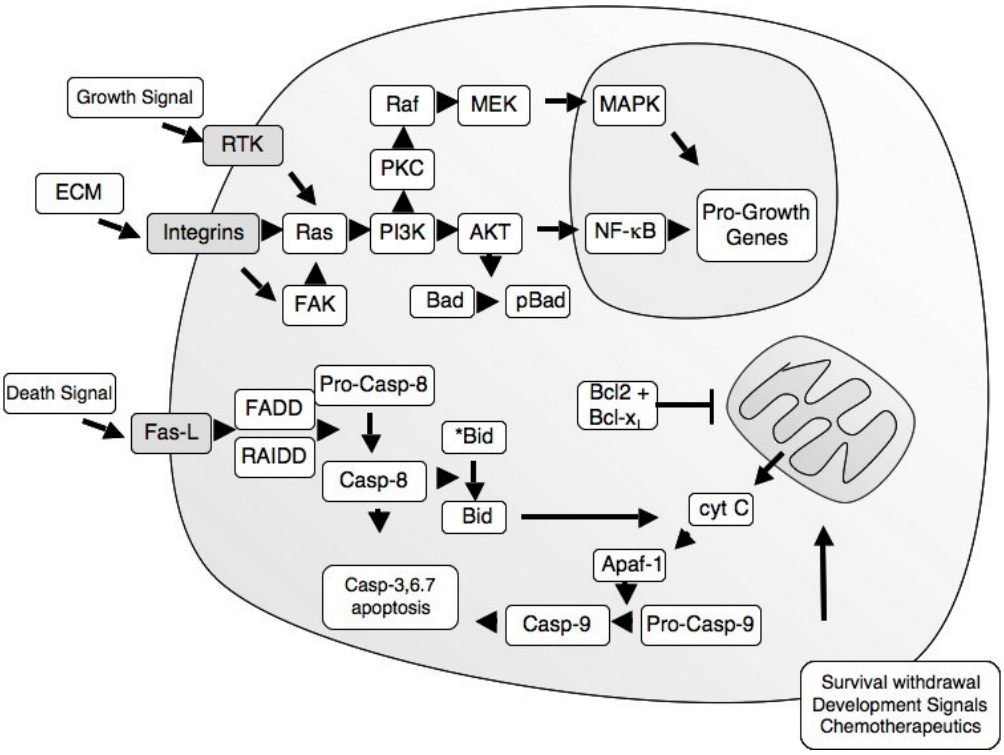

Figure 1.4 Simplified model of signaling pathways involved in cell proliferation and death. Pro-growth signals from the extracellular matrix (ECM) and growth factors such as TGF, VEGF activate Ras. Oncogenic Ras will therefore make the cell insensitive to changes in ECM and growth signals. Apoptosis can occur either by extrinsic signals from Fas-ligand or intrinsically through release of cytochrome C (cyt C). Figure adapted from Hanahan [116].

1.3.2 Detachment-Dependant Apoptosis: Anoikis

Apoptosis of epithelial cell as a result of detachment from the extra-cellular matrix (ECM) is termed anoikis, Greek for homelessness [117]. Anoikis occurs using both intrinsic and extrinsic apoptotic proteins and is a required process in certain tissues to remove aged and damaged cells. However, because of the number of cells that lose signaling via the ECM, over time cells can accumulate mutations and become resistant to anoikis [118]. The formation of a tumour is thought to be a stepwise process where normal cells gradually transition from normal to transformed phenotype [116]. There are several genetic checkpoints that have to be crossed before a cell becomes cancerous, but resistance to anoikis is of foremost concern in the development of epithelial cell-derived cancers. As a tumour rapidly outgrows or changes its environment, cancer cells must be able to grow in any environment by evading apoptosis/anoikis, having self-sufficient growth signaling and insensitivity to anti-growth signals [116, 119]. Since a tumour rapidly outgrows the surrounding vasculature, it becomes exposed to hypoxic conditions and requires increased angiogenesis in order to maintain aerobic growth [120]. Normal cells will cease division and become quiescent after many divisions, but a cancer cell must have a mechanism to have limitless replicative potential [121]. Finally, cancer cells often exhibit tissue invasion and metastatic potential [122]. The order in which these traits are acquired is not important, neither is what protein or system the cell uses to achieve the trait (be it the downregulation of a tumour suppressor or activation of an oncogene), but with each acquisition a cell becomes more aggressive and likely to be malignant.

Carcinomas are by far the most common malignant cancer, accounting for >90% of cancer from epithelial origin [123]. The formation of carcinoma or invasive malignant

tumours occurs by progression through several benign precursor lesions. The general trend heading toward malignancy is referred to as the epithelial to messenchymal transition (EMT) [123]. The EMT is characterized by changes in apical/basolateral to leading edge/lagging edge polarity that is coupled with the loss of cell adhesion molecules and upregulation of cell mobility [123]. Specifically, E-cadherin and cytokeratins are downregulated and N-cadherins and matrix metalloproteases are upregulated [124]. Cell mobility through pseudopodia is increased by the expression of smooth muscle γ-actin and vimentin that contribute to elaborate cytoskeleton formation [125]. All of these factors combined result in epithelial cells that degrade the basement substrate and are capable of invading surrounding tissue. These factors, combined with aberrant cell signaling, results in uncontrolled cell growth and metastasis throughout the body [123]. Following metastasis, cells resume attached growth in another location and begin to transform into a stationary tumour in response to a change in the ECM environment or extracellular signaling [123]. This model of tumour formation requires that either the genetic changes during the EMT are reversible after metastasis to a new tissue, or that a transformed stem cell metastasized with the tumour [126].

However, many cancers have been shown to arise without the transition to a messenchymal-like cell followed by re-differentiation [126, 127]. Another model of tumourigenesis involves the transformation of normal epithelial cells to a benign adenoma and then to malignant carcinoma [126, 127]. A clonal hyperproliferating neoplastic growth is called an adenoma [128]. Gradually, the adenoma grows in size, gains numerous genetic alterations and invades the basement membrane becoming a carcinoma that is now capable of metastatic growth. The acquisition of anoikis-resistance and mutation of the

adenomatous polyposis coli gene (APC) is often the first mutation to occur in a carcinoma [129]. This is visualized by a lack of contact growth inhibition, and partial invasion into surrounding tissues. Various other mutations can then occur, such as loss or mutation of the tumour supressor p53, and de-regulation of cell survival/death signaling.

When intestinal cells become detached from the basement membrane they lose survival signals from cell adhesion molecules, such as integrins [130]. These cells can be rescued from anoikis through embedding in a collagen gel [130]. Since survival of cancer cells often relies on anoikis-resistance, a common goal of anti-cancer pharmaceuticals is anoikis sensitization [131]. However, if the downstream signaling proteins for integrins, such as Ras, are mutated the cell can sustain growth and proliferation in detached conditions.

1.3.3 The Lipogenic Phenotype of Cancer Cells

Another hallmark of cancer cells is increased fatty acid synthesis [132]. Up-regulation of fatty acid synthase (FASN) and proteins involved in glycolysis such as hexokinase, glucose transporters, and pyruvate kinase has been linked to aggressive metastatic cancer [132, 133]. Once the tumour has outgrown the oxygen diffusion limit, it must adapt to hypoxic and nutrient poor conditions [134]. To adapt to this environment, cancer cells rely on increased glycolysis, resulting in a high output of lactate that acidifies the tissue. This bias toward glycolysis in cancer cells is called The Warburg effect, named after Otto Warburg who observed this phenomena in 1956 [135]. Specifically, The Warburg effect is defined as a shift within the cancer cell towards glycolysis even in the presence of oxygen [134].

A transformed cell will proliferate rapidly until the lesion reaches the oxygen diffusion limit. The cells must then adapt to hypoxic conditions by upregulating glycolysis as oxidative phosphorylation can no longer occur. The cancer cells upregulate glucose transporters (GLUT) and hexokinase with Akt , which accelerates glucose uptake to convert pyruvate to lactate to form ATP. Pyruvate is also used to generate citrate within the mitochondria but it cannot be used in the citric acid cycle and instead in converted to acetyl-CoA by ATP-citrate lyase [136]. This excess of acetyl-CoA is used by the upregulated FASN to synthesize fatty acids [136]. Glucose is also converted to ribulose through the pentose phosphate pathway producing NADPH, which is required for fatty acid synthesis. These metabolic changes reprogram the cell to provide energy through activated glycolysis, reduce fatty acid oxidation and stimulate fatty acid synthesis to provide membranes for cell division [136]. Under such circumstances cancer cells have a strong growth advantage and, with the addition of increased cell mobility, are capable of metastatsis.

Enzymes of the CDP-choline pathway are also upregulated in cancer cells [137]. Choline kinase is upregulated in many types of cancer including breast, colon, prostate, lung and others [29, 30, 138, 139]. After 4-6 h of H-Ras activation, choline kinase α expression is elevated and its activity increases 2-fold [140]. The activation of choline kinase α is compounded by increased by enhanced choline transport induced by H-Ras transformation [141]. A choline analogue hemicholinium-3 (HC-3) is an inhibitor of uptake and competitive inhibitor of choline kinase and blocks DNA synthesis by inhibiting signals from growth factors such as epidermal growth factor, platelet derived growth factor and basic fibroblast growth factor, but not growth signals from serum or insulin [139]. choline

kinase α inhibition by HC-3 or RNAi [142] does not interrupt these signals directly, but may be inhibiting the ability of phosphocholine (pChol) to activate raf/MAPK [143]. The source of the pChol could be PLC degradation of PC but it was found that H-Ras transformation had no effect on PLC activity [144]. This implies that pChol generated by choline kinase α is crucial in the regulation of cell cycle progression.

1.3.4 Ras Proteins and Apoptosis

Pro-growth signals can sometimes override anoikis/apoptotic signals, such as those produced from oncogenic forms of Ras protein (rat sarcoma protein) [145]. The Ras superfamily consists of over 170 Ras-related proteins that have roles in cell proliferation [145]. Ras proteins act as molecular switches that are active when bound to GTP and inactive when bound to GDP. The GTP/GDP status of Ras proteins is regulated by GTPase activating proteins (GAPs) and guanine nucleotide exchange factor (GEFs). [146]. If Ras possesses mutations that prevent the binding of GAPs or GTP hydrolysis, it remains in an active state. In this state, downstream proliferation signaling will persist in the absence of a stimulus. The transfection of normal cells with Ras mutated in this fashion results in the partial transformation of these cells so they can grow when detached from the ECM [147].

Three isoforms of the Ras, H-Ras, K-Ras and N-Ras, are ubiquitously expressed and have the same overall pro-growth functions [148, 149]. Relative to eachother H-Ras expression is increased in muscle and brain tissue, whereas gut and thymus have increased K-Ras, and N-Ras most highly expressed in the thymus [150]. Homozygous knockout of N-Ras in mice does not affect viability or development, while K-Ras knockout mice are not viable [151, 152]. Mice lacking H-Ras, or lacking both H-Ras and N-Ras, are normal and

fertile [152, 153]. Thus, although K-Ras is essential, the three Ras proteins most likely share many overlapping functions [153].

Ras proteins are often considered to be oncoproteins since the finding that constitutive activation bestows potent carcinogenic properties to cells [154]. Pro-growth signals from integrins, vascular endothelial growth factors and other growth factors that signal through receptor tyrosine kinases (RTK) and G-protein coupled receptors activate Ras, which in turn have pro-growth downstream effects [146, 154] (**Fig. 1.4**), primarily on PI3K, focal adhesion kinase (FAK), rac/rho and the mitogen activated protein kinase (MAPK) pathways [146].

Rat intestinal epithelial cells (IEC) transfected with an oncogenic constitutively active H-Ras from human bladder carcinoma fibroblasts (EJ cells) resulted in Ras-transformed IEC (IEC-Ras) that were not contact inhibited, grew in suspension and had reduced doubling times [147, 155]. These studies were some of the first to show that oncogenic Ras can induce the cancerous phenotype through transfection of otherwise normal cells.

1.4 The Role of CCTα in The Nucleoplasmic Reticulum

The transition from a normal to cancerous cell is accompanied by changes in cytoskeleton organization. Changes in nuclear cytoskeleton occurs through the remodeling of the intermediate filament family members lamin A/C and lamin B [156, 157]. Lamins form a filamentous network under the inner leaflet of the nuclear envelope to provide structural support as well as anchoring sites for chromatin and histones at the nuclear periphery [158]. In a mRNA screen of human colorectal cells, increased expression of

lamin A/C was correlated with a poor prognosis due to the increased mobility and invasiveness of these cancer cells [156, 157].

The nucleoplasmic reticulum is a series of invaginations of the inner and outer nuclear envelope. Nucleoplasmic reticulum tubules within the nucleus are supported by lamins and are thought to participate in nuclear processes such as calcium release, and DNA synthesis [159]. There are two types of nucleoplasmic reticulum, type I where only the inner nuclear membrane is invaginated, or type II where both the inner and outer nuclear envelope is invaginated [160]. The folded morphology of the nucleoplasmic reticulum allows the cytoplasm and ER to penetrate into the nucleus and increase potential contact sites [161, 162]. The appearance of the nucleoplasmic reticulum is seen in multiple tissues, but an increased amount of nucleoplasmic reticulum has also been observed in some cancer cells [161]. When comparing human adenoma and carcinoma cells, increasing changes to nuclear morphology and formation of the nucleoplasmic reticulum is linked proportionally to increasing malignancy and tumour progression [163, 164].

Regulation of the lamin cytoskeleton and nucleoplasmic reticulum is cell cycle-dependent. For example, lamins quickly associate with chromatin after mitosis, and DNA replication sites are found in close proximity to lamin-supported nucleoplasmic reticulum [159]. The most dramatic changes in nucleoplasmic reticulum structure occur from G1 to S phase when lamin transitions from a highly elaborate structure to almost nothing [165]. This disassembly of the nuclear cytoskeleton is due to cell cycle dependent kinases that phosphorylate lamin. Cytokinesis requires the dissociation of the nuclear envelope including lamins in order for the cell to separate its duplicate genomes and divide successfully.

CCTα has been shown to have an important role in the formation of the nucleoplasmic reticulum via a mechanism that involves both membrane binding and PC synthesis [166]. The activation of the CDP-choline pathway requires coordination of enzymes located in several cellular compartments (**Fig 1.1**). CCTα is normally localized to the nucleus away from choline kinase in the cytosol and CPT/CEPT in the ER. The activation of CCTα by oleate results in the formation of the nucleoplasmic reticulum [77]. Overexpression of CCTα has been shown to increase the amount of type II nucleoplasmic reticulum as well [160]. The formation of the nucleoplasmic reticulum also functions to bring choline kinase and CPT into close proximity with CCTα to potentially streamline PC synthesis.

When lamin A/C or B expression is knocked down, or mutated in Progeria Syndrome, the nucleoplasmic reticulum does not form [166, 167]. Mice that have a homozygous knockouts of lamin A/C appear normal at birth, but have growth defects, muscular dystrophy and die by day 10 [168]. Inhibition of nucleoplasmic reticulum formation through expression of a dominant negative lamin A resulted in the loss of the nucleoplasmic reticulum, as well as impaired DNA synthesis implying its direct involvement in cell cycle progression [169]. The reduction of lamin A/C protein levels do not affect CDP-choline pathway activity indicating that while the nucleoplasmic reticulum may activate PC synthesis it is not required [167]. Overall, the nucleoplasmic reticulum is implicated in cell division, cell cycle progression and CCTα activation, all of which are tied to tumour growth.

1.5 Aims for This Study

The resistance of cells to apoptosis is hypothesized to be partially contingent on increased lipogenesis to enable constant proliferation. Evidence exists for activation of PC biosynthetic enzymes, such as increased choline kinase and to a lesser extent CPT/CEPT, but the role of CCTα in tumourigenesis remains unclear. It is hypothesized that growth in detached conditions requires additional PC or other PC-derived signaling factors such as DAG and PA, through upregulation or activation of CCTα. My investigation of CCTα involvement in anoikis and apoptosis had 3 main objectives.

*Objective 1) Ras-transformation has many effects on cell signaling through its downstream effectors, leading to anoikis-resist*ance. The first objective was to determine how H-Ras transformation influences CCTα and CCTβ expression, activity, localization and role in anoikis sensitivity of IEC and IEC-Ras cells.

Objective 2) To understand whether CCTα is a causative factor in anoikis resistance by reducing expression with shRNA. Using clonogenic assays I determined how depletion of CCTα affected cell survival, as well as tumour growth using an *in vivo* mouse mouse model.

Objective 3) To correlate membrane binding activity of CCTα with its localization to the nuclear envelope. Transformed cells exhibit increased nucleoplasmic reticulum formation, therefore changes to nuclear morphology between IEC IEC-Ras was investigated by immunofluorescence.

CHAPTER 2 MATERIALS AND METHODS

2.1 Materials

Alexa-Flour® 488 goat anti-rabbit, and Alexa-Flour® 594 donkey anti-goat antibodies, puromycin, Dulbecco's modified Eagle's medium (DMEM), ampicillin, Thermoscript® and Lipofectamine 2000 transfection reagent were all purchased from Invitrogen (Burlington, ON). A rabbit anti-CCTα antibody was raised, purified, and purchased from Genscript (Scotch Plains, NJ). A rabbit anti-CCTβ antibody was generously provided by Dr. Suzanne Jackowski (St. Jude Children's Research Hospital, Memphis TN). Odyssey blocking buffer and IRDye® 800CW and 680LT were purchased from LI-COR Biosciences (Lincoln, NE). A rabbit anti-OSBP antibody was purified previously in our lab [170]. Horseradish peroxidase-conjugated goat anti-rabbit antibody, nitrocellulose (NC) membrane, glycine 40% acrylamide, and Tween-20 were purchased from Bio-Rad (Hercules, CA). A mouse anti-β-actin monoclonal antibody, bovine serum albumin (BSA), polybrene, oleic acid and all lentiviral shRNA pLKO.1 plasmids, shCCT1-4, shβ1-2, shGFP, non-targeting shRNA (shNT), and pLKO.1 empty vector were purchased from Sigma-Aldrich (St. Louis, MO). TLC-silica gel G plates were purchased from Analtech (Newark, DE). [^{3}H]Choline was purchased from Perkin-Elmer (Waltham, MA). Primers for mutagenesis were purchased from Integrated DNA Technologies (Coralville, IA). Goat anti-lamin A/C and mouse anti-lamin A/C antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Plasmid purification kits were purchased from Quiagen (Missisauga, ON). Mowiol® 4-88 reagent was purchased EMD Biosciences (La Jolla, CA). SurfactAmps® X-100 was purchased from Thermo Scientific (Rockford, IL).

Biomax XAR film for autoradiography was purchased Kodak (Toronto, ON). Fetal calf serum (FCS) was purchased from PAA Cell Culture Company (Etobicoke, ON). Immobilon Western Chemiluminescent HRP substrate was purchased from Millipore (Billerica, MA). Eight-week-old female athymic mice (BALB/c) were purchased from Charles River Laboratories (Wilmington, MA). Sea Plaque agarose was purchased from Cambrex (Rockland, ME).

2.2 Cell Culture

All cells (IEC-18, IEC-Ras3, IEC-Ras4, IEC-Ras7, Hela, and HEK293T) were incubated at 37°C in a humidified 5% CO_2 atmosphere. IEC-18, IEC-Ras3, IEC-Ras4, and IEC-Ras7 cells were grown in IEC-MEM (α-MEM plus 5% FBS, 3.6 g/L D-glucose, 12.74 μg/mL insulin 600 μg/mL penicillin, 100 μg/mL streptomycin and 2.92 mg/mL glutamine). HEK 293T and Hela cells were cultured in CCL107 media (D-MEM with 10% FBS). For [^{3}H]Choline pulse-labeling experiments, cells were cultured in choline free D-MEM.

2.3 **Plasmid Transfection**

When 70% confluent, 35 mm dishes of HeLa cells, or IEC/IEC-Ras were transfected by incubation with 2 μg of DNA and 4 μL of lipofectamine. Lipofectamine and DNA were combined and incubated in 200 μL DMEM for 30 min prior to addition to cells in 0.8 mL of an appropriate culture media. After 18 h cells were then used in further experiments.

2.4 Lentiviral Production and Infection

A lentiviral delivery system was used to express shRNA for selectable knockdown of a target mRNA. Viral particles are produced by co-transfection of plasmids encoding packaging factors (Δ8.2), envelope protein vesicular stomatitis virus glycoprotein (VSVG) and an shRNA into replication competent HEK293T cells with polybrene, a cationic polymer that aids viral entry. The packaging vector synthesizes capsid proteins that bind the recombinant RNA from the coding vector. After assembly, the recombinant viruses bud from the host cell plasma membrane coated with the viral envelope protein L-VSVG. This viral particle can now infect target cells and insert the desired shRNA coding sequence into the host genome. After being produced in replication competent cells, these particles cannot replicate further since non-essential virulent genes have been deleted (vpr, vif, vpu and nef) and the promoter enhancer region in the U3 promoter has been deleted.

HEK293T cells ($2x10^6$ cells/mL) in a 60mm dish were transfected with 3 μg of pLKO.1-shRNA, 2.7 μg of Δ 8.2 plasmid, 0.3 μg of L-VSVG plasmid (6 μg DNA total) with 14 μL polyethyleneimine (PEI) transfection reagent in 400 μL D-MEM. This transfection mix was incubated at room temperature for 15 min and added to 2 mL of HEK293T cells in CCL107 media for 48 h to produce viral particles. Knockdown of CCTα was performed using the following shRNA sequences: shCCT1 (5'- CCGGCCTAAGGA CATCTACAAGAACTCGAGTTCTTTGTAGATGTCCTTAGGTTTTTG-3'), shCCT2 (5'-CCGGCCCGAGAGTTCATTGGAAGTTCTCGAGAACTTCCAATGAACTCTCGG GTTTTTG-3'), shCCT3 (5'-CCGGCCTGTGAGAGTTTATGCGGATCTCGAGATCC GCATAAACTCTCACAGGTTTTTG-3') and shCCT 4 (5'- CCGGGTTGACTT TAGTAAGCCCTATCTCGAGATAGGGCTTACTAAAGTCAACTTTTTG-3'). Knockdown of CCTβ was performed using the following shRNA sequences: shβ1 (5'-

CCGGGCATGTTTGTTCCAAC ACAAACTCGAGTTTGTGTTGGAACAAACATG CTTTTTG-3') shβ2 (5'-CCGGGCT ACTTGTTGGTAGGAGTTTCTCGAGAAACTC CTACCAACAAGTAGCTTTTTG-3'). Control vectors were pLKO.1 empty that lacks a targeting sequence, shNT (5'- CCGGCAACAAGATGAAGAGCACCAACTCGAGTT GGTGCTCTTCATCTTGTTG TTTTT-3') and shGFP (5'-CCGGTACAACAGCCAC AACGTCTATCTCGAGATAGA CGTTGTGGCTGTTGTATTTTT-3')

Virus-containing media was harvested, filtered through a 0.45um filter and 1 μg/mL polybrene was added. The viral media was then applied to a culture of 70% confluent IEC or IEC-Ras4 target cells for 4 h after which 7 mL IEC-MEM was added. After 24 h, 1 μg/mL and 2 μg/mL puromycin was added to IEC18 and Ras34 cells, respectively, for 48-72 h to select for virus-infected cells. Puromycin was also added to non-infected cells of equal density (canary dishes) to gauge when all non-infected cells have been killed. Knockdown was confirmed by Western blot for each batch of cells.

2.5 Immunofluorescence Microscopy

Cells were trypsinized and seeded at 70% confluence onto 35mm dishes containing sterile glass cover slips (0.15 mm). At the end of the experiment the cover slips were washed twice with PBS (10mM sodium phosphate pH 7.4, 225 mM NaCl and 2 mM magnesium chloride), fixed with 4% (w/v) paraformaldehyde in PBS for 15 min and incubated with 0.5% Triton-X100 for 10 min at 4°C. Antibodies were diluted in PBS-1% (w/v) BSA and applied as indicated in the figure legends and were diluted in PBS-1% (w/v) BSA. All antibodies were incubated with cover slips at room temperature for 1 h. Secondary AlexaFluor-conjugated antibodies were used at 1:4000 in PBS-1% (w/v) BSA

and were incubated at room temperature for 1 h. Cover slips were then mounted on glass slides with 5 μL of Mowiol® 4-88. Images were captured using a Zeiss LSM510 META confocal microscope at 0.6-0.7 μm thickness with a 100X oil immersion (1.4 NA) objective lens and LSM510 Meta software. Wide-field images were captured using a Zeiss Axiovert 300M fluorescent microscope equipped with an AxioCamHRm CCD camera, 100X or 40X oil immersion (1.4 NA) objective lens, and Axiovision Software.

2.6 Western Blotting of Cell Lysate

Two alternative methods for visualizing protein expression were used in this study that are different in detection methods. As our lab only had access to a Licor Odyssey® IR Imaging System in the later part of my work, some Western blots in this study use ECL and film exposure.

Total cell lysates for Western blotting were prepared as follows. Cells were washed twice with cold PBS before being lysis in SDS Buffer (12.5% SDS, 30 mM Tris-HCl [pH 6.8], 12.5% glycerol and 0.01% bromophenol blue). Samples were then dismembranated by sonication at 60 Hz for 20 sec, and heated to 90°C for 5 min, before being loaded into an SDS-(8%) polyacrylamide gel. Conditions for gel electrophoresis are included in figure legends and SDS-PAGE running buffer (3 mM SDS, 200 mM glycine, 25 mM Tris-base). Following electrophoresis, proteins were transferred to nitrocellulose membranes in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 100V for 1 h.

Western blots to be imaged on the Odyssey Infrared Imaging System were incubated with a 1:5 dilution of the Odyssey blocking buffer in TBS-Tween (20 mM Tris-HCl, [pH 7.4] 500mM NaCl, 0.05% Tween 20) for 60 min before immunoblotting. Primary

antibody dilutions were applied as indicated in the figure legends and for 1 h at room temperature. All secondary antibodies were used at 1:15000 dilution in blocking buffer for 1 h at room temperature. Fluorescence was visualized at 680 or 800 nm in the IR spectrum using a Licor Odyssey and quantified using Odyssey Application Software v3.0.

Western blots visualized by ECL were incubated in 5% (w/v) skim milk in TBS-Tween (Blotto) for 60 min at room temperature before immunoblotting. Horseradish peroxidase (HRP)-conjugated secondary antibodies were diluted 1:10000 in Blotto were incubated for 1 h at room temperature. Primary antibodies were diluted as mentioned in figure legends, and for 1 h at room temperature. Membranes were visualized by exposure to film and band intensities were quantified by densitometry using ImageJ software v1.4.

2.7 Measurement of PC and Metabolites by [^{3}H]Choline Incorporation

Two alternative methods of [^{3}H]choline radiolabeling were performed, steady-state and a 3 h pulse. The steady state method measures [^{3}H]choline incorporation after 24 h and is representative of an equilibrium condition for PC and its metabolites. Labeling for 3 h pulse measures the rate of synthesis and non-steady-state levels of PC metabolites over a 3 h period.

Cells were incubated with 1.5 μCi/mL [^{3}H]choline for 3 or 24 h (indicated in the figure legends), washed twice in PBS, scraped from the dish and resuspended in 1 mL of methanol:water, 5:4, (v/v), 200 μL of which was taken for Lowry protein quantification [171]. This was followed by 3 mL of $CHCl_3$ and phase separation by centrifugation at 3000 rpm for 5 min. The aqueous phase was collected by aspiration, while the organic phase was washed twice with ideal upper phase (methanol/0.58% NaCl/$CHCl_3$, 45/46/3, (v/v)), dried

under nitrogen, resuspended in 1 mL chloroform and 200 μL was counted by liquid scintillation counting (LSC). The aqueous fraction was also dried with nitrogen, resuspended in 100 μL water, and 20 μL was applied to TLC plates along with standards for CDP-choline, GPC and phosphocholine. The TLC plate was resolved using water:ethanol:ammonium hydroxide, (50:95:6 v/v). Standards were visualized by spraying the TLC plates with 1% phosphomolbydate in chloroform:methanol, (1:1 v/v) followed by 1% (w/v) stannous chloride in 3N HCl. Samples were collected by scraping the appropriate areas, and [^{3}H]choline radioactivity was measured by LSC.

2.8 Clonogenicity Assay for Cell Survival

This assay involves culturing a relatively small population of cells in detached conditions on Sea Plaque agarose-coated plates followed by transfer to plastic adherent conditions to measure viability and colony-forming activity. Cells that survived detachment will resume growth when transferred back to adherent conditions and form colonies. Surviving colonies can then be compared to plastic dishes seeded with an identical amount of cells to calculate a percent survival. This method was adapted from Rofstad [172].

IEC-18 and IEC-Ras cells were trypsinized and 500 cells were seeded onto 60 mm dishes coated with 2 mL of 1% (w/v) Sea Plaque agarose (SP-agarose) in α-MEM. IEC-MEM was added to the trypsinized cells to a total of 2 mL. As a control, 500 cells were also seeded onto 60 mm dishes without SP-agarose to compare colony formation. Cells on SP-agarose were transferred to plastic dishes after 24, 48 and 72 h, and the SP-agarose surface was washed with 2 mL IEC-MEM to ensure all cells were removed. Transferred cells were allowed to form colonies for 3-4 days, after which they were washed with PBS,

fixed with methanol:acetic acid, (2:1 v/v) and stained with Crystal Violet (1.4% (w/v) Crystal Violet in 95% ethanol and 0.04 M HCl). Colonies were counted and survival was calculated as percent of adherent control.

2.9 Soft Agar Assay

This assay is similar to that of Section 2.8, but tests different characteristics by measuring colony growth in a suspension of soft agar. Cells that are growth arrested after detachment but viable if cultured in adherent conditions will not form colonies in this soft agar assay.

Trypsinized IEC-Ras (500 cells) were seeded in 60 mm dish cells in 0.3% (w/v) bacto-agar in IEC-MEM on 60 mm dishes coated in 2 mL 0.5% (w/v) agar. The cells were grown in suspension for 4-5 days to allow colony formation and counted by microscopy. Plastic control dishes were prepared and used as in Section 2.8. IEC cannot be used with this assay as they do not form colonies at any time.

2.10 Tumour Formation Assay

While the two previous methods give an effective measure of cell survival *in vitro*, they cannot replicate the complexity of systemic mechanisms at work in an *in vivo* model. Immunocompromised (athymic) BALB/c mice were used in this study as this allowed for tumour growth without variable immune responses in each specimen. As well, the IEC-Ras cells used are from rat and would normally cause a rejection response. This system is the assay of choice for measuring tumour growth *in vivo* [173].

Athymic BALB/c mice were housed in sterile conditions under quarantine for 1 week upon arrival to the Carleton Animal Care Facility. Mice were then injected with IEC-Ras4 cells infected with virus encoding shGFP, shCCT1 or shCCT3 (as prepared in Section 2.4) by subcutaneous flank injection on the right side. Tumour growth was monitored daily using a skin-fold caliper, and volume was calculated using an ellipsoidal volume approximation ($0.5*(LxW^2)$) [173]. Mice were sacrificed after 15 days or if tumours grew too large (>1cm^2) or became ulcerated. Additionally, the Carleton Animal Care Facility (CACF) staff would euthanize mice if the specimen was moribund because of a debilitating condition. A certified CACF technician performed euthanization of the mice by intraperitoneal injection of Euthanyl (240 mg/ml Pentobarbital Sodium USP, supplied by CDMV). All procedures and protocols were certified by the Dalhousie University Committee on Laboratory Animals (protocol number: 11-022).

2.11 Statistical Analysis

Unless otherwise stated in the figure legend, data presented is the mean of 3 independent experiments with error bars representing the standard error of mean (SEM). Statistical significance was evaluated using the student two-tailed t-test. Significance is reported if the p-value was < 0.1 (*), 0.05 (**), and 0.01 (***).

CHAPTER 3 RESULTS

3.1 The Role of H-Ras in the Regulation of CCTα

The detachment of epithelial cells from the basement membrane results in the loss of pro-survival signaling through integrins, H-Ras and downstream kinases resulting in anoikis. In the intestine, epithelial cells are constantly being sloughed off and regenerated to prevent buildup of damaged or non-functional cells [117]. IEC-Ras clones IEC-Ras3, IEC-Ras4 and IEC-Ras7 were generated by transfection of IEC with activated H-Ras cloned from human bladder carcinoma [147]. These IEC-Ras cells are resistant to anoikis by constitutive activation of pro-survival signals that act downstream of H-Ras. Anoikis resistance in IEC-Ras has been reported previously by Rak *et al.* [174] and is useful as a cell culture model of metastatic tumours. Resistance to anoikis can be shown using a clonogenicity assay where cells are grown in detached conditions and then transferred to plastic dishes where their ability to attach and form colonies can be measured (**Fig. 3.1.1**)[174]. IEC cells rapidly die by anoikis following detachment, therefore cells do not survive to form colonies when placed back in adherent conditions. On the other hand, IEC-Ras 3, 4 and 7 have a constitutively active H-Ras and are 50-80% resistant to anoikis and continue growth in detached conditions. The initial loss of colony formation by IEC-Ras at 24 and 48 is most likely due to a loss of a subpopulation of cells that have adapted for adherent conditions.

The growth rates of IEC-Ras are quite different from one another. IEC-Ras3 and IEC-Ras4 have doubling times that are 3-fold less than IEC, and IEC-Ras7 doubled twice as quickly as IEC-Ras3 and IEC-Ras4. The relative doubling times seem to be correlated

with colony formation during detachment (**Fig. 3.1.1**). The rapidly dividing IEC-Ras7 has the highest colony formation, followed by IEC-Ras4, and IEC-Ras3. So although all IEC-Ras are similar in generation and phenotype there are differences between them.

This study investigated whether or not CTTα has a role in anoikis resistance of IEC-Ras. Although it is known that oncogenic H-Ras provides anoikis resistance, the specific downstream effectors of resistance are not identified. Studies of IEC-Ras have found that anti-apoptotic proteins IAP, Bcl-x_L are upregulated, while proapoptotic proteins such as Bcl2 and Bak are downregulated [175-177]. Previous studies have found that various proteins responsible for lipid synthesis, such as FASN, ATP citrate lyase and glycolytic enzymes are upregulated in transformed cells, so it is reasonable to assume that CCTα could be among them [29, 96, 141]. CCTα expression was investigated by analysis of cell lysate from IEC and the IEC-Ras cell lines using SDS-PAGE and Western blotting (**Fig 3.1.2**). The expression of CCTα is greatly increased in IEC-Ras with ~30-fold increased expression in IEC-Ras3, ~10-fold expression in IEC-Ras4, and 15-fold in IEC-Ras7. The expression of CCTβ remains unchanged between IEC and all IEC-Ras suggesting that CCTα not CCTβ is involved in H-Ras anoikis resistance.

Continuing this investigation, changes to CCT expression were measured in IEC-Ras that were grown in detached conditions for up to 72 h following detachment (**Fig 3.1.3**). Domain P in CCT is known to act as a negative regulatory domain when phosphorylated; this phosphorylation can be visualized as an apparent increase in molecular mass by SDS- PAGE. Although IEC are quickly dying from anoikis under these conditions and lose protein expression compared to IEC-Ras, the trend of increasing

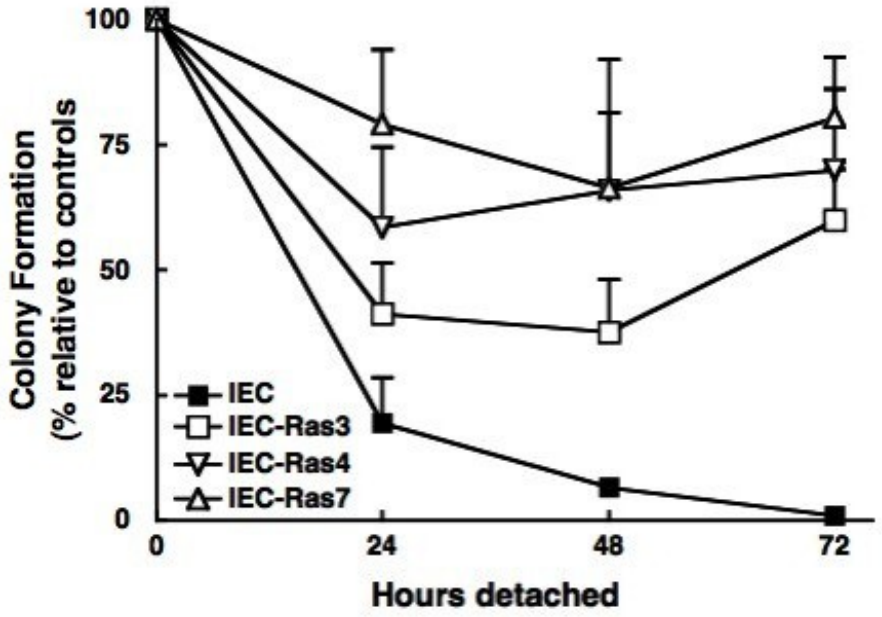

Figure 3.1.1: IEC-Ras cells grown on SP-agarose are resistant to anoikis. IEC, IEC-Ras3, IEC-Ras4, and IEC-Ras7 cells were grown in dishes coated with 1% SP-agarose to simulate detached conditions. After 24, 48 or 72 h cell colony formation was measured compared to cells grown on a plastic dish (Section 2.8). Data presented is the average from 3 separate experiments with error bars corresponding to SEM.

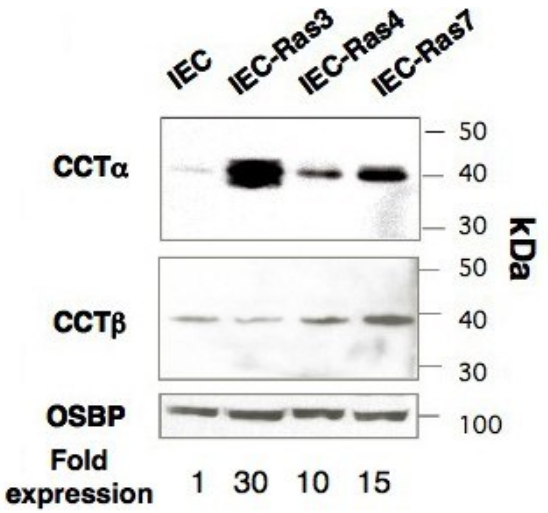

Figure 3.1.2: CCTα expression level is increased in IEC-Ras cells. Cell lysates from IEC and IEC-Ras cells were harvested, lysed, resolved by SDS-PAGE, and immunoblotted for CCTα, CCTβ and OSBP as described in Section 2.5. Expression quantification of CCTα is indicated below the panels as fold expression compared to IEC (n=1). Antibodies used: Rabbit anti-OSBP (1:10000) was used as a loading control, rabbit anti-CCTα (1:2000) and rabbit anti-CCTβ (1:1000).

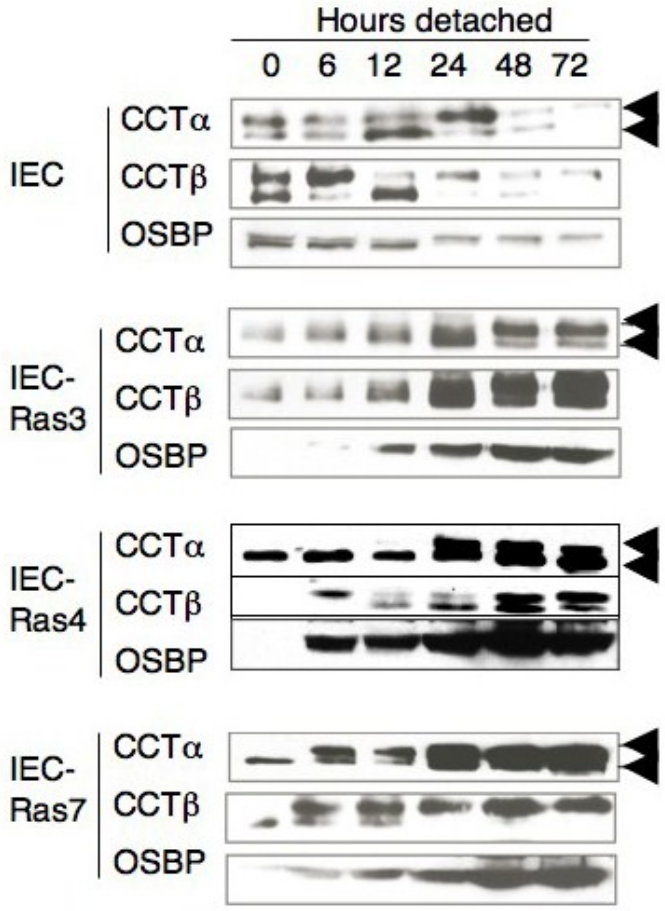

Figure 3.1.3: CCTα is phosphorylated after prolonged detachment. Cell lysates from IEC and IEC-Ras under detached conditions were harvested, lysed, resolved by SDS-PAGE, and immunoblotted for CCTα, CCTβ and OSBP as described in 2.5 of the Materials and Methods. The IEC-Ras signal is strong compared to IEC so 4 times as much IEC lysate was loaded into the gel. The arrows denote the upper phosphorylated form of CCT while the lower is the unphosphorylated form. Antibodies used: Rabbit anti-OSBP (1:10000) was used as a loading control, rabbit anti-CCTα (1:2000) and rabbit anti-CCTβ (1:1000).

phosphorylation during detachment is common in all cell lines. IEC have both phospho-forms of CCT at all times, while IEC-Ras favour the unphosphorylated form until detached. As IEC and IEC-Ras are cultured in detached conditions the phosphorylation status of CCTα and CCTβ shifts to a high molecular mass phosphorylated form. This suggests that CCT, and therefore for the CDP-choline pathway, may be less active in detached conditions.

From Western blots it is clear that there is upregulation of CCTα in IEC-Ras so the catalytic activity of the CDP-choline pathway was investigated through observation of the [^{3}H]choline labeled metabolites and products phosphorylcholine (pChol), CDP-choline, glycerophosphocholine (GPC) and PC after 24 h. While CCTα catalyzes only one step of the pathway its inhibition would result in a buildup of its precursor pChol, and its activation would appear as increased PC and/or GPC due to increased synthesis and degradation, respectively [78, 134]. What is observed in IEC after 24 h of [^{3}H]choline incorporation is a large pool of labeled pChol, with low GPC, and similar PC relative to IEC-Ras (**Fig 3.1.4**). IEC-Ras have similar [^{3}H]choline-labeled PC levels, with high GPC and low pChol, indicative of increased flux through the CDP-choline pathway. When [^{3}H]choline incorporation was measured after a 3 h [^{3}H]choline labeling period incorporation into PC was similar between IEC and IEC-Ras, IEC-Ras had relatively high incorporation into GPC, and IEC relatively high incorporation into pChol (**Fig 3.1.5).** Radioactivity measured in steady-state is more indicative of total metabolite levels within the cell, while the 3 h pulse label experiment measures relative rates of formation of each metabolite. This data suggests that the CDP-choline pathway in IEC-Ras is more active than in IEC leading to

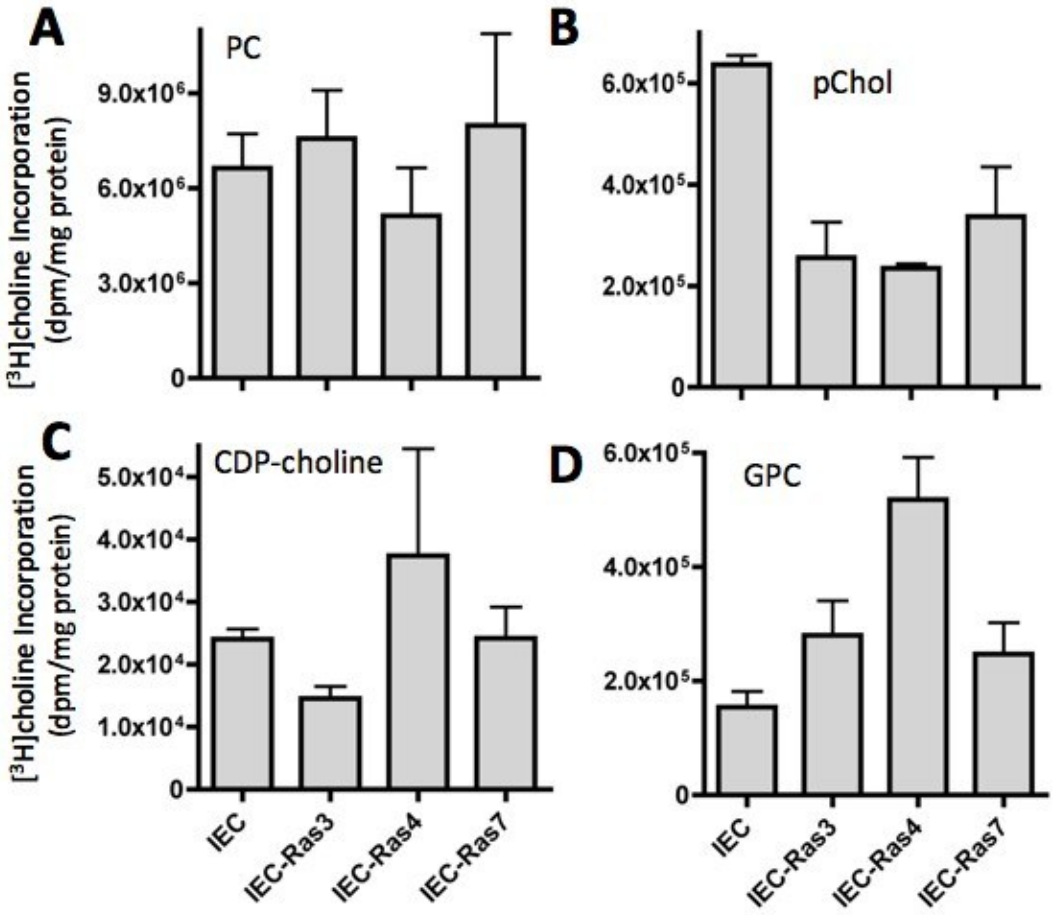

Figure 3.1.4: IEC-Ras have higher GPC and lower pChol steady-state levels. [^{3}H]Choline incorporation into PC **(A),** GPC **(B),** CDP-choline **(C)** and pChol **(D)** was measured in IEC and IEC-Ras as discussed in Section 2.7. Isotope incorporation was measured after 24 h incubation with [^{3}H]choline and, values presented are the average of 5 independent experiments with error bars representing SEM.

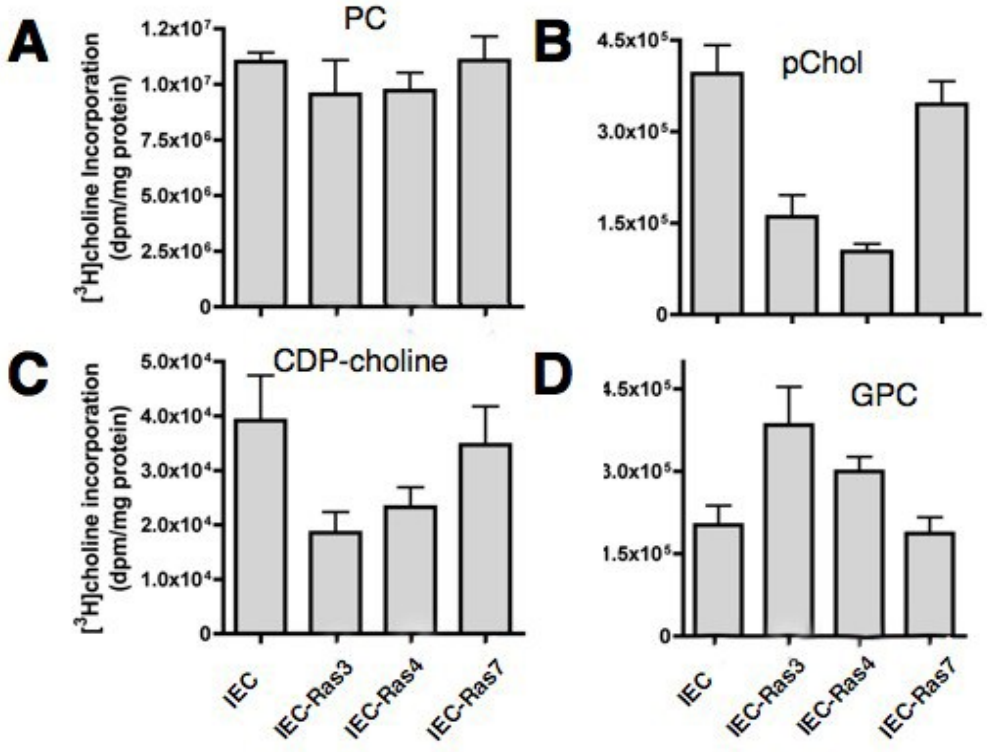

Figure 3.1.5 IEC-Ras have higher GPC and lower pChol incorporation in a short term [^{3}H]Choline pulse label. [^{3}H]Choline incorporation into PC **(A),** GPC **(B),** CDP-choline **(C)** and pChol **(D)** was measured in IEC and IEC-Ras as described in Section 2.7. Metabolite levels were measured after 2 h incubation in choline-depleted media and a 3 h [^{3}H]choline incubation. Data presented is the average from 3 separate experiments with errors bars representing SEM.

increased flux through the CDP-choline pathway, where PC does not buildup because of high turnover to GPC and fatty acids.

During detachment, the phosphorylation state of CCT increased, potentially resulting in a reduction of CCT activity (**Fig 3.1.3**). To investigate changes to flux through the CDP-choline pathway during detachment IEC and IEC-Ras cells were incubated with [^{3}H]choline in detached conditions. The measurement of [^{3}H]choline incorporation into PC and choline metabolites under detached conditions is deceptive because as protein levels fall during anoikis so does the incorporation due to IEC cell death, which would inflate radioactivity incorporation due to reduced protein. Because of these factors [^{3}H]choline incorporation for cell cultured on plastic dishes was compared to that for detached conditions (**Fig 3.1.6).** Under detached conditions IEC cells rapidly lose [^{3}H]choline incorporation into PC and all metabolites most likely due to these cells undergoing anoikis. IEC-Ras instead have approximately 2-fold higher radiolabeled PC levels compared to adherent controls, and also have an initial sharp reduction of pChol and GPC. The loss of pChol incorporation indicates high flux through the pathway, and the reduction of GPC incorporation indicates less PC degradation. As well, the relative PC levels in IEC-Ras increase over time when detached in spite of increased phosphorylation. This data suggests that flux through the CDP-choline pathway is increased in IEC-Ras upon detachment.

The activation status of CCT can also be identified by monitoring the distribution of inactive soluble and active membrane-bound form of CCT between the nucleoplasm and the nuclear envelope [167]. In this study the location and degree of activation of CCTα was visualized using immunofluorescence (IF) microscopy with antibodies specific to lamin A/C (a nuclear marker) and concanavalin A (ConA, an ER marker) (**Fig. 3.1.7)**. As

expected, CCTα is localized within the nucleus in each cell line. In IEC, CCTα is localized diffusely throughout the nucleus with little co-localization with lamin A/C at the nuclear envelope. In IEC-Ras cells CCTα is more localized to the periphery of the nucleus and nuclear envelope. In addition, the nuclear lamina in IEC-Ras adopts a highly folded morphology that co-localizes with CCTα. The distribution of CCTα is heterogeneous in IEC-Ras with IEC-Ras4 having the most co-localization of CCTα with the nuclear envelope, and IEC-Ras3 appearing mostly similar to IEC. Nucleoplasmic reticulum tubules in the nucleus would have been visualized by ConA, but it does not appear as though the nucleoplasmic reticulum is formed in any of these cells. These findings suggest that CCTα exists in its membrane-bound active state in IEC-Ras and this is correlated with changes to nuclear morphology.

CCTα is known to be activated by type II lipids that induce membrane curvature and anionic lipids such as oleate [76]. Since it has been observed that IEC-Ras have increased CCTα expression and increased nuclear envelope localization, it was investigated if anoikis resistance could be conferred to IEC cells by overexpressing CCTα and/or increasing activity through oleate treatment (**Fig 3.1.8)**. The addition of oleate and/or the ~10-fold overexpression of CCTα did not have any effect on the anoikis-resistance of IEC. This suggests that the increased CCTα expression and activity is not sufficient for anoikis resistance displayed by IEC-Ras.

3.2 Knockdown of CCTα and its Effects on Anoikis Resistance in IEC-Ras and the CDP-Choline Pathway

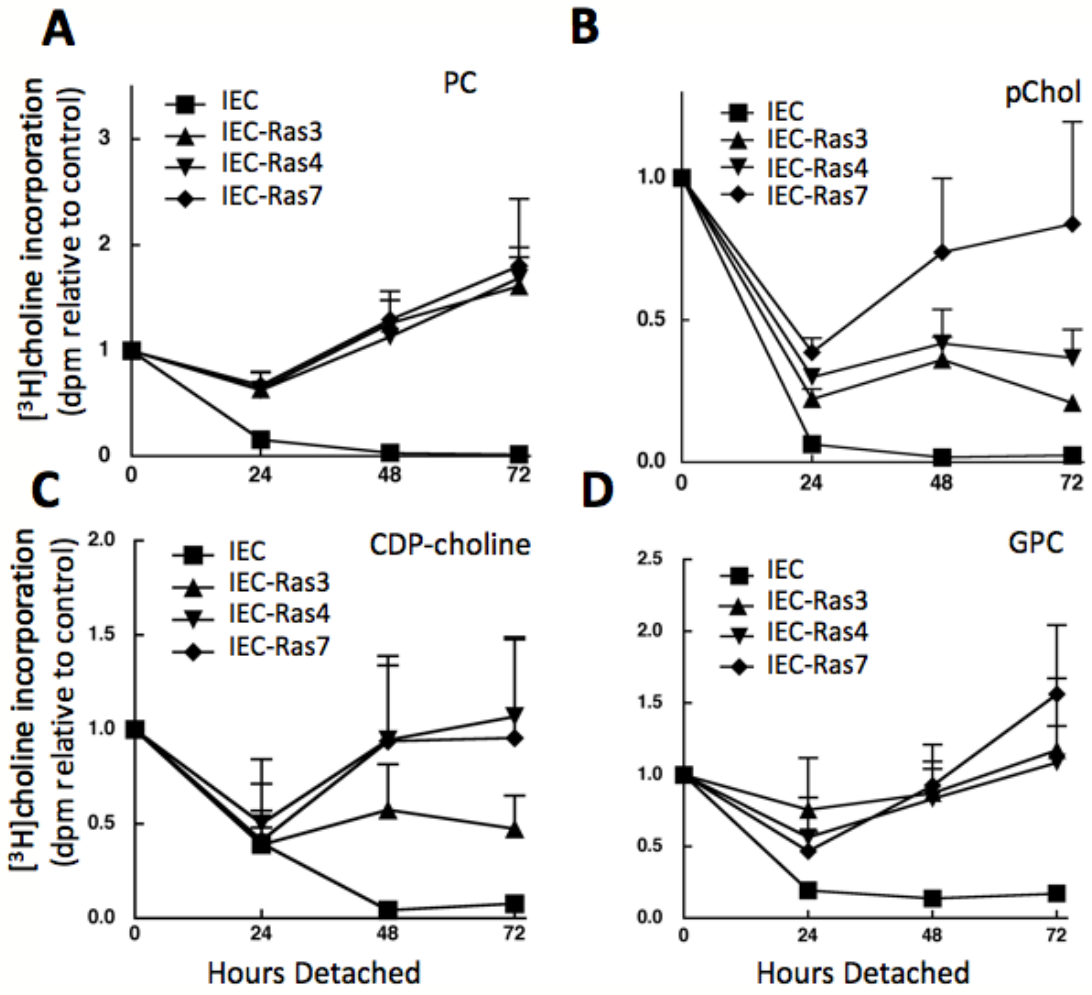

Figure 3.1.6: Increased [^{3}H]choline labeling of PC detached IEC-Ras. [^{3}H]Choline incorporation into PC **(A),** GPC **(B),** CDP-choline **(C)** and pChol **(D)** was measured in IEC and IEC-Ras as described in Section 2.7. IEC, and IEC-Ras cells were grown on plastic (control) on or SP-agarose coated dishes with [^{3}H]choline for the indicated time. The data presented is the average relative radioactivity from 5 separate experiments with error bars representing the SEM.

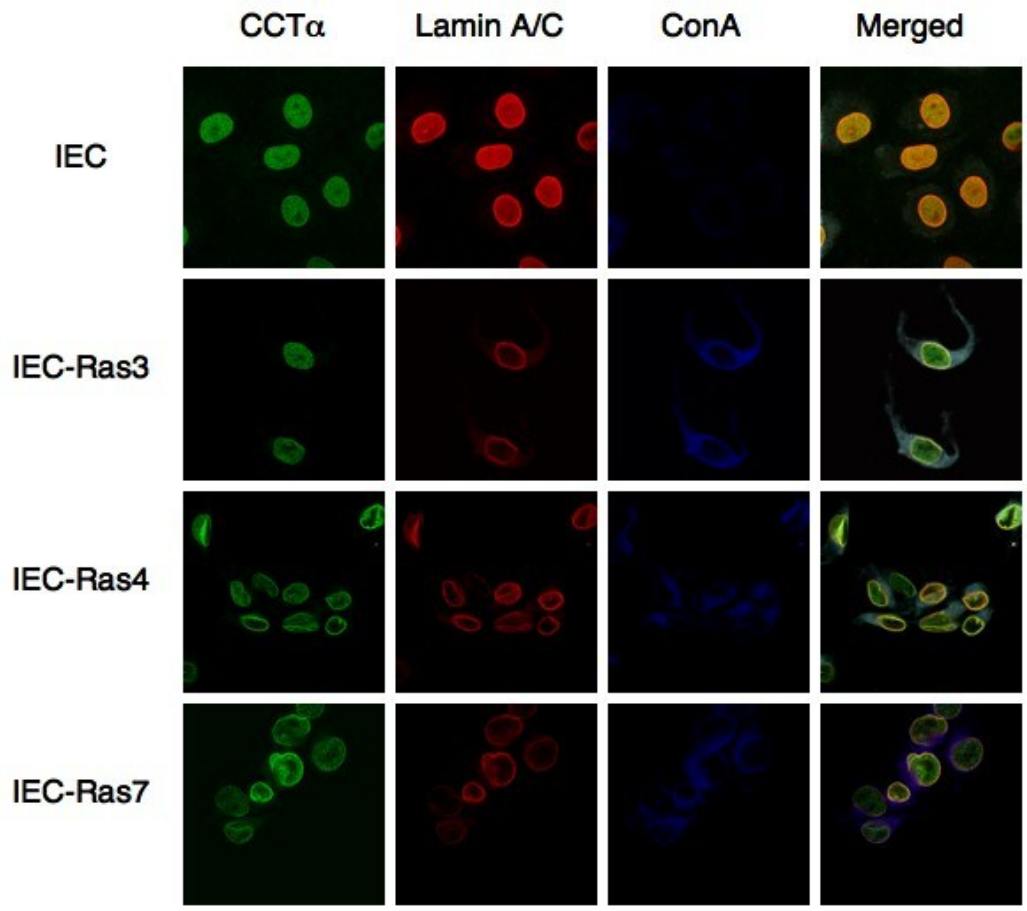

Figure 3.1.7: CCTα is localized to the nucleoplasm and nuclear envelope in IEC-Ras. Coverslips with IEC and IEC-Ras3, 4 and 7 cells were fixed, permeabilized and incubated with rabbit anti-CCTα antibodies diluted at 1:1000, goat anti-laminA/C antibodies diluted at 1:1000, and ConA-Alexaflour-633 used at 1:500. Cells were and mounted on slides to be viewed by confocal microscopy (Section 2.5).

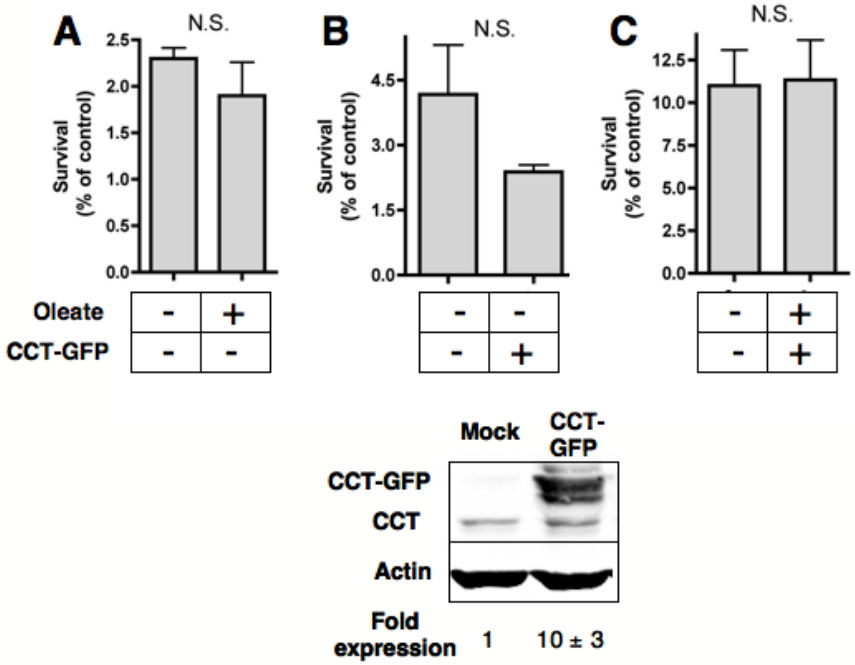

Figure 3.1.8: Activation and/or overexpression of CCTα does not grant anoikis resistance to IEC. IEC cells were grown to 70% confluency before being transfected with a plasmid coding CCTα-GFP (Section 2.3). Once seeded onto SP-agarose at 500 cells/60mm dish the media was supplemented with 300 μM oleate during 72 h detachment. All cells were processed by the clonogenicity assay following the indicated treatments (Section 2.8). Successful transfection of CCTα-GFP was confirmed by fluorescence microscopy and Western blotting. Quantification of overexpression relative to endogenous CCTα was done using densitometry analysis. Values presented are averages with SEM (N=3). All values are not significantly different (N.S.) from control.

To determine if the CDP-choline pathway plays a role in the anoikis resistance of IEC-Ras, and to investigate the role of CCTα and CCTβ expression of both isoforms were reduced by expression of short hairpin RNA (shRNA). The shRNA was delivered to the cell by lentivirus coding shRNA and puromycin resistance. This enabled the antibiotic selection of cells that had the desired protein knocked-down. Two shRNA were selected for each CCT isoform based on optimum knockdown of the protein as detected by Western blot (**Fig. 3.2.1 A)**. Knockdown of CCTα in IEC after transduction with shCCT 1-4 were 91%, 85%, 94%, and 55% respectively. Knockdown of CCTα in IEC-Ras after transduction with shCCT 1-4 were 78%, 0%, 74%, and 79% respectively. The knockdown of CCTα in IEC and IEC-Ras was most successful using shCCT1 and shCCT3 so these shRNAs were chosen for future experiments. Additionally, all IEC-Ras lines available were tested for knockdown using shCCT1 (**Fig. 3.2.1 C).** IEC-Ras4 was chosen for the remainder of this study as the level of CCTα expression after knockdown was similar to untreated IEC. Reduction of CCTα could have triggered the upregulation of CCTβ, however its expression in cells lacking CCTα was unchanged by transduction of shCCT1 (**Fig 3.2.1B**). Overall, subsequent knockdown experiments were performed using IEC and IEC-Ras4 with shCCT1 and shCCT3.

The first parameter tested after CCTα knockdown was the [^{3}H]choline incorporation into metabolites of the CDP-choline pathway. Without CCTα, the synthesis of PC should be reduced and pChol should be increased compared to cells expressing non-targeting shRNA. Finally, GPC levels should fall, reflecting reduced degradation as a result of decreased PC synthesized. When the metabolite levels in cells lacking CCTα were

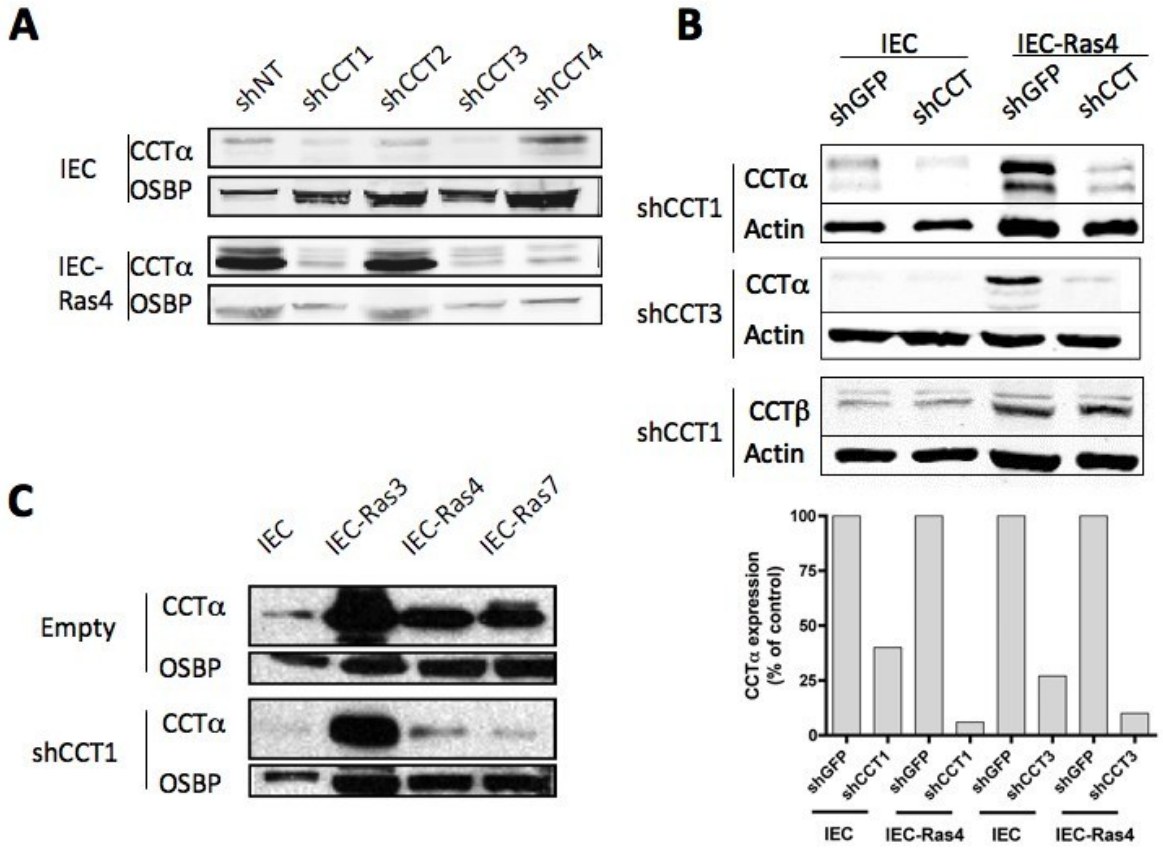

Figure 3.2.1: CCTα expression is reduced by transduction using lentivirus encoding shCCT. Lentivirus encoding shRNA against CCTα, non-targeting shRNA (shNT, control), shRNA against GFP (control) or empty shRNA (control) was applied to IEC and IEC-Ras cells and were then selected with puromycin for 72 h (Section 2.4). Cells were then harvested, lysed and immunoblotted against CCTα (1:2000), CCTβ (1:1000), and OSBP (1:10000) and visualized by **(A,B)** Odyssey Infrared Imaging System or **(C)** chemiluminescence (Section 2.6). Expression was measured using ImageJ densitometry. (**A**) All shRNA efficacies were tested after transduction in IEC and IEC-Ras4. (**B**) knockdown CCTα by shCCT1 and 3 was quantified compared to actin in IEC and IEC-Ras4. CCTβ expression is unchanged by shCCT1 transduction. (**C**) Transduction of shCCT1 reduced CCTα expression in all IEC cell lines.

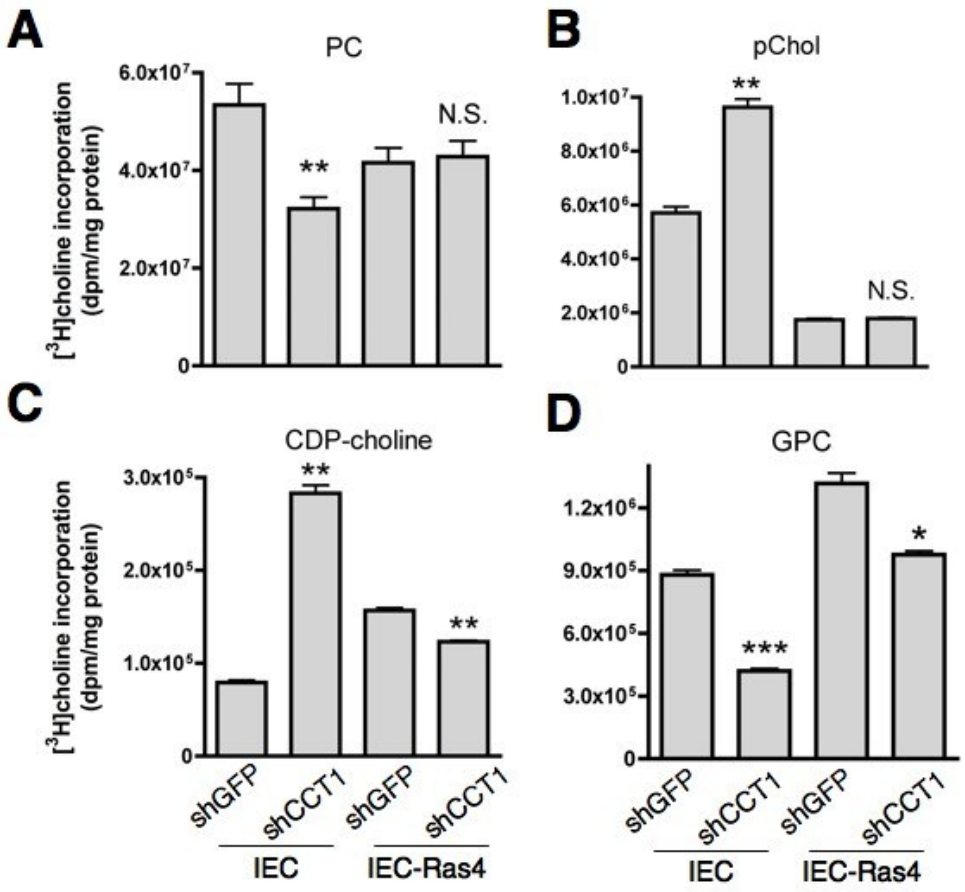

Figure 3.2.2: Knockdown of CCTα does not reduce PC synthesis in IEC-Ras4. IEC and IEC-Ras were transduced with shGFP, shCCT1 or shCCT3 were selected for 72 h in puromycin. Cells were then grown in choline depleted D-MEM for 2 h, followed by [H^3]choline labeling for 3 h. Cells were harvested and [^{3}H]choline incorporation into **(A)** PC, **(B)** GPC, **(C)** CDP-choline, and **(D)** pChol was measured and standardized to total protein. Data presented is the average of 3 independent experiments with error bars representing SEM. Values that are not significantly different are labeled N.S.

analyzed the levels of PC were reduced in IEC but not in IEC-Ras cells (**Fig 3.2.2A**). This could be because elevated CCTα expression in IEC-Ras may be sufficient to maintain PC levels even though 90% of CCTα expression is knocked down. The radiolabeled pChol pool was increased in IEC but similar in IEC-Ras4 compared to shGFP controls (**Fig 3.2.2B**). The levels of GPC are reduced following CCTα knockdown in IEC and IEC-Ras4 indicating that although PC levels are unchanged, degradation of existing PC has slowed **(Fig. 3.2.2D)**. Overall, reduction of CCTα expression had the predicted effect on incorporation in IEC, but IEC-Ras4 maintained similar PC synthesis while reducing PC degradation (GPC) slightly.

Experiments were then undertaken to test whether reduction of CCTα sensitized IEC-Ras to anoikis using the clonogenicity and soft agar assay in cell culture, as well as an *in vivo* tumour growth assay. The clonogenicity assay tests the ability for cells to form colonies after detachment for 0-72 h while the soft agar assay measures the ability to form colonies while detached. The subtle difference is that some transformed cells, such as BT549 breast carcinoma, undergo growth arrest in detached conditions to prevent death by apoptosis [178]. When these cell are placed in acceptable conditions their growth resumes [178]. In this situation, growth arrested cells would appear as colonies after transfer from SP-agarose and culturing on plastic dishes, but will not form colonies on soft agar. In this cell culture model, IEC-Ras cells lacking CCTα expression had approximately 50% survival in detached conditions compared to controls in both survival assays (**Fig 3.2.3 A and B)**. IEC cells were sensitive to anoikis in all cases as expected. These results, combined with the previous finding that CCTα knockdown in IEC-Ras causes no change to

PC synthesis, suggesting that CCTα is necessary for anoikis resistance independent of changes in PC synthesis and choline metabolites.

While the reduction of CCTα may sensitize cells to anoikis in a cell culture model, its effect in an *in vivo* system was also investigated. IEC-Ras4 cells expressing shCCT1 or shCCT3 were injected subcutaneously into the right flank of female 8-week-old BALB/c immunocompromised (athymic) mice (**Fig. 3.2.4**). The recorded tumour growth was not measured past day 12 as by that point many of the mice either displayed ulcerated tumours or other complications requiring euthanization. While early tumour volumes (~days 1-7) for IEC-Ras4 expressing shCCT1 and shCCT3 were similar in size, late tumours (~days 8-12) displayed a significant ~50% and ~25% reduction in tumour size. These results suggest that CCTα knockdown significantly reduces the growth of Ras-transformed cells in tumours.

While it is thought that CCTβ does not play a role in anoikis resistance, the effect of its reduction on cell survival under detached conditions and the activity of the CDP-choline pathway was tested. The selection of shCCTβ1 and shCCTβ2 was done through comparison of commercially available shRNA to the *rattus norvegicus* gene sequence for *pcyt1b*. Transduction of shRNA targeting CCTβ was done in an identical fashion to CCTα. IEC and IEC-Ras4 with reduced CCTβ expression did not have any difference in survival in detached conditions (**Fig. 3.2.5A)**. Confirmation of knockdown for the clonogenicity assay and following metabolic labeling assay was done by Western blot (**Fig. 3.2.5B).**

Metabolic labeling experiments using a 3 h pulse with [^{3}H]choline were performed in cells lacking CCTβ (**Fig. 3.2.6)**. There was no significant difference in incorporation any

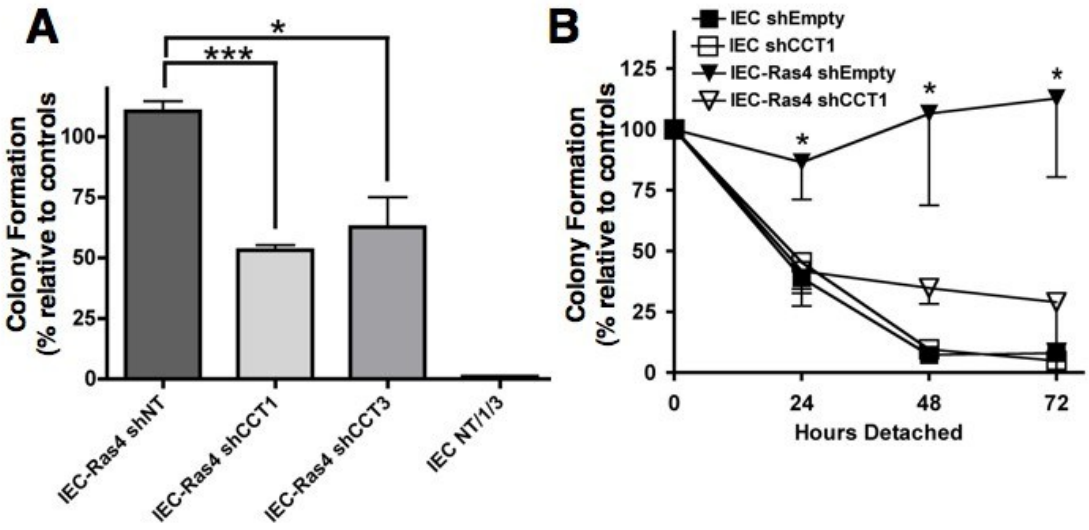

Figure 3.2.3: Knockdown of CCTα sensitizes IEC-Ras4 to anoikis. Lentivirus encoding shRNA against CCTα (1 or 3), empty shRNA or GFP (control) were applied to IEC-Ras cells and grown under selection with puromycin for 72 h. Cells were seeded at 500 cells/60mm dish on **(A)** 0.3% agar on 0.5 agar and colonies counted by microscope after 5 days (N=6) or **(B)** SP-agarose for 72 h before being transferred to plastic dishes, cultured for 3-4 days, stained, and colonies counted (N=6).

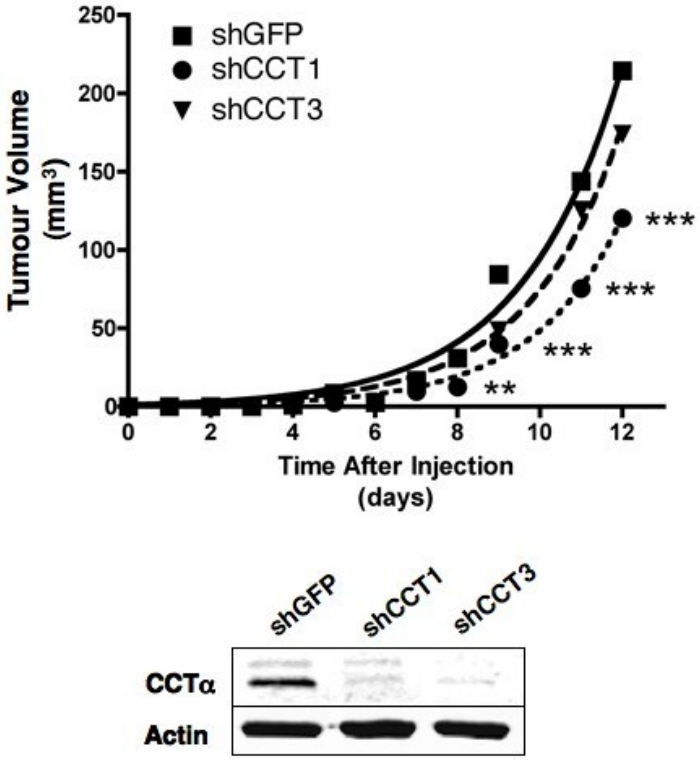

Figure 3.2.4: Tumour growth in mice is reduced upon CCTα silencing in IEC-Ras4. IEC-Ras4 ($2x10^5$ cells) expressing shCCT1, shCCT3 or shGFP were resuspended in sterile PBS and injected subcutaneously into the right flank of athymic mice (Section 2.10). Tumour growth was measured daily using a skin-fold caliper. Data presented is an average of 8-12 mice in 3 separate experiments. Error bars represent the SEM for each day. Both shCCT1 and shCCT3 were different from shGFP controls at the same level of significance at days 9, 11 and 12, at day 8 only shCCT1 was significantly different. A representative Western blot for CCTα knockdown is presented above.

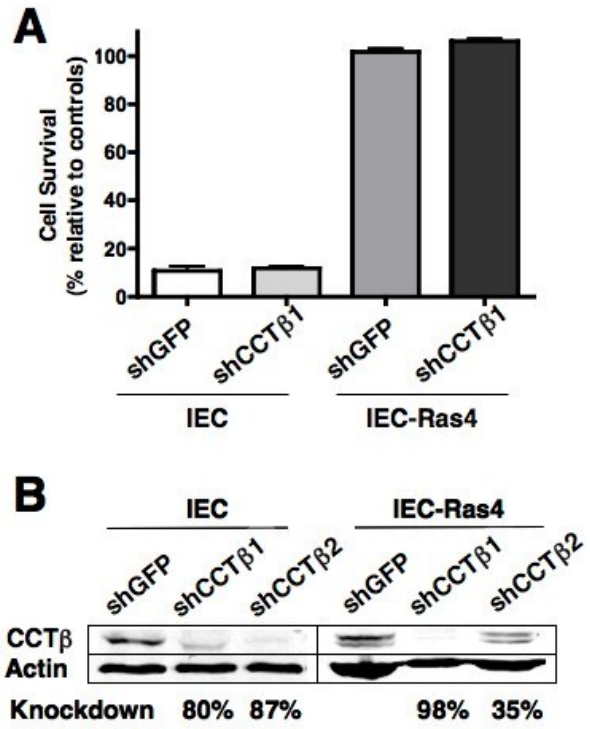

Figure 3.2.5: Knockdown of CCTβ does not sensitize IEC-Ras4 to anoikis. (A) IEC and IEC-Ras4 cells were seeded at 500 cells/60 mm dish. Cells were grown for 72 h on SP-agarose, before being transferred to plastic dishes to form colonies, stained and counted (Section 2.8). **(B)** IEC and IEC-Ras4 cells were infected with virus encoding shRNA against GFP or CCTβ, and selected for 72 h in puromycin. Cells were then lysed, resolved by SDS-PAGE and immunoblotted using CCTβ (1:1000) and actin (1:10000) antibodies. Protein expression was visualized and quantified with the Odyssey Infrared Imaging System.

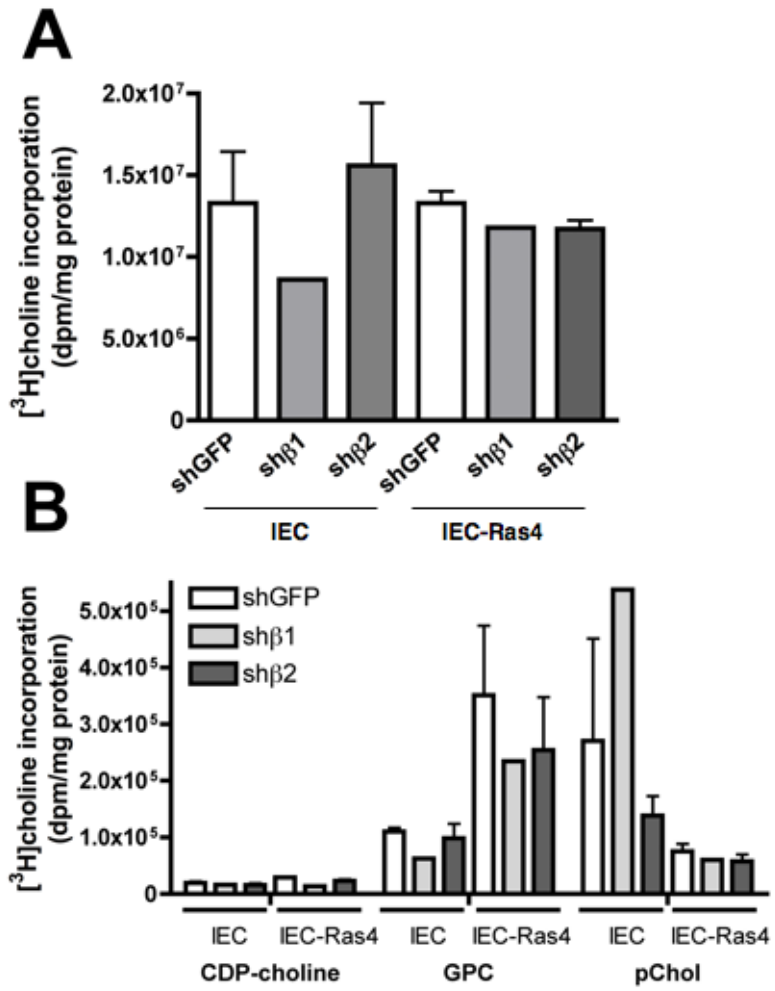

Figure 3.2.6: Synthesis of choline metabolites is not affected by the knockdown of CCTβ. IEC and IEC-Ras cells infected with virus encoding shGFP, or shCCTβ (1 or 2) were selected for 72 h in puromycin. [^{3}H]Choline incorporation into PC **(A),** GPC, CDP-choline and pChol **(B)** was measured in IEC and IEC-Ras as described in Section 2.7. The degree of knockdown was confirmed by Western blot (**Fig 3.2.5**). Data presented are the averages of 3 separate experiments with error bars representing SEM. Data for shCCTβ1 is from a single experiment.

of the metabolites between control and IEC and IEC-Ras4 expressing shCCTβ2. These results are not surprising as CCTβ expression is not upregulated in IEC-Ras4.

CHAPTER 4 DISCUSSION

4.1 IEC-Ras Have Increased Anoikis Resistance and CCTα Expression

Oncogenic H-Ras is expressed in many types of epithelial cancers. Although mutations of H-Ras and K-Ras are more common (50%) than N-Ras (~5%), each mutated isoform contributes to the transformation of cells [179]. H-Ras and K-Ras increase lesion formation by promoting proliferation and suppression of differentiation, while N-Ras does so through resistance to apoptosis [179-181]. Knockdown or genetic disruption of Ras proteins reduces the tumourigenic potential of cells through sensitization to apoptosis [179, 182, 183]. As oncogenic H-Ras is required for transformation, manipulation of its downstream effectors may attenuate cancer growth.

Many enzymes are upregulated in cancer cells but of particular interest to this study are lipogenic enzymes like choline kinase, FASN, ATP-citrate lyase and enzymes involved in glycolysis such as GLUT and hexokinase [134, 141, 184]. Ras-transformation results in increased pChol metabolism through upregulation of choline kinase via the downstream activators PI3K and AKT [139, 141, 144, 185]. FASN production of fatty acids is also an essential requirement of transformed cells, as reduction of its activity inhibits tumour growth [134]. Glycolytic enzymes are required for tumour growth in hypoxic environments where the vasculature cannot continue to supply oxygen to support oxidative phosphorylation [184].

The IEC-Ras clones used in this study are capable of growth in detached conditions and exhibited increased CCTα expression (**Fig 3.1.1 and Fig 3.1.2**). The overexpression of CCTα in IEC-Ras is hypothesized to correlate with increased generation of PC needed to

sustain membrane synthesis and generation of signaling lipids like DAG, fatty acids and PA [186]. Although I did not measure choline kinase in this study, concurrent upregulation might be expected in order to supply pChol required by additional CCTα activation (**Fig. 3.1.3 and Fig. 3.1.4**). However, PC levels are not increased in IEC-Ras as measured by [^{3}H]choline incorporation. Instead IEC and IEC-Ras compared have similar [^{3}H]PC levels with reduced [^{3}H]pChol and increased [^{3}H]GPC (**Fig. 3.1.3 and Fig. 3.1.4**), indicating that the synthesis of PC may be increased, but the amount of PC is unchanged since degradation is also increased. Ras-transformed fibroblasts (NIH-3T3) have also been shown to have upregulated CCTα expression coupled with increased degradation of PC to GPC [185, 187]. The degradation of PC to GPC also results in the generation of fatty acids, an activator of CCTα and substrate for other lipid biosynthetic pathways. Thus, there seems to be a futile cycle of increased PC synthesis and turnover that could be driven by upregulation of the biosynthetic or degradative arm of the cycle. Overexpression of PC-PLC in NIH-3T3 cells results in the sustained activation of ERK1 and 2 in the absence of growth signals, suggesting that degradation alone could drive cell proliferation. In addition, expression of a dominant negative PLC abolished this [187]. PLC generation of DAG from PC is required in Ras-transformed cells to maintain proliferative signals [188, 189]. This suggests that the constant generation and degradation PC supplies the cancer cell with membrane components, as well as signaling mediators.

IEC-Ras cells have decreased [^{3}H]pChol incorporation resulting from increased expression of CCTα. IEC display relatively increased [^{3}H]choline incorporation into pChol indicating that CCTα is less active in IEC causing a bottleneck in the CDP-choline pathway (**Fig. 3.1.3 and Fig. 3.1.4**). The incorporation of [^{3}H]choline into CDP-choline is

relatively low in comparison to other metabolites resulting in variable measurements. This is because CPT quickly converts CDP-choline produced by CCTα to PC. Therefore, the influence of cellular transformation on CDP-choline levels cannot be properly analyzed by isotope incorporation.

Although there is evidence for CCTα activation and increased PC synthesis the enzyme exists in a more phosphorylated state when cultured under detached conditions (**Fig. 3.1.2; Fig. 3.1.5**). Even though phosphorylation has a negative effect on CCTα activity, overall increased expression and membrane binding may be enough in IEC-Ras to counter these effects (**Fig. 3.1.6**). In HeLa cells, increased phosphorylation of CCTα did not significantly reduce membrane binding and localization under oleate activated conditions [81]. This data suggests that although CCTα may be highly phosphorylated in detached conditions, activation through membrane association counteracts this inhibition.

This study has shown that the expression of oncogenic H-Ras in IEC cells causes the upregulation of CCTα but not CCTβ (**Fig. 3.1.2)**. Although CCT isoforms have the same enzymatic activity, the difference in localization could result in different levels of activity. If the catalytic activity of CCT were required for transformation, then one would anticipate that both the α and β isoforms would be upregulated in IEC-Ras. If the localization of the CCT isoform is a factor in transformation, then only one isoform would be specifically upregulated. Based on this reasoning and the finding that H-Ras transformation only has effects only on CCTα expression and activity, its role in transformation is likely reliant on localization and not catalytic activity.

Overexpression and activation of CCTα did not result in any resistance to anoikis in IEC (**Fig. 3.1.7)**. The inability of CCT alone to induce anoikis resistance was not surprising

since modulating the expression of other proteins downstream of Ras, such as PI3K in RIE-1 kidney epithelial cells, does not sensitize cells to anoikis [190]. The proteins and pathways mutated in each cancer cells are different and often involve many bifurcating pathways. For example, unlike IEC-Ras, DLD-1 K-Ras transformed human colorectal cells do not have activated PI3K and AKT [190]. The increase of CCTα expression and potentially flux through the CDP-choline pathway is not sufficient to induce resistance to anoikis as activation of multiple signaling pathways is required to maintain an oncogenic phenotype [154, 191].

4.2 Reduction of CCTα Expression Results in Anoikis Sensitivity

A logical approach to combat any disease is to downregulate or remove factors that promote disease. The knockdown or inhibition of signaling or metabolic enzymes that are upregulated in cancer cells has been the main strategy in the development of chemotherapeutics. The small molecule cerulenin (C57) is one such example that inhibits FASN and reduces tumour growth [192]. Similar pleiotropic effects of inhibiting Ras downstream targets would also be valuable to combat tumourigenesis [193]. Perhaps because CCTα is upregulated in many types of cancer, its reduction will also reduce or prevent cancer (**Fig. 4.1)** [194].

In this study, shRNAs specific for CCTα were used to reduce expression (**Fig 3.2.1**). The knockdown of CCTα in IEC-Ras4 resulted in expression similar to that of IEC, so it was chosen as a representative cell line for the remainder of the knockdown experiments. This meant that if CCTα was a pivotal factor in anoikis resistance, its knockdown in IEC-Ras4 should result in cells that are phenotypically similar to IEC. The

Gene	Fold Change	P-value
PCYT1A	4.38	1.25E-09
PCYT1B	1.58	0.078
ACTA1	-1.19	0.898
ACTA2	-1.73	0.995

Figure 4.1: Pcyt1a is upregulated in human cancer cells. mRNA levels for human *pcyt1a* and *pcyt1b* were compared between normal and cancer cells using the Oncomine online database [194]. The genes encoding actin *acta1* and *acta2* were used as a control.

reduction of PC synthesis has previously been shown to cause growth arrest and apoptosis in MT58 cells that do not express CCTβ, therefore the knockdown of CCTα could cause anoikis sensitization due to loss of PC [64]. Interestingly, CCTα silencing did not reduce PC synthesis in IEC-Ras4 (**Fig. 3.2.2A**). However, flux through the CDP-choline pathway may be reduced since there is a decrease in the amount of [^{3}H]GPC produced in both IEC and IEC-Ras knockdown cells (**Fig. 3.2.2D**). The levels of [^{3}H]pChol are increased in IEC lacking CCTα, showing that CCTα activity is reduced, causing a bottleneck in the CDP-choline pathway (**Fig. 3.2.2B**). This maintenance of PC synthesis in IEC-Ras4 after reduction of CCTα expression may be explained by a reserve of soluble inactive CCTα in the nucleus and expression of CCTβ [64]. As hypothesized earlier, CCTβ may be able to provide sufficient catalytic activity to maintain PC and could compensate for the loss of CCTα. When CCTα was knocked down, there was no compensation by upregulation of CCTβ (**Fig. 3.2.1B)**. This suggests that PC synthesis would be reduced in IEC-Ras4 only after reduction of CCTα below the wild-type threshold.

Although the knockdown of CCTα had little effect on PC synthesis, it had a significant effect on anoikis resistance (**Fig. 3.2.3**). When anoikis resistance was tested by the clonogenicity and soft agar growth assays it was found that IEC-Ras expressing shCCTs had approximately 50% less colony formation compared to shNT or shGFP controls. This is particularly intriguing as the levels of [^{3}H]choline incorporation into PC are unchanged. To further illustrate this effect, IEC-Ras cells with shCCT1, shCCT3 or shGFP were injected into athymic mice and tumour growth was measured. In this assay as well, the loss of CCTα expression resulted in approximately 40% less tumour growth (**Fig.**

3.2.4). These results suggest that although IEC-Ras cells do not have deficient PC production, the loss of CCTα induces anoikis sensitivity.

The expression of CCTβ was not upregulated in IEC-Ras cells, but in order to investigate if it was a factor in anoikis-resistance expression was reduced by an shRNA. The knockdown of CCTβ knockdown was shown to have no effect on IEC or IEC-Ras survival in detached conditions (**Fig. 3.2.5**). The rate of PC synthesis in CCTβ knockdown cells was also investigated and did not have any significant effect on metabolic labeling of PC or other CDP-choline pathway metabolites (**Fig. 3.2.6**). This suggests that CCTα and CCTβ have overlapping and perhaps redundant activity with respect to PC synthesis, but unique activities beyond just catalysis in the CDP-choline pathway.

One possible explanation of these results is that elevated expression of CCTα in IEC-Ras does not serve a metabolic function, but instead plays a role in the nucleus, perhaps in the formation of the nucleoplasmic reticulum. The nucleoplasmic reticulum is often found in cancer, and rapidly dividing cells [195, 196]. The disruption of the nucleoplasmic reticulum by reduction of lamin A/C expression does not alter the synthesis of lipids, specifically PC [167]. Therefore reducing the amount of CCTα may affect anoikis resistance not by affecting PC synthesis but through a change in nuclear morphology [162, 197]. As nuclear morphology is altered in IEC-Ras by CCTα, and CCTα is upregulated upon H-Ras transformation, the transformed phenotype could be linked to nucleoplasmic reticulum formation, enhanced nuclear signaling and manipulation of cell cycle progression [78, 196].

4.3 Conclusion

IEC-Ras have increased expression and activation of CCTα in the absence of CCTβ upregulation. Reduction of CCTα expression in IEC-Ras4 results in sensitization to anoikis without changes to PC synthesis. As CCTα is a nuclear localized protein involved in formation of the nucleoplasmic reticulum, it is thought that the knockdown of CCTα could sensitize cells to anoikis by its absence from the nucleus. This paradigm also explains why knockdown of CCTβ has no effect as it is not present in the nucleus (**Fig. 4.2**).

The knockdown of CCTα did not fully sensitize IEC-Ras cells to anoikis in cell culture or *in vivo*. This is because oncogenic H-Ras regulates multiple proliferative pathways of which CCTα is only one component. Alternatively, increased activation of CCTα cannot grant anoikis resistance to normal IEC, since transformation into a cancer cell requires mutations in more than a single downstream effector. Overall these data indicate that CCTα does not grant anoikis resistance but is necessary for the maintenance of H-Ras transformation of IEC-Ras cells.

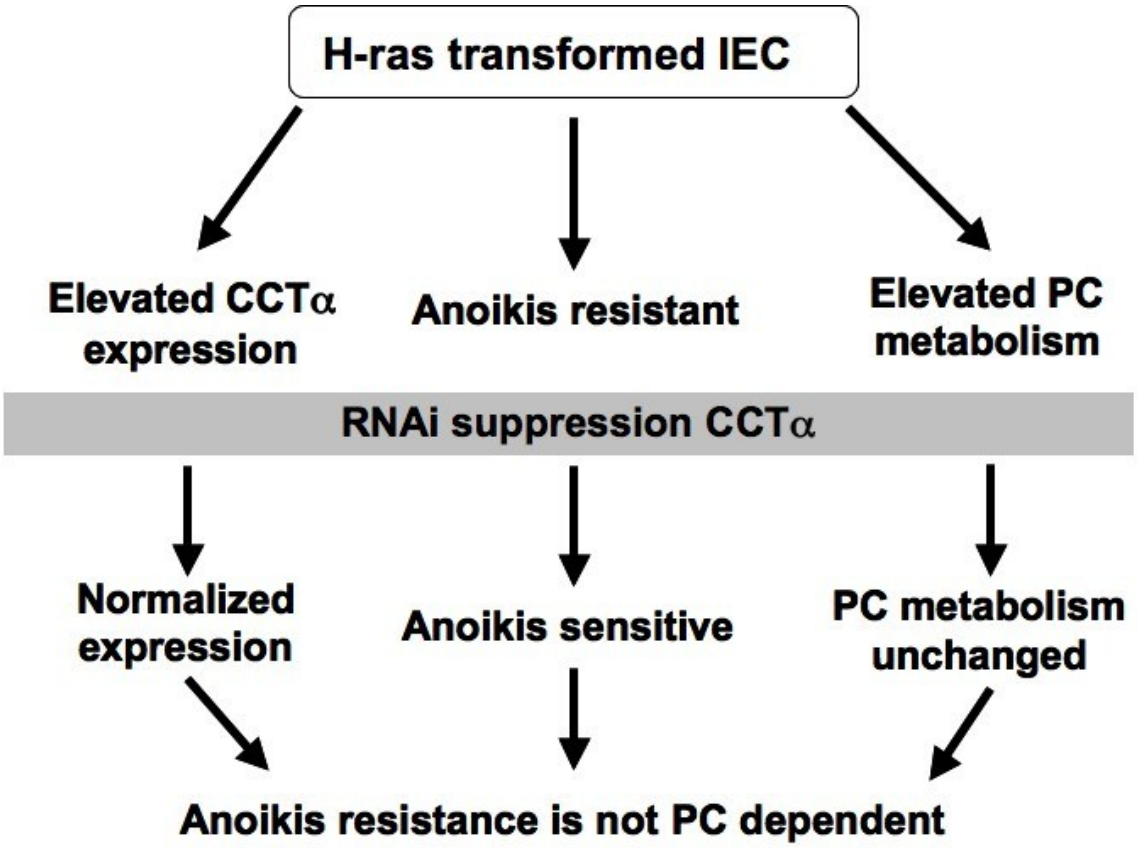

Figure 4.2: **Anoikis resistance of IEC-Ras is not PC dependent.** H-Ras transformed IEC display increased CCTα expression without any change in CCTβ expression. IEC-Ras are anoikis resistant and have slightly elevated PC metabolism. Following reduction of CCTα expression by RNAi, IEC-Ras have similar CCTα expression to that of IEC. This results in anoikis sensitivity in the absence of changes to PC metabolism. Therefore, anoikis resistance is not PC dependent.

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