

ROLE OF C/EBP β ON QUIESCENCE-SPECIFIC GENE EXPRESSION IN CEF

**CHARACTERIZING THE ROLE OF
CCAAT/ENHANCER BINDING PROTEIN β
ON QUIESCENCE-SPECIFIC GENE EXPRESSION
IN CHICKEN EMBRYO FIBROBLASTS**

By

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Descriptive Note

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Abstract

CCAAT/enhancer binding protein (C/EBP) family of transcription factors regulate cellular fates such as proliferation and differentiation. During conditions like serum-depletion or contact inhibition, cells can exit the cell cycle and enter a state of quiescence. Our studies have focused on characterizing the role of the C/EBP family in cellular survival and quiescence in chicken embryonic fibroblasts (CEF).

Treating CEF with a dominant negative mutant of C/EBP β (designated $\Delta 184$ -C/EBP β) increased cell survival during prolonged starvation. These cells also developed large autophagosomes characteristic of autophagy, a process cells utilize to acquire energy by degrading their own organelles. In yeast, a key mediator of autophagy is ATG8, which is essential for autophagosome formation and maintenance. Starved CEF expressing $\Delta 184$ -C/EBP β had elevated levels of a vertebrate homologue of ATG8, γ -aminobutyric acid receptor-associated protein (GABARAP). Hence, we attempted to determine whether GABARAP plays a role in regulating cellular survival in starved CEF. However, over-expression and shRNAi studies indicate that GABARAP is not required for survival but instead may regulate apoptosis in CEF during starvation.

We also attempted to determine the effects of the C/EBP family in quiescence-specific gene expression. p20K is a well-characterized quiescence-specific gene in CEF, whose expression is regulated by C/EBP β and cellular density. Recent findings suggest that p20K expression is negatively regulated by another member of the C/EBP family, CHOP10. Gene-profiling studies also discerned that contact-inhibited CEF display a signature of hypoxia, where genes such as HIF1 α and carbonic anhydrase IX are activated.

Further analyses show that hypoxia can activate p20K expression in a C/EBP β -dependent manner and this induction is repressed by CHOP10. Additionally, HIF1 α may also be regulated by C/EBP β , since its dominant negative mutant repressed HIF1 α expression in CEF treated with hypoxia mimetics. Therefore, these findings suggest the existence of a C/EBP β -HIF1 α axis in the regulation of p20K expression in CEF.

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List of Abbreviations

AMP	Adenosine Monophosphate
ANOVA	Analysis of Variance
ApoAI	Apolipoprotein A-1
AAR	Amino Acid Response
AARE	Amino Acid Response Element
Arf	ADP-ribosylation Factor
ARF	Alternative Reading Frame
ARNT	Aryl-Hydrocarbon Receptor Nuclear Transporter
AP-1	Activator Protein-1
ATF	Activating Transcription Factor
ATG	Autophagy Related Gene
APP	Alzheimer's β -amyloid precursor protein
BAK	Bcl-2 homologous antagonist/killer
bHLH	Basic Helix-Loop-Helix
BH3	BCL-2 Homology Domain 3
BIRC2	Baculoviral IAP repeat-containing 2 gene
Bmp2	Bone Morphogenetic Protein 2
BNIP3	Bcl-2/ adenovirus E1B 19 kDa-interacting protein 3
bZIP	Basic Leucine Zipper
CA	Carbonic Anhydrase
Ca ²⁺	Calcium Ions
CBP	CREB Binding Protein
CDC	Cell Division Cycle
CDK	Cyclin-Dependent Kinase
cDNA	copy DNA
C/EBP	CCAAT Enhancer Binding Protein
CEF	Chicken Embryo fibroblasts

CHM	Chicken Heart Mesenchymal
CHOP10	C/EBP Homologous Protein 10
cTAP2	Cellular inhibitor of apoptosis 2
C.I.	Contact Inhibition
CIP1	CDK Interacting Protein 1
CKI	CDK Inhibitor
CMA	Chaperone-mediate autophagy
CoCl ₂	Cobalt Chloride
CRE	cAMP responsive elements
C-TAD	C-terminal Transactivation Domain
DAPK	Death Associated Protein Kinase
DAPI	4',6-Diamidino-2-Phenylindole
DKK1	Dickkopf 1
DDX	DEAD box polypeptide
DMOG	Dimethyloxalylglycine
DNA	Deoxyribonucleic Acid
DOC	Downstream of CHOP
ECM	Extracellular Matrix
eIF2 α	Eukaryotic Translation Initiation Factor 2 α
EM	Electron Microscopy
EOR	ER overload response
ER	Endoplasmic Reticulum
ERSE	ER stress response elements
ERAD	ER Associated Degradation Protein
ERK	Extracellular signal-regulated kinase
ERO1 α	ER oxidase 1 α
FABP4	Fatty Acid Binding Protein 4
FIH	Factor Inhibiting HIF
G ₀	Reversible Growth-Arrest

GADD	Growth-Arrest and DNA Damage-Inducible
GAS	Growth Arrest-Specific
GABARAP	γ -aminobutyric Acid Receptor A associated Protein
GATE-16	Golgi ATPase Enhancer 16
GFP	Green Fluorescent Protein
GS3K β	Glycogen synthase kinase 3 β
HDAC	Histone Deacetylase
HES	Hairy/enhancer-of-split
HEY	Hes-related with YRPW motif
HIF	Hypoxia Inducible Factor
HRE	Hypoxia Response Elements
ID	Inhibitors of DNA Binding
IL	Interleukin
INK4	Inhibitors of Kinase 4
IRE-1	Inositol requiring 1
JNK	c-Jun N-terminal Kinase
KIP	Kinase Inhibitor Protein
LAP	Liver Activating Protein
LIP	Liver Inhibitory Protein
LTR	Long Terminal Repeat
MAF	Musculoaponeurotic Fibrosaroma
MAP1LC3	Microtubule Associated Protein Light Chain 3
MAPK	Mitogen Activated Protein Kinase
MDM2	Mouse Double Minute-2
MEF	Mouse Embryo Fibroblasts
MEM	Minimal Essential Medium
MCE	Mitotic Clonal Expansion
mRNA	Messenger RNA
mTOR	Mammalian Target of Rapamycin

NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NICD	Notch Intracellular Domain
NOXA	D-nopaline dehydrogenase A
N-TAD	N-terminal Transactivation Domain
ODD	Oxygen Degradation Domain
PAS	PER-ARNT-SIM
PBS	Phosphate Buffer Saline
PDGF	Platelet-derived growth factor
PDGF α R	Platelet-derived growth factor α receptor
PE	Phosphatidylethanolamine
PERK	PRK-like ER stress kinase
PGK-1	Phosphoglycerate Kinase-1
PHD	Prolyl Hydroxylases
PO	Propylene Oxide
PI3K	Phosphatidyl Inositol 3 Kinase
PCAF	p300/CBP Associated Factor
PLIER	Probe Logarithmic Intensity Error
PPAR γ	Peroxisome Proliferator-Activated Receptor γ
pRb	Retinoblastoma Protein
PUMA	p53 Up-regulated Modulator of Apoptosis
REDD1	Regulated in Development and DNA damage responses 1 protein
ROS	Reactive Oxygen Species
RSK	Ribosomal protein S-6 Kinase
RSV	Rous Sarcoma Virus
RXR	Retinoid X Receptor
SARPs	Secreted Apoptosis Related Proteins
SDS	Sodium Dodecyl Sulfate
SIM	Single Minded Protein
SNAT2	Sodium-dependent neutral amino acid transporter 2

Snf1p	AMP-activated protein kinase
Src	Sarcoma
SRU	Src-responsive unit
TdT	Terminal deoxynucleotidyl Transferase
TNF α	Tumour necrosis factor α
TUNEL	Terminal deoxynucleotidyl Transferase dUTP Nick-End Labeling
Tzb	Trastuzumab
QRU	Quiescence-Responsive Unit
RCASBP	Replication Competent ALV LTR with a Splice Acceptor Bryan Polymerase
RNA	Ribonucleic Acid
RNAi	RNA Interference
shRNA	Short Hairpin RNA
TAD	Transactivation Domain
TGF β 3	Transforming Growth Factor β 3
TOR	Target of Rapamycin
SFRP	Secreted Fizzled Related Protein
SNAT2	Sodium-dependent Neutral Amino acid Transporter 2
UPR	Unfolded Protein Response
VEGF	Vascular Endothelial Growth Factor
VHL	von Hippel Lindau
XBP-1	X-box binding protein -1

Literature Review

Overview

The following literature review covers topics that are discussed in my Masters thesis. The first section of the introduction addresses the process of cell cycle arrest with a particular emphasis on reversible growth arrest or G_0 . The next section describes the activities of the CCAAT/Enhancer Binding Protein (C/EBP) family of transcription factors and their regulation of various cellular processes. Subsequent sections give a brief description of the hypoxia response pathway and the process of autophagy.

1. Cellular growth arrest

Cells have to make critical decisions regarding the entry to or exit from the cell cycle. Different environmental factors can induce cycling cells to exit the cell cycle into one of several forms of growth arrest [reviewed in (Malumbres and Barbacid, 2001)]. Depending on the initiating signal, cells can either go into quiescence (G_0), a reversible form of growth arrest, or they can enter irreversible forms of growth arrest like senescence or terminal differentiation (Malumbres and Barbacid, 2001). Cells growth-arrested in G_0 , in response to anti-proliferative signals, are said to be reversible as they can re-enter the cell cycle when conditions are favourable [Fig.1; (Malumbres and Barbacid, 2001)]. In response to excessive stress caused by DNA damage, telomere shortening or oxidative stress, cells can growth arrest by entering a state of senescence (Blagosklonny, 2006; Itahana et al., 2002). Signaling pathways, such as the retinoic acid receptor pathway, can also induce cells to permanently growth arrest by undergoing terminal-differentiation (Langmann et al., 2005). Terminally-differentiated and senescent cells are

different from 'quiescent cells' in that they are incapable of re-entering the cell cycle and thus are said to be in a state of irreversible growth arrest (Noda et al., 1994; Poupponnot et al., 1995). However, if stressful conditions render cells irreparably damaged, they can bypass growth arrest and undergo apoptosis (Li et al., 2006).

1.1 Properties of cells in growth-arrest

Quiescent cells are different from cycling cells in many ways; they do not synthesize DNA or have mitotic spindle formation (Coller et al., 2006). Eukaryotic and prokaryotic quiescent cells can also exist through extended intervals of nutrient deficiency without undergoing apoptosis [reviewed in (Gray et al., 2004)]. In yeast, quiescent cells do not undergo cell division and have reduced levels of transcription and protein synthesis (Gray et al., 2004). These cells are also autophagic and have thicker cell walls, thus making them more resilient towards environmental stresses. Moreover, the expression of a particular subset of genes is repressed in these cells, while another subset is activated (Gray et al., 2004). For example, genes that code for ribosomal proteins are repressed, and stress-related genes, such as heat shock protein *HSP26* and ubiquitin coding *UBI4*, are activated (Gray et al., 2004). Similarly, in human fibroblasts, genes stimulated in response to serum-addition are repressed following serum-starvation, while genes activated by starvation are repressed by serum-addition. Genes activated following serum-stimulation include transcription factors involved in promoting proliferation and RNA metabolism, such as FOS and MYC (Liu et al., 2007). In contrast, genes induced upon serum-starvation were comprised of components that regulate immune responses (e.g. IL6), metabolism of extracellular matrix (ECM) (e.g. laminin α 4), and growth-inhibitory

factors (e.g. Myc-antagonist MXI1) (Liu et al., 2007). Additionally, these serum-deprivation induced genes are not activated by other stresses such as ER stress, oxidative stress, or heat shock, thus reiterating that the regulation of these genes is specific to the response to serum-withdrawal (Liu et al., 2007).

Senescent cells are similar to quiescent cells in terms of having no cell division and being resistant to apoptosis [reviewed in (Campisi et al., 1996)]. They also have similar programs of gene expression in response to growth factor stimulation. For example, quiescent and senescent human fibroblasts had elevated levels of a negative regulator of the cell cycle, the cyclin-dependent kinase inhibitor p21. p21 levels were further increased following mitogenic stimulation (Noda et al., 1994). However, unlike quiescent cells, senescent cells were unable to re-enter the cell cycle following mitogenic stimulation (Noda et al., 1994). The inability of senescent fibroblasts to re-enter the cell cycle is partly due to increased levels of p21, which in response to mitogen-stimulation remain elevated with respect to levels in proliferating cells (Noda et al., 1994). Whereas, serum-stimulation of cells quiescent by starvation caused a reduction in p21 expression to levels seen in proliferating cells prior to S-phase entry (Noda et al., 1994). In contrast to quiescent cells, senescent cells also possess distinct characteristics such as a flat and enlarged morphology, accumulation of focal adhesions, increased β -galactosidase activity, and formation of heterochromatin foci (Rastogi et al., 2006).

1.2 Regulation of different forms of growth arrest

The cell cycle is sustained by the activities of complexes consisting of cyclin dependent kinases (CDKs), like CDK4, CDK6, and CDK2, and their regulatory subunits,

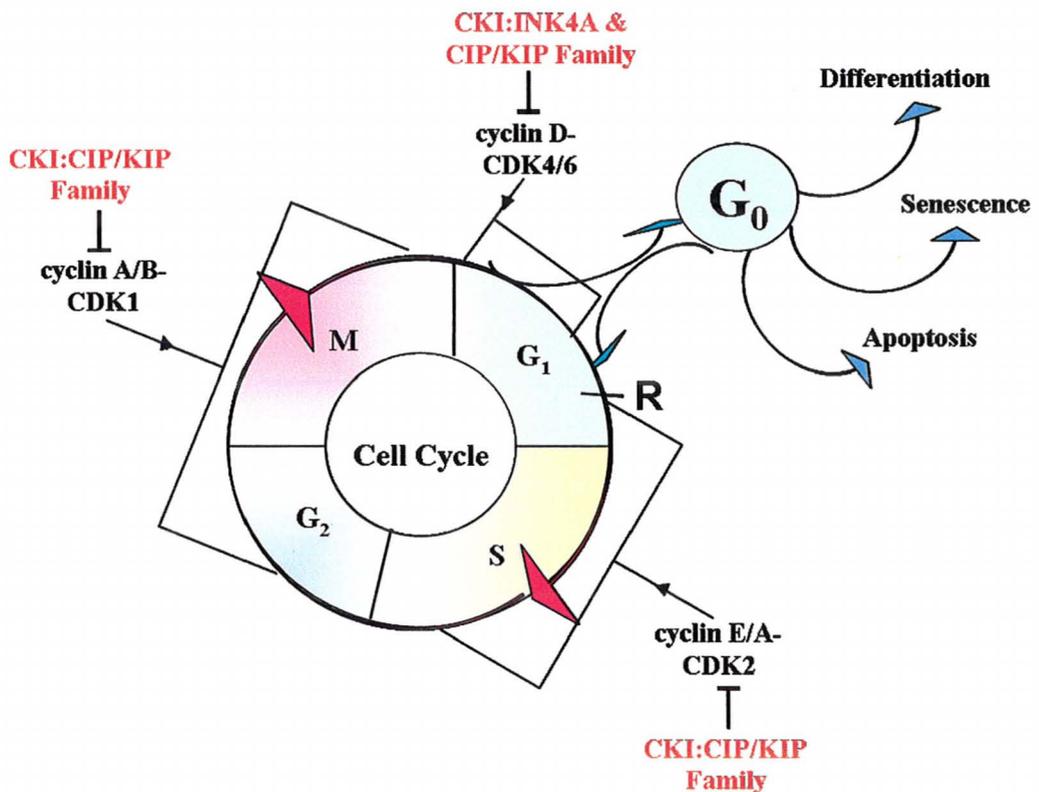
the cyclins, such as cyclin D, cyclin E, and cyclin A [Fig.1; reviewed in (Malumbres and Barbacid, 2001)]. The CDK4/6 forms complexes with D-type cyclins to regulate the early G1 phase, while CDK2-cyclin E complexes promote the G1 to S-phase transition [Fig 1; (Malumbres and Barbacid, 2001)]. The activity of CDKs can be inactivated by CDK inhibitors (CKIs). CKIs include members of the CDK interacting protein or kinase inhibitory protein (CIP/KIP) family, namely p21, p27 and p57, and members of the inhibitors of kinase 4 (INK4) family that consists of p15, p16, p18, and p14 [Fig.1; (Chassot et al., 2008; Pajalunga et al., 2007; Serrano et al., 1993; Xiong et al., 1993). Alternative splicing of the same locus on human chromosome 9p21 gives rise to CKIs p16 and p14 (Quelle et al., 1995). p14 is also known as alternative reading frame (ARF) protein and is referred to as p19 in mice (Quelle et al., 1995). The CIP/KIP family blocks the activity of all cyclin-CDK complexes, including the cyclin D-CDK and cyclin E-CDK complexes (Chassot et al., 2008; Pajalunga et al., 2007; Xiong et al., 1993). However, the activity of the INK4 family is more specific in that they exclusively inhibit the activity of CDK4 and CDK6 complexes [reviewed in (Canepa et al., 2007); (Serrano et al., 1993)].

Other important regulators of cell cycle arrest and apoptosis are the tumour suppressors, retinoblastoma protein (pRB) and p53. pRB forms complexes with the E2F transcription factors, which inhibits their ability to induce the transcription of genes required for S-phase entry, like cyclin E [reviewed in (Vidal and Koff, 2000)]. pRB is sequentially phosphorylated by the CDK4/CDK6-cyclin D and CDK2-cyclin E complexes, which induce the disassociation of the pRB-E2F complexes (Connell-Crowley et al., 1997; Lundberg and Weinberg, 1998). This releases E2F to promote cell cycle progres-

sion by inducing gene expression for S-phase entry (Vidal and Koff, 2000). CKIs can regulate the activity of pRB. For example, p16 and p21 inhibits the activity of CDK4 and CDK6, thereby allowing pRB to remain bound to E2F and promote cell cycle arrest (Serrano et al., 1993; Xiong et al., 1993).

p53 is a transcription factor that can induce cell-cycle arrest or apoptosis in response to different cellular stresses such as DNA damage, aberrant cell proliferation, and hypoxia [reviewed in (Hammond and Giaccia, 2005; Meek, 2004)]. p53 expression and function is negatively regulated by an E3 ligase, MDM2 (Meek, 2004). MDM2 regulates p53 in several ways: it can target p53 for proteosomal degradation or block p53 activity by binding to its transactivation domain or relocate p53 to the cytoplasm (Meek, 2004). The regulation of p53-MDM2 interaction is very complex and different stresses can disrupt the p53-MDM2 loop in distinct ways. For example, the hypoxia-inducible factor-1 α (HIF1 α) protein regulates the hypoxia-mediated stabilization of p53, by blocking MDM2-induced p53 degradation and nuclear export (Chen et al., 2003). During genotoxic stress, p53 levels are stabilized by post-translational modifications, like the phosphorylation of a serine-15 residue located within its N-terminus that disrupts the p53-MDM2 interaction (Shieh et al., 1997; Siliciano et al., 1997).

Fig.1 – Mammalian cell cycle. The cell cycle (**M** – Mitosis Phase, **S** – DNA Synthesis Phase, **G1/G2** – GAP phase) is regulated by different **cyclins** (regulatory subunit) and cyclin dependent kinases (**CDKs** – catalytic subunit). Early to mid G1 phase is regulated by cyclin D-CDK4/6 complexes. The G1-S boundary is controlled by the cyclin E-CDK2 complex, while the S-phase is regulated by cyclinA-CDK2 complex. The G2-M-phase is modulated by the activity of cyclin A/B-CDK1 complexes. The cyclin-CDK complexes are negatively regulated by CDK inhibitors (CKIs), which consist of two families. The CIP/KIP family (p21, p27, p57) can regulate the activity of all cyclin-CDK complexes, while the INK4 family (p15, p16, p18, p19) inhibits the activity of CDK4/6-cyclin D complexes. Once past the restriction point, depicted by 'R', cells are committed to complete one round of cell division.



1.2.1 Regulation of irreversible forms of growth arrest

The individual functions of CKIs, pRb and p53 proteins are essential for instigating irreversible forms of growth-arrest like senescence (Pajalunga et al., 2007; Shay et al., 1991). CKIs can also regulate cell cycle arrest by modulating the activity of p53 and pRb proteins. p16 can promote senescence via pRb, by inhibiting the CDK4/CDK6-mediated phosphorylation of pRb, thus stabilizing the RB-E2F complexes that promote growth arrest (Serrano et al., 1993). Growth arrest due to senescence can also be mediated by p53 via p14/ARF. For example, p14/ARF is essential for the stabilization of p53, as it sequesters MDM2 away, thereby releasing p53 to promote replicative growth-arrest (Weber et al., 1999).

p53 can also mediate senescence by inducing p21 expression. In this scenario, the transcriptional activity of p53 is increased during senescence and it in turn activates the transcription of p21, which induces senescent growth-arrest (Atadja et al., 1995; el-Deiry et al., 1993; Tang et al., 1998; Weber et al., 1999). Therefore, p21 is an important regulator of replicative growth arrest. Forced expression of p21 in fibrosarcoma cells, induces senescence and activates the expression of senescence-specific genes, which includes ECM components like integrin β 3, and genes commonly expressed in age-related diseases, like Alzheimer's β -amyloid precursor protein (APP) that contributes to the formation of Alzheimer's amyloid plaques (Chang et al., 2000). Furthermore, p21 expression in these cells actively repressed a set of proliferation-related genes involved in controlling DNA replication, DNA repair, and mitosis, thus promoting cell cycle arrest due to senescence (Chang et al., 2000).

In conditions of severe cellular stress, p53 can be activated to promote apoptosis to prevent the build-up of damaged cells. p53 can induce apoptosis by mediating the expression of pro-apoptotic genes such as PUMA and NOXA. In conditions of ER stress in mouse embryo fibroblasts (MEF), p53 was shown to mediate apoptosis by up-regulating the transcription of PUMA and NOXA (Li et al., 2006). The expression of PUMA and NOXA were inhibited in p53 deficient MEF and these cells were more resistant to apoptosis during ER stress (Li et al., 2006). In these studies, PUMA and NOXA was responsible for activating the mitochondrial apoptotic pathway by inducing downstream effectors, BAK and caspase 7 (Li et al., 2006). Thus, in response stressful stimuli, p53 can mediate growth arrest or apoptosis in several ways.

1.2.2 Regulation of reversible growth arrest

Stem cells and many somatic cells exist in a state of reversible growth arrest (Coller et al., 2006). Entry into or exit from quiescence can be triggered by environmental factors such as the availability of essential nutrients or anti-mitogenic signals [reviewed in (Gray et al., 2004)]. The decision to enter the cell cycle or quiescence is made during the G₁ phase of the cell cycle (Gray et al., 2004). When an adequate supply of nutrients is present, the cells will pass the point of no return, called the restriction point, and commit to the cell cycle [Fig.1, (Gray et al., 2004)]. However, in the case of insufficient nutrient levels, the cells are unable to pass the restriction point and will go into the G₀ phase instead and are able to exit G₀ when nutrient supplies are replenished [Fig.1, (Gray et al., 2004)]. Hence, the entry into and exit from quiescence is regulated by external environmental conditions.

In culture, adherent cells can be stimulated to enter G_0 either by serum-starvation, where cells are cultured in serum-free starvation media at subconfluent levels, or by growing cells to contact inhibition, such that they growth arrest due to high cell density (Coller et al., 2006; Gos et al., 2005). Starved cells can be induced to re-enter the cell cycle by the addition of mitogens, while passaging the contact- inhibited cells enables them to re-enter the cycle (Gos et al., 2005). The paths to acquiring G_0 occur differentially, depending on the initiating factor. In human diploid fibroblasts, distinct programs of gene expression are activated in response to the different initiators of growth arrest, suggesting that different forms of G_0 exist (Coller et al., 2006). Therefore, different genes were activated in response to quiescence by contact inhibition versus quiescence by serum-starvation.

Several signaling pathways regulate entry into and exit from G_0 . In yeast, pathways that may retard entry into quiescence include conserved pro-proliferative/survival pathways like the Target of Rapamycin (TOR) pathway; while, pathways that induce G_0 entry include growth inhibitory pathways, such as the Snf1p AMP-activated protein kinase pathway [reviewed in (Gray et al., 2004)]. Exiting the state of quiescence is regulated by Gcs1p protein in yeast, since *gcs1* mutants were defective in exiting G_0 upon the re-addition of nutrients (Gray et al., 2004). The Gcs1p protein, encoded by the *GCS1* gene, functions as a GTPase-activating protein (GAP) that interacts with ADP-ribosylation factor (Arf) proteins involved in regulating vesicular transport in cycling cells (Gray et al., 2004). Moreover, as the Arf proteins are involved in vesicular transport it is possible that the process of vesicular trafficking plays an important role in the exit

from quiescence and re-entry to the cell cycle (Gray et al., 2004).

The CKIs and p53 are important regulators of G₀. In human fibroblasts, p53 was shown to be involved in instigating and maintaining cells in G₀ upon serum-withdrawal by inducing the expression of p21 (Itahana et al., 2002). As mentioned previously, the phosphorylation of the p53 serine-15 residue in its N-terminus by kinases, like ATM kinase, disrupts the p53-MDM2 interaction, thereby allowing p53 to accumulate post-translationally under conditions of genotoxic stress (Shieh et al., 1997). However, in quiescent human fibroblasts, an increase in p53 mRNA levels occurred prior to the elevation of protein levels (Itahana et al., 2002). Moreover, ATM-mediated phosphorylation of serine-15 residue was not required for p53 protein induction (Itahana et al., 2002). These observations suggest that the mode of induction of p53 during serum-starvation is unique from the path activating p53 under conditions of genotoxic stress.

Studies also indicate that p21 and p27 are important regulators of G₀. Human fibroblasts lacking p21 expression and activity had higher levels of DNA synthesis and were compromised in their ability to enter G₀ following serum-withdrawal and contact inhibition, suggesting that p21 is necessary for the onset of quiescence (Perucca et al., 2009). Findings of Ladha *et al.* suggest that p27 is also required for entry into G₀, and along with p21, can maintain cells in G₀ (Ladha et al., 1998). Additionally, entry into and remaining in G₀ required the repression of cyclin D1 levels, since the inactivation of p27 in serum-starved murine fibroblasts expressing cyclin D1 led to re-initiation of DNA synthesis and exit from G₀, whereas the same was not seen in control cells that lacked the expression of cyclin D1 (Ladha et al., 1998). Similarly, in human dermal fibroblasts, p27

was found to inhibit cyclin A-CDK and cyclin D1-CDK complex activity (Chassot et al., 2008). However, the knockdown of p27 or a double knockdown of p27 and p21 was not sufficient to inhibit cell cycle exit into G_0 due to contact-inhibition or serum-starvation (Chassot et al., 2008). The down-regulation of cyclin D1 levels was also required for cell cycle arrest in these conditions (Chassot et al., 2008). Therefore, these studies show that the attainment of G_0 is regulated by the activity of CKIs and the inactivation of cell cycle components. In particular, p21 and p27 are major players in regulating G_0 and cyclin D1 is the main cell cycle protein implicated in hindering growth arrest by stimulating re-entry into the cell cycle.

1.3 Growth-arrest specific gene expression

The G_0 state is characterized by distinct patterns of gene expression. Genes induced during mitogenesis are repressed during growth arrest while a separate set of genes is activated (Kim et al., 1999; Liu et al., 2007). These growth arrest induced genes are collectively known as growth arrest-specific genes (GAS) or as growth arrest and DNA damage-inducible (GADD) or quiescence-specific genes (Kim et al., 1999). Although the function of GAS gene products is not well characterized, their products include protein components of the extracellular matrix (ECM) like collagen $\alpha 1$, collagen $\alpha 2$, and the proteoglycan decorin (Coppock et al., 1993; Mauviel et al., 1995), as well as proteins that bind lipids with high affinity, such as the p20K lipocalin protein (Kim et al., 1999).

GAS gene products also include factors that can enhance cellular survival and those that are negative regulators of cell cycle progression (Kim et al., 1999; Lih et al., 1996). For example, GAS gene products like the secreted apoptosis related proteins

(SARPs) are secreted only in quiescent mouse C3H/10T1/2 cells and not in exponentially growing cells (Melkonyan et al., 1997). The SARPs allow quiescent cells to resist apoptotic stimuli and hence protects quiescent cells from succumbing to cell death (Melkonyan et al., 1997). The *gas-1* is another example of a GAS gene that is expressed only in quiescent cells and its expression is down-regulated in cycling and transformed cells (Del Sal et al., 1992). Its product is a transmembrane protein acting as a negative regulator of the cell cycle, as its over-expression in cycling NIH3T3 murine cells inhibits DNA synthesis and blocks entry into S-phase (Del Sal et al., 1992). Finally, CKI p27 is also a GAS gene that functions to promote G₀ arrest in response to high cellular density (Rivard et al., 1996; Zhang et al., 2000).

In addition to preventing cellular proliferation, GAS gene products can also act as positive regulators for re-entry into the cell cycle and promoting cellular growth (Lih et al., 1996). Lih *et al.* characterized one such GAS gene, the platelet-derived growth factor α -receptor (PDGF α R), which was found to accumulate following serum-starvation (Lih et al., 1996). PDGF α R mRNA levels increased following serum-depletion and are reduced following serum addition or transformation of serum-starved NIH3T3 cells (Lih et al., 1996). Thus, PDGF α R is a GAS gene regulated at the transcriptional level, whose gene product may prime the cell for re-entry into the cell cycle from G₀ upon mitogenic stimulation by platelet-derived growth factor (PDGF) (Lih et al., 1996).

1.3.1 GAS gene expression in CEF

p20K is a GAS gene, which encodes a secretory protein that is expressed in quiescent chicken embryo fibroblasts (CEF) and in chicken heart mesenchymal (CHM) cells

(Bedard et al., 1987; Bedard et al., 1989). p20K belongs to the lipocalin family of lipid binding proteins. Thus far, its function is unknown but it has been shown to bind poly-unsaturated fatty acids with high affinity (Cancedda et al., 1996; Kim et al., 1999). p20K expression is stimulated in quiescent CHM cells and is down-regulated in the presence of mitogens (Bedard et al., 1989). p20K is also expressed in starved CEF, albeit modestly, but is highly activated in contact-inhibited CEF cells; thus p20K is a marker for contact inhibition (Kim et al., 1999; Mao et al., 1993). Further analysis shows that p20K is a quiescence-specific GAS gene, since its expression was observed exclusively in conditions of reversible growth arrest but not in senescent or apoptotic CEF (Kim et al., 1999). In fact, apoptosis induced by non-steroidal anti-inflammatory drugs in CEFs led to the repression of p20K expression (Lu et al., 1995).

ApoA1 is another GAS gene in CEF whose expression is regulated by quiescence (T. Miyake, MSc Thesis). However, unlike p20K it was shown to be regulated by serum-starvation and not by contact inhibition (T. Miyake, MSc Thesis). Moreover, Miyake showed that ApoA1 levels were upregulated in response to lipid starvation, whereas CHOP10 [a member of the CAAT/Enhancer Binding Protein (C/EBP) family] was activated specifically in response to amino acid starvation and ER-stress (Tetsuaki M., MSc Thesis). Hence, these observations show that different GAS genes are activated in response to different stimuli. In CEF, p20K expression can be used a marker for contact inhibition, while ApoA1 and CHOP10 levels would be good indicators of lipid and amino acid starvation respectively.

1.4 Regulation of GAS genes

The regulatory mechanisms controlling GAS gene expression are largely undefined; however, it has been shown that several GAS genes are regulated either transcriptionally or post-transcriptionally during growth arrest. The common feature of GAS genes is that their expression is repressed in response to serum-stimulation and activated in quiescence. For example, in confluent mouse embryonic fibroblasts (MEFs), the expression of GAS genes, *gas-2*, *gas-3*, *gas-5*, and *gas-1* mRNA were repressed following serum-stimulation (Ciccarelli et al., 1990). Further analyses of serum-stimulated quiescent MEF showed that while the expression of *gas-1* was transcriptional, the regulation of *gas-2*, *gas-3*, and *gas-5* was post-transcriptional. *p27* expression is also regulated by high cellular density in murine fibroblasts (Yanagisawa et al., 1999). Although its expression is reported to be regulated post-transcriptionally in most cases, there is evidence showing that it is regulated transcriptionally as well (Millard et al., 1997; Yanagisawa et al., 1999). In murine fibroblasts, high cell density induced *p27* expression at the mRNA level, which was repressed in response to serum-addition (Yanagisawa et al., 1999). Overall, very little is known about the regulatory elements and transcriptional factors that regulate GAS gene expression. The regulation of *p20K* GAS gene has been studied extensively in CEF and can provide information as to how different factors can regulate GAS gene expression.

1.4.1 Regulation of *p20K* expression

In CEF, *p20K* expression is induced by high cell density and its expression is regulated at the transcriptional level. Contact inhibition activates the transcription of

p20K via a 48 base pair region in its promoter called the quiescence-responsive unit (QRU) (Kim et al., 1999). Previously, it has been shown that a member of the C/EBP family of transcription factors, C/EBP β , is a key regulator of p20K expression in contact-inhibited cells (Kim et al., 1999). The p20K QRU contains two C/EBP binding sites and C/EBP β has been shown to interact with these sites to activate its expression (Gagliardi et al., 2003). The proximal C/EBP element (site B: TTCCGTAAG) in the QRU represents the consensus C/EBP binding site, while the distal C/EBP β element (site A: TGTTGCAAG) was found to resemble the sequence of sites that are recognized by CHOP10 heterodimers (Kim et al., 1999). Over-expressing a dominant negative mutant of C/EBP β , Δ 184-C/EBP β , repressed p20K levels during contact inhibition (Gagliardi et al., 2003; Kim et al., 1999). Whereas, the over-expression of C/EBP β led to a massive induction of p20K in actively cycling CEF (Gagliardi et al., 2003; Kim et al., 1999). These results indicate that C/EBP β is essential for inducing p20K transcription during contact inhibition.

p20K expression was also shown to be negatively regulated by linoleic acid and peroxisome proliferator-activated receptor γ 2 protein (PPAR γ 2), both of which can induce adipogenesis (Kim et al., 1999). Moreover, additional studies showed that in cycling CEF, C/EBP β -dependent p20K expression was repressed by members of the activator protein-1 (AP-1) family, namely c-Jun, JunD, and Fra-2 (Gagliardi et al., 2003). Over-expression of c-Jun or JunD or Fra-2 inhibited density-dependent growth arrest and p20K accumulation in CEF (Gagliardi et al., 2003). Additionally, inducing AP-1 activity via phorbol esters treatment in cells over-expressing C/EBP β significantly diminished p20K

levels, again implying that AP-1 negatively regulates p20K induction by C/EBP β (Gagliardi et al., 2003). Hence, p20K expression is regulated by different cell cycle stages, as its expression is activated by conditions of reversible growth-arrest and repressed following mitogenic stimulation or in conditions of irreversible growth-arrest like senescence (Gagliardi et al., 2003; Kim et al., 1999). Thus, it is a marker of quiescence-specific growth arrest and contact inhibition.

2. CAAT/enhancer binding (C/EBP) family

2.1 Structure of C/EBP

The CAAT/enhancer binding proteins (C/EBP) are a family of basic leucine zipper (bZIP) transcription factors that regulate several aspects of cellular fate, such as proliferation, differentiation, inflammation, and growth-arrest [reviewed in (Ramji and Foka, 2002)]. There are six members of the C/EBP family, C/EBP α , C/EBP β , C/EBP γ , C/EBP δ , C/EBP ϵ , and C/EBP ζ , which is also known as C/EBP homologous protein 10 (CHOP10) (Ramji and Foka, 2002). All the family members have a highly conserved C-terminal region, which consists of a basic DNA binding domain followed by a leucine zipper that is required for dimerization (Landschulz et al., 1989; Vinson et al., 1989). Therefore, other than CHOP10, all C/EBP family members are able to form homo- or heterodimers and are able to regulate transcription of target genes carrying C/EBP binding sites (Ramji and Foka, 2002; Williams et al., 1991). The typical consensus sequence that the C/EBP family members bind to is 5'-T(T/G)NNGNAA(T/G)-3' (Kim et al., 1999). C/EBP ζ /CHOP10 is distinct from the other C/EBP family members in that it does not have a conventional basic region (Ron and Habener, 1992). Its basic domain contains

a glycine substitution in place of an asparagine residue and a conserved aliphatic and basic residue were replaced by two proline residues (Ron and Habener, 1992). The amino acid substitutions cause a misalignment of the leucine residues within the CHOP10 zipper domain, thereby preventing it from forming homodimers and disables it from interacting with the canonical C/EBP binding sites of target genes [Fig.3; (Ron and Habener, 1992)]. C/EBP transcription factors also have a transactivation domain (TAD) in their N-terminus, which is essential for controlling the transcription of target genes [Fig 2; (Ramji and Foka, 2002)]. However, C/EBP γ only contains the bZIP domain and by forming heterodimers with other C/EBP members, can inhibit their functions [Fig.2, (Cooper et al., 1995)].

C/EBP β and C/EBP γ are ubiquitously expressed, whereas C/EBP α expression is more specific, although it is more widely expressed than C/EBP ϵ and C/EBP δ . C/EBP ϵ is only expressed in hematopoietic cells and the expression of C/EBP δ is specifically induced by stress or inflammation [(Akira et al., 1990; Antonson and Xanthopoulos, 1995; Davies et al., 2000; Yamanaka et al., 1997), reviewed in (Johnson, 2005)]. CHOP10 exists at very low levels in normal conditions and its levels are massively up-regulated during various types of cellular stress, such as ER stress and oxidative stress (Wang et al., 1996).

Fig.2 – Schematic representation of the protein structure of C/EBP family members.

The CCAAT/Enhancer Binding Proteins (C/EBP) are bZIP transcription factors. This figure depicts the basic structure of the six C/EBP family members. Most members have a transactivation domain (TAD), regulatory domains (RD), basic DNA binding domain (Basic DBD), and leucine zipper (LZ). The TAD is required to activate transcription of target genes, while the RD represents sites of post-translational modifications that can alter their function. The basic DBD is required to bind target DNA and the leucine zipper (LZ) allows members to form homo-/heterodimers. C/EBP α -, β -, δ -, ϵ -members have similar structures. C/EBP γ lacks a TAD, while C/EBP ζ (CHOP10) has glycine and proline substitutions within its DBD.

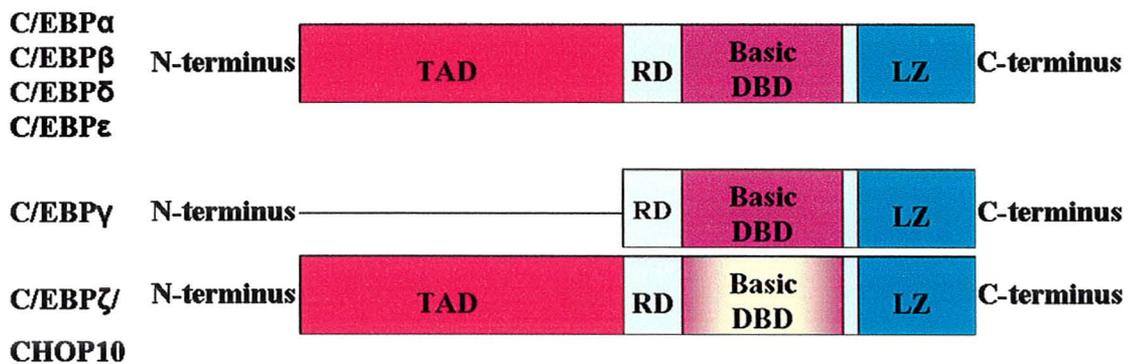
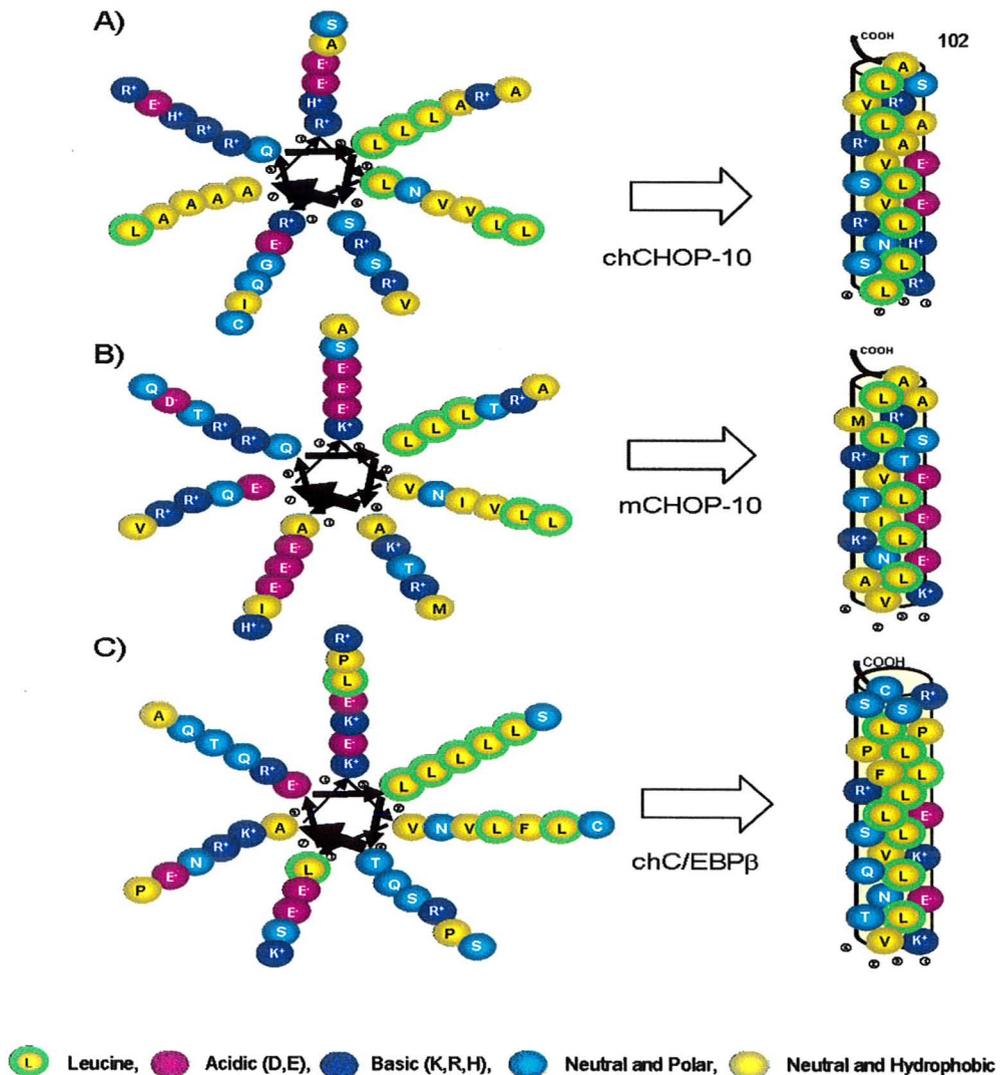


Fig.3 - The 3D structure of leucine zipper domain of C/EBP proteins. [This figure was prepared by T. Miyake (Masters Thesis)]. **A)** The leucine zipper domain of chicken CHOP10 (chCHOp10). **B)** mouse CHOP10 (mCHOP10) and **C)** chicken C/EBP β (chC/EBP β) were constructed according to the heptad repeat α -helix model. Each amino acid was colour-coded based on their characteristic. The alignment of five leucine residues seen in C/EBP β is disrupted in chicken and mouse CHOP10.



2.2 Functions of C/EBP transcription factors

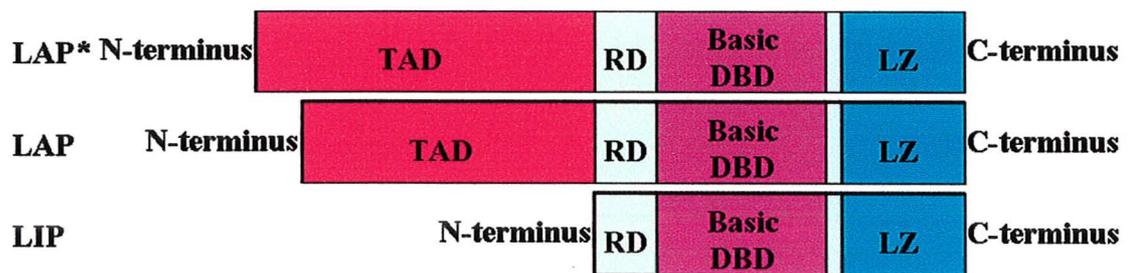
The C/EBP family members regulate various aspects of cellular fate. C/EBP α and C/EBP β are essential for regulating the differentiation of several cell types including adipocytes, neuronal cells, hepatocytes, granulocytes, and osteocytes [reviewed in (Nerlov, 2007)]. Moreover, several studies have confirmed the antiproliferative functions of C/EBP α and it has been implicated as a tumour suppressor in several cell types (Halmos et al., 2002; Nerlov, 2007). Unlike C/EBP α , the effect of C/EBP β in the control of cellular proliferation is complex, as it can either have anti-proliferative or pro-proliferative roles in a cell-type and context-specific manner (Nerlov, 2007). C/EBP δ also functions in negatively regulating cellular proliferation (Johnson, 2005). Similar to C/EBP α , C/EBP ϵ functions to promote cell differentiation and negatively regulates cell proliferation (Johnson, 2005; Yamanaka et al., 1997). CHOP10 is involved in regulating stress-induced responses such as apoptosis or growth arrest and also functions as an inhibitor of other C/EBP family members [(Zinszner et al., 1998), reviewed in (Oyadomari and Mori, 2004)]. Since my research focused on the activity of C/EBP β and CHOP10 in cellular quiescence, I will go on to further describe the regulation and functions of these two family members.

2.3 Regulation of C/EBP β and its diverse functions

C/EBP β mRNA can be translated into three isoforms: Liver activating protein* (LAP*), liver activating protein (LAP), and liver inhibitory protein (LIP), where LAP and LIP are the major forms found in cells (Cao et al., 1991; Ramji and Foka, 2002). The two

LAP forms contain the bZIP and TAD domains (Descombes et al., 1990; Ramji and Foka, 2002). LAP* is the full length form of C/EBP β (38 kDa), whereas LAP (35 kDa) lacks 21 amino acids from the N-terminus [Fig.4; (Boggs and Reisman, 2007)]. The LAP* form is a more potent activator of gene expression than the LAP form, as the additional 21 nucleotides in the LAP* form is capable of recruiting the SWI/SNF chromatin remodelling complex and hence can transactivate a set of genes that LAP cannot (Kowenz-Leutz and Leutz, 1999). Finally, the LIP (21 kDa) contains only the bZIP domain and is therefore only able to form homo- or heterodimers but is unable to induce transcription [Fig.4; (Descombes and Schibler, 1991)]. C/EBP β is constitutively expressed in the intestine, liver, adipose tissue, kidney, lung, spleen, and myelomonocytic cells (Ramji and Foka, 2002). The function of C/EBP β can be regulated by changes in the LAP to LIP ratio, post-translational modifications or by protein-protein interactions (Ramji and Foka, 2002).

Fig.4 – Schematic representation of the different isoforms of C/EBP β transcription factor. The transactivation domain (TAD) is required to activate transcription of target genes. The regulatory domain (RD) regulates its various cellular activities, while the basic DNA binding domain (Basic DBD) is required to bind target DNA. The leucine zipper (LZ) allows it to form homo-/heterodimers. The activator forms of C/EBP β are the LAP* and LAP (Liver-activating protein), which activate gene expression. On the other hand, LIP (Liver inhibitory protein) is an inhibitor of gene expression as it lacks a TAD.



2.3.1 Regulation of C/EBP β expression

Expression of C/EBP β can be regulated by various cellular conditions. For example, in chicken embryo fibroblasts (CEF), C/EBP β expression was induced at the transcriptional level following contact inhibition, and it was also suggested that C/EBP β might be a GAS gene (Kim et al., 1999). During amino acid starvation, cells mount the amino acid response pathway, thereby inducing the transcription of genes containing amino acid response elements (AARE) (Chen et al., 2005). In human hepatoma cells, histidine starvation led to an increase in C/EBP β transcription via an AARE sequence located within a 93bp regulatory region downstream of the protein coding sequence and this seem to occur in a ATF4-dependent manner (Chen et al., 2005). Moreover in cervical cancer cells, ER stress stimulated by hydrogen peroxide treatment, induced expression of C/EBP β (Jin et al., 2009). This expression of C/EBP β was also dependent on ATF4, since loss of ATF4 led to a reduction in C/EBP β protein and mRNA levels (Jin et al., 2009). Therefore, different cellular conditions can activate C/EBP β expression.

2.3.2 Post-translational modifications

Different post-translational modifications of C/EBP β can add to its functional repertoire. For example, phosphorylation of C/EBP β by extracellular signal-regulated kinase (ERK), cyclin A/CDK2, and GS3K- β at residues Thr-188 and Thr-179/Ser-184 respectively is required to activate its DNA binding ability and to promote adipogenesis of murine 3T3-L1 preadipocytes (Li et al., 2007; Park and Park, 2010; Tang et al., 2005). During serum-starvation, phosphorylation at residues Ser-64 and Ser-189 is diminished in murine NIH 3T3 fibroblasts (Shuman et al., 2004). These phosphorylation events were

regulated by CDK1 and CDK2 in a cell-cycle dependent manner and was essential for the HA-RasV12-mediated transformation of fibroblasts (Shuman et al., 2004). In hepatic stellate cells, ribosomal protein S-6 kinase (RSK)-mediated phosphorylation of C/EBP β at threonine-217 was essential for the proliferation and survival of these cells in response to CCl₄-induced oxidative stress (Buck et al., 2001). This phosphorylation event was shown to mediate the interaction of C/EBP β with procaspases 1 and 8, such that C/EBP β could block their activity by sequestering them away (Buck et al., 2001). Another example implying the significance of post-translational modifications of C/EBP β is depicted in a study, where Kowenz-Leutz *et al.* propose that the transcriptional activities of C/EBP β is held in check by intra-molecular folding between two inhibitory regions within the N-terminus and the transactivation domain (Kowenz-Leutz et al., 1994). Phosphorylation of C/EBP β at specific sites in these regulatory domains can disrupt the inhibitory folding of C/EBP β , thus de-repressing its transactivation activity (Kowenz-Leutz et al., 1994). This model was supported by the observation that deletion of the regulatory domain enhanced C/EBP β DNA binding and function, presumably by disrupting the intra-molecular folding.

The function of C/EBP β can also be regulated by acetylation. Studies performed on human embryonic kidney cells, Chinese hamster ovary cells and murine preadipocytes demonstrate that C/EBP β is acetylated at several lysine residues by co-activators p300/PCAF (Cesena et al., 2007). Acetylation of a particular residue, Lysine-39 (Lys-39), found within its transactivation domain, is required for the C/EBP β -dependent activation of target genes, like *c-fos* (Cesena et al., 2007). Further studies showed that Lys-39 acety-

lation is regulated by p300 and HDAC1 and is required for the transcription of genes involved in adipogenesis (Cesena et al., 2008). p300 acetylates Lys-39 and activates the transcriptional activity of C/EBP β , while HDAC1 deacetylates it and represses transcription of target genes such as C/EBP α and PPAR γ (Cesena et al., 2008). In mouse embryonic cells, GCN5 was found to acetylate C/EBP β at lysine residues 98, 101, and 102 upon stimulation by the hormone glucocorticoid and this is important for promoting preadipocyte differentiation (Wiper-Bergeron et al., 2007). Acetylation of these residues led to an increase in transactivation potential of C/EBP β and its dissociation from co-repressor complex containing HDAC1 (Wiper-Bergeron et al., 2007). Overall, these studies demonstrate that C/EBP β activity can be differentially regulated by post-translational events, which in turn contributes to the diversity of its functions.

2.3.3 Protein-Protein Interactions

C/EBP β is implicated in regulating different aspects of cellular processes. For example, it has been shown to have pro-survival roles in some cases and promote cell death in others (Haltermann et al., 2008; Li et al., 2008; Yoon et al., 2007). Moreover, C/EBP β can promote different forms of growth arrest in cells such as senescence, differentiation, and reversible growth-arrest (Kim et al., 1999; Sebastian and Johnson, 2009; Tang et al., 2004). How C/EBP β is able to control multiple facets of cellular fate is partly dependent on its interacting protein, which can modify the pattern of gene expression regulated by C/EBP β in response to various cellular conditions (Ramji and Foka, 2002).

2.3.3.1 Role of C/EBP β in cellular proliferation

In CEF, C/EBP β may be a negative regulator of proliferation as expression of a dominant negative mutant of C/EBP β (Δ 184-C/EBP β) led to increased proliferation in cells (Gagliardi et al., 2003). Inhibiting the function of activator protein-1 (AP-1) member, c-Jun, abrogated the enhanced proliferation of the Δ 184-C/EBP β cells (Gagliardi et al., 2003). Therefore, the enhanced proliferation of Δ 184-C/EBP β -expressing CEF was mediated by the AP-1 family, which is also known to modulate proliferation via cyclin D1 expression and phosphorylation of pRb (Gagliardi et al., 2003). In addition, the dominant negative mutant of C/EBP β had elevated levels of AP-1 members, c-Jun, JunD, and Fra-2, and cyclin D1, suggesting that C/EBP β may regulate cell proliferation via AP-1 family members and cyclin D1 in actively dividing CEF (Gagliardi et al., 2003). The over-expression of AP-1 members activated cell proliferation, prevented growth-arrest by contact inhibition, and instead caused cells to go straight into apoptosis (Gagliardi et al., 2003). This observation was restated in MEFs lacking C/EBP β function, as they were also found to have a proliferative advantage (Gagliardi et al., 2003). Therefore, these findings suggest that in embryonic fibroblasts, C/EBP β negatively regulates cell proliferation (Gagliardi et al., 2003).

Additionally, C/EBP β has different roles during different cellular stages. Under cycling conditions, C/EBP β cooperates with AP-1 and controls the expression of G₀/G₁ transition genes like CEF-4 (Gagliardi et al., 2003). However, in contact-inhibited cells C/EBP β antagonizes the expression of AP-1 members, activates the expression of p20K and promotes G₀ (Gagliardi et al., 2003; Kim et al., 1999). Hence, C/EBP β and AP-1 ap-

appear to have opposite functions in the control of growth arrest in CEF, where in the absence of AP-1 C/EBP β can promote growth arrest and in the presence of AP-1 mediate gene expression of the G₀/G₁ transition (Gagliardi et al., 2003).

In addition to its activity in promoting cell cycle progression as cyclin/CDK complexes, cyclin D1 has also been shown to modify the function of transcription factors (Lamb et al., 2003). For example, cyclin D1 was shown to activate estrogen receptor-mediated gene expression independently of its CDK binding (Zwijssen et al., 1997). Since high expression of C/EBP β was found in human epithelial cancers possessing a signature for cyclin D1 over-expression, it was proposed that C/EBP β regulates cyclin D1-induced gene expression (Lamb et al., 2003). In the absence of cyclin D1, it was found that C/EBP β repressed the expression of cyclin D1 target gene promoters (Lamb et al., 2003). However, in conditions of cyclin D1 over-expression, C/EBP β was able to induce the transcription of these target genes (Lamb et al., 2003). Moreover, over-expression of cyclin D1 on its own was not able to activate target gene transcription, suggesting that cyclin D1 functions to activate gene expression by counteracting the inhibitory effects of C/EBP β and enhancing its transcriptional activity (Lamb et al., 2003). Therefore, interactions of C/EBP β with pro-survival factors like AP-1 or cyclin D1 can alleviate the growth-inhibitory effects of C/EBP β thereby enhancing cell survival.

2.3.3.2 Role of C/EBP β in the expression of G₀/G₁ transition genes

Several lines of evidence indicate that C/EBP β can regulate the expression of G₀/G₁ transition genes like cytokines. Studies in human monocytic cells and mouse embryonic carcinoma cells showed that C/EBP β can cooperate with NF- κ B, particularly its

p65 subunit, to activate the expression of cytokines interleukin-6 and interleukin-8 (IL-6 and IL-8) (Matsusaka et al., 1993). Both cytokines were shown to contain a C/EBP site and a NF- κ B site that were both equally important for transcription (Matsusaka et al., 1993). Moreover, C/EBP β was found to directly interact with the p65 subunit of NF- κ B via its bZIP domain and the Rel homology domain of p65 (Matsusaka et al., 1993).

C/EBP β can also interact with AP-1 to regulate cytokine-induced gene expression. In human fibroblasts, cytokines like tumour necrosis factor α (TNF α) and interleukin 1 (IL-1), can stimulate the expression of a member of the hyaluronan-binding protein family, TSG-6 (Klampfer et al., 1994). The TSG-6 promoter consisted of an AP-1 site and a C/EBP site that were important for the cytokine-mediated gene expression (Klampfer et al., 1994). Therefore, the activation of TSG-6 gene was regulated by AP-1, in a cooperative manner with C/EBP β (Klampfer et al., 1994). The inhibitory isoform of C/EBP β (LIP) was found to repress AP-1 mediated transcription of TSG-6, whereas the activator form (LAP) activated transcription by AP-1 (Klampfer et al., 1994).

Moreover, C/EBP β also regulates G₀-G₁ transition genes during Rous sarcoma virus (RSV)-mediated transformation (Gagliardi et al., 2001). C/EBP β was required for the transcriptional activation of the chemokine, CEF-4 in v-Src-transformed CEF (Gagliardi et al., 2001). However, it was not required for the induction or maintenance of the transformed phenotype (Gagliardi et al., 2001). A region within the CEF-4 promoter contained two C/EBP sites, an AP-1 site, and an NF- κ B site, which was collectively termed as the Src-responsive unit (SRU) (Gagliardi et al., 2001). C/EBP β was shown to cooperate with AP-1 and NF- κ B via the SRU to activate CEF-4 expression in v-src-transformed CEF

(Gagliardi et al., 2001). Hence, these findings indicate that through its interactions with NF- κ B and/or AP-1, C/EBP β can regulate genes of the G₀/G₁ transition.

2.3.3.3 Function of C/EBP β during adipogenesis

C/EBP β has been shown to have critical roles in promoting the differentiation of several cell types including osteoblasts, keratinocytes, and adipocytes (Lopez et al., 2009; Tominaga et al., 2008; Zuo et al., 2006). Terminal differentiation of mouse pre-adipocytes is comprised of a signaling cascade, whereupon C/EBP β is induced almost immediately following the induction of differentiation (Tang et al., 2004). However, there is lag period before C/EBP β can acquire its DNA binding activity to activate C/EBP α transcription (Tang et al., 2004). This delay in C/EBP α expression is important as C/EBP α is mainly anti-proliferative and cells undergoing terminal differentiation must first undergo two rounds of cell division from G₀, referred to as mitotic clonal expansion (MCE), following differentiation stimulation in order to become terminally differentiated (Tang et al., 2004). C/EBP β activates the expression of C/EBP α via a C/EBP site within its promoter. C/EBP α in turn promotes its own transcription, and the transcription of the nuclear factor, peroxisome proliferator-activated receptor (PPAR γ), which also contains C/EBP elements in its proximal promoter (Tang et al., 2004). In turn, C/EBP α and PPAR γ cooperate to activate the expression of adipocyte-specific genes, thus promoting adipogenesis (Tang et al., 2004).

PPAR γ and retinoid X receptors (RXR) form heterodimers and regulate the expression of target genes involved in lipid metabolism in response to ligand stimulation (Examples of ligands: RXR ligand - 9-cis-retinoic acid and PPAR γ – polyunsaturated

fatty acid) (Hamm et al., 2001). PPAR γ is present as two isoforms within the cell, PPAR γ 1 and PPAR γ 2, where the two are very similar in structure, with the exception that PPAR γ 2 has an extra 30 nucleotides at its N-terminus (Hamm et al., 2001). C/EBP β was shown to activate PPAR γ 2 activity in mouse pre-adipocytes in response to 3-isobutyl-1-methylxanthine-stimulation and this was attenuated in the presence of the LIP isoform (Hamm et al., 2001). Moreover, in non-adipogenic mouse fibroblasts C/EBP β was found to bind to the C/EBP α promoter but unable to activate transcription, possibly due to the additional binding of an HDAC1 co-repressor (Zuo et al., 2006). Induction of PPAR γ 2 was required to mediate the removal of HDAC1 from the C/EBP β -promoter complex in order for C/EBP β to activate C/EBP α expression (Zuo et al., 2006). Therefore, these studies demonstrate that C/EBP β , C/EBP α , and PPAR γ cooperate with each other to regulate adipogenesis.

In contact-inhibited CEFs, the density dependent-expression of p20K was repressed following over-expression of PPAR γ 2 or treatment with linoleic acid, an essential fatty acid that can bind p20K and activate PPAR γ 2 (Kim et al., 1999). Hence in CEF, it appears that p20K is repressed during adipogenesis by PPAR γ ; however, this inhibition was reduced when either C/EBP β or C/EBP α were over-expressed simultaneously with PPAR γ 2 (Kim et al., 1999). Thus, this represents another example, where the interaction of C/EBP β with other transcription factors controls the expression of p20K.

2.3.3.4 C/EBP β controls expression of genes involved in the ER-stress response

The endoplasmic reticulum (ER) is the location where protein synthesis and protein folding occur [reviewed in (Oyadomari and Mori, 2004)]. Any disruption of these

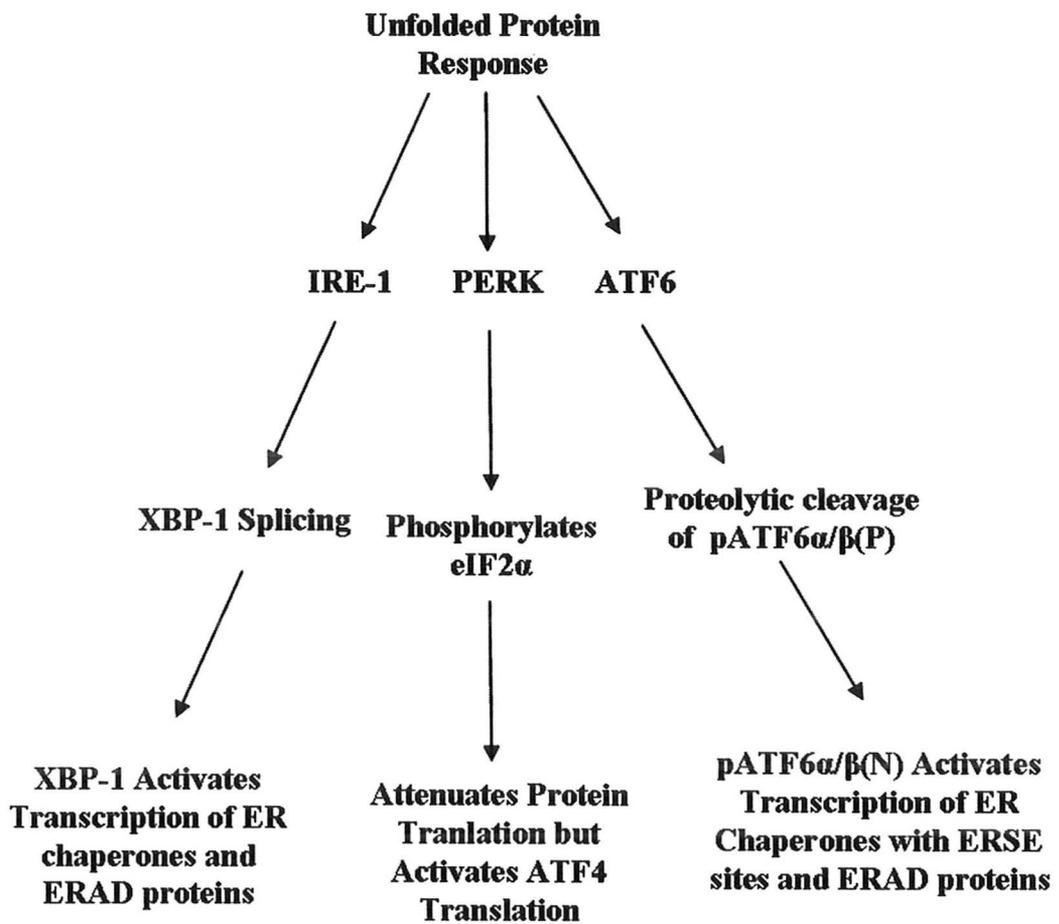
processes can induce the ER stress response pathway to protect the cell from adverse effects (Oyadomari and Mori, 2004). This pathway predominantly mediates four distinct responses. One response is to inhibit protein translation to reduce the protein load and accumulation of misfolded proteins within the ER (Oyadomari and Mori, 2004). Cells can also mount the unfolded protein response (UPR), to counter the accumulation of unfolded proteins within the ER (Oyadomari and Mori, 2004). The UPR regulates the expression of ER stress response genes including ER chaperone proteins, like BiP/Grp78, to facilitate protein folding and ER components that promote proteasomal-degradation of unfolded proteins, termed ER associated degradation proteins (ERAD) (Kaneko et al., 2003; Yamamoto et al., 2007). Moreover, build up of malfolded proteins in the ER can instigate the ER overload response (EOR), to enhance cell survival by stimulating the NF- κ B pathway, as it can activate anti-apoptotic genes like Bcl-X_L (Kaneko et al., 2003). Finally, if all these responses are unsuccessful to alleviate ER stress, the pro-apoptotic response is instigated to protect the cell from deleterious effects caused by the accumulation of damaged cells (Jin et al., 2009; Oyadomari and Mori, 2004). Mediators of ER stress-induced apoptosis include CHOP10, c-Jun N-terminal kinase (JNK), and a ER-specific caspase, caspase 12 that is associated with the ER membrane (Oyadomari and Mori, 2004).

To reduce ER stress due to the build-up of unfolded proteins within the ER, the UPR activates a program of gene expression via three pathways to protect the cell. The UPR pathways consists of the activating transcription factor 6 (ATF6) pathway, inositol requiring 1 (IRE-1) pathway, and the PRK-like endoplasmic reticulum kinase (PERK)

pathway (Oyadomari and Mori, 2004). Under normal cellular environments, the BiP chaperone protein binds to each of these ER transmembrane proteins and represses their function (Bertolotti et al., 2000). At the induction of cellular stress, PERK/ATF6/IRE-1 are released from BiP and are able to mount the UPR response, thus indicating that BiP acts as a sensor of ER stress (Bertolotti et al., 2000).

IRE-1 is a ER transmembrane endo-ribonuclease that is responsible for splicing an intron from its effector gene, bZIP transcription factor X-box binding protein -1 (XBP-1), thereby stabilizing it to activate transcription during ER stress (Calton et al., 2002). XBP-1 is a multifunctional transcription factor that controls expression of genes involved in secretory pathway, protein folding and degradation [Fig.6; (Adachi et al., 2008)]. ER transmembrane transcription factor ATF6 contains two members, pATF6 α (P) and pATF6 β (P) (Adachi et al., 2008). ER stress stimulates the re-location of pATF6 α/β (P) to the golgi apparatus, where they undergo proteolytic cleavage, thus expressing a free form of the protein, pATF6 α/β (N), which can then enter the nucleus to activate transcription [Fig.6; (Adachi et al., 2008)]. Most ER chaperones contain ER stress response elements (ERSE) in their promoter to which ATF6 members can bind to (Yamamoto et al., 2007). Thus, activated ATF6 regulates the expression of proteins maintaining ER quality control, hence facilitating the relief of ER stress [Fig.6; (Adachi et al., 2008)]. Specifically it is a major contributor in controlling the expression of ER chaperones, like BiP/Grp78 via their ERSE sites, and in conjunction with XBP-1 regulates ERAD proteins as well (Adachi et al., 2008; Yamamoto et al., 2007).

Fig.5 – The unfolded protein response pathway. The figure depicts the three pathways that are activated by the unfolded protein response (UPR) to alleviate ER stress. In response to the accumulation of malformed proteins in the ER, the UPR induces the PERK pathway, which attenuates protein translation but also activates ATF4 expression. ATF4 can in turn induce stress response genes like CHOP10. The IRE-1 and ATF6 pathways are also activated, which can promote the expression of ER molecule chaperones to facilitate protein folding and ER associated degradation proteins (ERAD) .



PERK is a ER transmembrane kinase that promotes cell survival during ER stress by inhibiting protein translation [Fig.6; (Harding et al., 2000)]. By phosphorylating the Serine-51 residue of eukaryotic translation initiation factor 2 α , eIF2 α , PERK mediates attenuation of protein synthesis (Hamanaka et al., 2005). Another kinase, GCN2, can also phosphorylate eIF2 α following ER stress to repress protein translation and contribute to cell cycle arrest via the loss of cyclin D1 translation (Hamanaka et al., 2005). Recently, eIF2 α phosphorylation has been implicated in interfering with the transcription of ribosomal RNA during ER stress, which constitutes an immense burden on the cell (DuRose et al., 2009). These effects emphasize that eIF2 α phosphorylation by UPR is essential to protect the cell from ER stress and restore homeostasis.

However, during ER stress, PERK can also activate the expression of its downstream effector, activating transcription factor 4 (ATF4) via eIF2 α phosphorylation (Oyadomari and Mori, 2004). ATF4 is a transcription factor that can bind ATF-cAMP responsive elements (CRE) and to amino acid response elements (AARE) in target genes (Oyadomari and Mori, 2004). An example of a downstream effector of the PERK-ATF4 pathway is the regulated in development and DNA damage responses 1 protein (Redd1), whose expression in mammalian cells during ER stress requires the presence of both PERK-induced phosphorylation of eIF2 α and ATF4 activation (Whitney et al., 2009). Studies in several human cell lines showed that oxidative and ER stress led to the repression of mammalian target of rapamycin (mTOR) mediated protein synthesis and the induction of ER stress effector genes, CHOP10 and BiP/Grp78 (Jin et al., 2009). In these

conditions, ATF4 inhibits the mTOR pathway by the C/EBP β -dependent induction of Redd1 (Jin et al., 2009). ATF4 and C/EBP β cooperatively bind to the C/EBP binding site of the Redd1 promoter to activate its expression (Jin et al., 2009). This represents a mechanism through which C/EBP β and ATF4 are able to affect protein translation by repressing the mTOR pathway, which is an important regulator of protein translation (Jin et al., 2009).

Studies from C/EBP β ^{-/-} MEFs over-expressing the inhibitory LIP isoform suggest that upon prolonged ER stress, LIP inhibits ATF4-mediated transcription but not transcription induced by the ATF6 or IRE-1 pathways (Li et al., 2008). Additionally, the expression of ATF4 downstream genes, like CHOP10, were diminished in C/EBP β ^{-/-} MEFs, while another set was activated including amino acid transporters, like sodium-dependent neutral amino acid transporter 2 (SNAT2), and those involved in amino acid synthesis like asparagine synthase (Li et al., 2008). These mutant cells were also associated with sustained phosphorylation of eIF2 α (Li et al., 2008). These studies suggest that C/EBP β can also positively regulate ATF4-mediated cell death and interfere with its transactivation of genes involved in amino acid metabolism (Li et al., 2008). Therefore, these studies indicate that depending on the stage and degree of ER stress, C/EBP β can either cooperate or interfere with ATF4-mediated gene expression.

2.3.3.5 Summary

Overall, the examples stated above signify how different C/EBP β interacting factors can alter C/EBP β dependent gene expression in response to proliferation, G₀-G₁ transition, differentiation, and ER stress. Moreover, these protein interactions either did not

affect C/EBP β -mediated expression of p20K or in the case of PPAR γ and AP-1, inhibited p20K transcription. p20K is a GAS gene that is highly expressed by contact inhibition in a C/EBP β -dependent manner; however, the binding partner of C/EBP β to control its expression during G₀ is currently unknown (Kim et al., 1999). Therefore, during my Masters, we sought to identify potential binding partner(s) or conditions promoting the activity of C/EBP β in the regulation of p20K at high cell density (contact inhibition).

Moreover, p20K levels are not massively activated during serum-deprivation, which indicates that C/EBP β may interact with another binding partner that suppresses p20K under these conditions (Kim et al., 1999). Since C/EBP binding site A within the p20K promoter partially corresponds to the CHOP10-C/EBP binding sequence, and CHOP10 is upregulated by serum-starvation, it implies that CHOP10 may be a suitable candidate for a C/EBP β interacting partner under starvation conditions (Miyake, T., Masters Thesis). Therefore, we attempted to determine which protein(s) interact with C/EBP β , at least functionally, to regulate its function during starvation conditions as well.

2.3.4 Role of dominant negative mutant of C/EBP β

The dominant negative mutant of C/EBP β , Δ 184-C/EBP β , lacks the transactivating regions of C/EBP β , but it is able to homodimerize or heterodimerize with endogenous C/EBP family members, bind DNA, and block transcriptional activation (Gagliardi et al., 2003). Expression of this C/EBP β mutant in CEF amplified the level of cell proliferation and also allowed them to reach greater cell densities without impairing their ability to contact inhibit (Gagliardi et al., 2003). It also enhanced the transformed phenotype of v-Src transformed CEF, thus implying that C/EBP β negatively regulates

transformation and proliferation of CEF *in vitro* (Gagliardi et al., 2001). Moreover, our studies showed that $\Delta 184\text{-C/EBP}\beta$ repressed the induction of ER stress-response and pro-apoptotic CHOP10 during serum-starvation (Results Section, Chapter 1). This alludes to a role for C/EBP β in regulating CHOP10 expression and apoptosis in times of ER stress.

2.4 Regulation of CHOP10 expression and function

2.4.1 Regulation of CHOP10

CHOP10 is regulated by various stresses, like hypoxia, DNA damage, glucose starvation, ER stress, and growth arrest (Lee et al., 2009; Marciniak et al., 2004; Wolfgang et al., 1997). For example, in rat cardiac myocytes, hypoxia led to an elevation in CHOP10 levels (Lee et al., 2009). In human epithelial cervical cancer cells, hepatocellular carcinoma cells, and human colon adenocarcinoma cells, the expression of CHOP10 was upregulated at the transcriptional and translational level following leucine deprivation (Bruhat et al., 1997). Additionally, studies in various mammalian cells showed that the levels of CHOP10 are diminished in actively dividing cells and upregulated in growth-arrested cells (Fornace et al., 1989; Ron and Habener, 1992).

The CHOP10 promoter contains an amino acid response element (AARE) that is essential for CHOP10 activation in response to amino acid starvation (Bruhat et al., 2000). The AARE is also a composite of ATF/CREB and C/EBP consensus sequences (Bruhat et al., 2000). During ER stress, PERK-mediated induction of ATF4 activates CHOP10 expression by binding to its AARE site (Li et al., 2008). Moreover, leucine starvation enhances the transcription of CHOP10 as well as its mRNA stability (Bruhat et

al., 1997). Further studies indicate that during amino acid limitation, CHOP10 mRNA levels are positively regulated by ATF2, since the expression of CHOP10 mRNA was repressed in ATF2 deficient cells in response to amino acid deprivation, but not ER stress (Bruhat et al., 2000). A stress-induced transcriptional repressor, ATF3, could down-regulate CHOP10 mRNA levels and interact with the CHOP10 promoter via its AP-1 and composite C/EBP-ATF sites (Wolfgang et al., 1997). Additionally, CHOP10 can be phosphorylated at two Serine residues, Ser-78 and Ser-81, by mitogen-activated protein kinase (MAPK) p38, which amplifies its pro-apoptotic activity during ER-stress (Oyadomari and Mori, 2004). Hence, the above examples reiterate that CHOP10 is a stress-inducible gene, whose expression and function is regulated by the ATF family of transcription factors.

2.4.2 Role of CHOP10 in response to cellular stress

CHOP10 has a role in regulating apoptosis and growth-arrest under cellular stress (Lee et al., 2009; Marciniak et al., 2004). Although CHOP10 is unable to bind DNA as homodimers, it is able to form heterodimers with other members of the C/EBP family and inhibit C/EBP members from binding to the canonical C/EBP binding sites of target genes and activating them (Ron and Habener, 1992). For example, in hepatocellular carcinoma cells CHOP10 was shown to inhibit C/EBP β DNA binding ability and interferes with its transcriptional activity (Ron and Habener, 1992). Furthermore, during conditions of cellular stress, CHOP10-C/EBP heterodimers can interact with an alternative DNA consensus sequence: 5'-(A/G)(A/G)(A/G)TGCAAT(A/C)CCC-3' and initiate transcription of target genes, referred to as downstream of CHOP (DOC) genes (Ubeda et al.,

1996).

CHOP10 can promote apoptosis by repressing the expression of anti-apoptotic Bcl-2 and activating the expression of pro-apoptotic genes (Zinszner et al., 1998; Li et al., 2008). Also, CHOP10 promotes cell death in ER stress by activating genes such as gadd34, which is the regulatory subunit of a phosphatase complex of eukaryotic translation initiation factor 2 α (eIF2 α), and ER oxidase 1 α (ERO1 α) that promotes formation of disulfide bonds in nascent proteins (Marciniak et al., 2004). The activation of GADD34 phosphatase causes the re-instigation of protein translation by reversing the PERK-induced repression of translation initiating factor eIF2 α , which in turn causes additional ER stress and promotes cell death (Marciniak et al., 2004). Similarly, CHOP10 increases the oxidizing state of the cells during ER stress by activating ERO1 α , thereby inducing cell death (Marciniak et al., 2004). These studies reiterate that CHOP10 is an active mediator of apoptosis in response to different forms of stress.

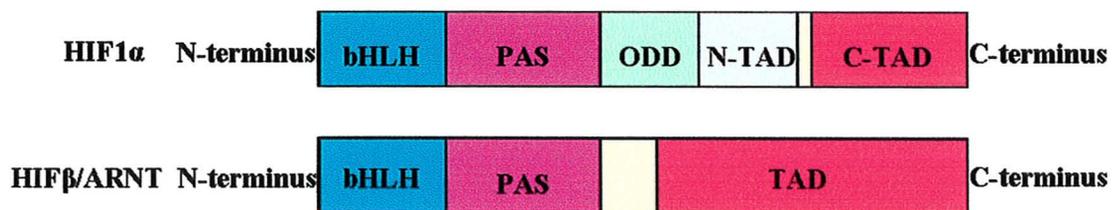
3. Hypoxia response pathway

3.1 Hypoxia inducible factors

Cells can become stressed under conditions of hypoxia or low oxygen (O₂) levels. In order to promote survival during hypoxic conditions, cells activate a specific set of genes that allow the cell to cope with the effects of diminished O₂ levels [reviewed in (Rankin and Giaccia, 2008)]. For example, hypoxia response genes include angiogenic factors like vascular endothelial growth factor (VEGF) and glycolytic enzymes phosphoglycerate kinase 1 (PGK-1), which are activated by diminished oxygen levels (Salceda et al., 1996). To compensate for low oxygen supply, VEGF can promote the

formation of new blood vessels to restore O₂ supply (Rankin and Giaccia, 2008). Regulation of gene expression in response to hypoxia is controlled by the hypoxia-inducible factors (HIFs), which belong to the PER-ARNT-SIM (PAS) family of basic helix-loop-helix (bHLH) transcription factors (Wang et al., 1995; Wood et al., 1996). HIFs consists of two subunits, the α and β subunits, which can heterodimerize resulting in the activated form of HIF during restricted oxygen levels (Wang and Semenza, 1995; Wood et al., 1996). The α and β subunits are structurally similar in that they both contain bHLH, PAS, and transactivation (TAD) domains (Jiang et al., 1996). However, the α subunit is distinct in that it has an additional TAD domain closer to the N-terminal (N-TAD) and an oxygen-dependent degradation domain (ODD) that acts as a sensor for oxygen levels in the cell (Huang et al., 1998; Jiang et al., 1996). The basic DNA binding region of HIF is required for DNA binding and the helix-loop-helix domain is required for dimerization [reviewed in (Kewley et al., 2004)]. HIFs also contain a PAS domain, which acts as a second dimerization domain and confers target gene specificity. The C-terminal transactivation domain (C-TAD) of the α -subunit mediates the HIF interaction with transcriptional co-activators, like p300/CBP, which is required to activate the transcription of target genes (Rankin and Giaccia, 2008).

Fig.6 – Schematic representation of the regulatory units of hypoxia inducible factor (HIF). The activated form of HIF1 contain heterodimers of the α and β subunits. HIF β is also known as Aryl-hydrocarbon receptor nuclear transporter (ARNT). Each subunit contains a transactivation domain (TAD), which is required to activate transcription of target genes. They also have a PER-ARNT-SIM (PAS) domain and a basic helix-loop-helix (bHLH) domain, which are required for dimerization. The PAS also provides target specificity, allow binding to hypoxia response elements (HRE) in target genes. HIF1 α has an additional TAD, one closer to the C-terminus and the other closer to the N-terminus (C-TAD & N-TAD). The oxygen-dependent degradation domain (ODD) in HIF1 α acts as a sensor to oxygen levels and is required for the degradation of HIF1 α under normoxia and stabilization during hypoxia.



3.2 Regulation of hypoxia inducible factors

The HIF activity is regulated by the stability of the α -subunit, whereas the β -subunit is constitutively expressed (Huang et al., 1998; Jiang et al., 1996). Low oxygen levels increase the stability of the α -subunit, allowing for the α -subunit to accumulate and heterodimerize with the β subunit to activate the hypoxia response pathway (Huang et al., 1998; Jiang et al., 1996). HIF heterodimers bind to specific DNA sequences found in target genes known as the hypoxia response elements (HRE) and promotes their transcription via its interaction with co-activators p300/CBP [reviewed in (Rankin and Giaccia, 2008)]. The core HRE sequence is RCGTG, where R can be A or G; however the flanking sequences are also important for target gene expression [reviewed in (Wenger et al., 2005)].

During normoxia (normal O₂ levels), the HIF1 α subunit is hydroxylated at specific proline residues located in the ODD by prolyl-4-hydroxylases (PHDs) (Jaakkola et al., 2001). This enables its interaction with the von Hippel-Lindau protein (pVHL), which is the recognition component of an E3 ubiquitin ligase complex (Jaakkola et al., 2001). Interaction of the α subunit with pVHL ubiquitinates HIF1 α and targets it for degradation by the proteasome (Jaakkola et al., 2001). In addition to regulating the stability of HIF1 α , any residual activity of HIF1 α is inhibited by the hydroxylation of an asparagine residue (N803) in the C-terminal TAD by an asparaginyl hydroxylase known as the factor inhibiting HIF1 (FIH-1) (Mahon et al., 2001). This hydroxylation event prevents the C-TAD from interacting with p300/CBP and further represses the function of HIF1 α (Lando et al., 2002a; Lando et al., 2002b). Therefore, in order to stimulate HIF1 α activity, both

these hydroxylation events must be inhibited. This occurs during hypoxia starting at O₂ levels lower than 6% and reaches a maximal level at 0.5% O₂ levels (Rankin and Giaccia, 2008). HIF1 α activity can also be stabilized by treating the cells with an FIH-1 inhibitor dimethyloxallyglycine (DMOG) and other hypoxia mimetics like cobalt chloride (CoCl₂) that also function to prevent prolyl and asparaginyl hydroxylase activity (Gustafsson et al., 2005; Zheng et al., 2008). Conversely, the addition of FeCl₂ has been shown to enhance prolyl-4-hydroxylase activity, thereby increasing the interaction of pVHL and HIF1 α and subsequent degradation of HIF1 α (Gustafsson et al., 2005).

3.3 Regulation of hypoxia by HIF1 α and other transcription factors

In addition to the HIFs, several other transcription factors have been implicated in regulating the response to hypoxia and these include C/EBP β , CHOP10, AP-1, NF- κ B, and p53 [reviewed in (Cummins and Taylor, 2005)]. For example, AP-1 interacts with other transcription factors, including HIF1 α , in the regulation of hypoxia-responsive genes such as VEGF and tyrosine hydroxylase (Millhorn et al., 1997; Salnikow et al., 2002). Hypoxia may also regulate cell death via the p53 protein. Studies have shown that HIF1 α may directly interact with p53 (Hansson et al., 2002). Furthermore, increased p53 stabilization was attributed to HIF1 α , since it bound to the E3 ligase of p53, MDM2 and sequestered it away from p53, thereby allowing p53 levels to increase and enabling it to perform its functions (Chen et al., 2003). Hypoxia-induced regulation of the expression of IL-6 was found to be mediated by C/EBP β in cardiac myocytes but not in fibroblasts (Matsui et al., 1999). CHOP10 expression is also elevated following hypoxia in a HIF1 α -independent manner, however further studies are required to characterize the hypoxia-

induced regulation of CHOP10 (Carriere et al., 2004; Chen et al., 2000).

In addition to mediating the survival response to low oxygen levels, HIF1 α can also regulate other cellular processes, such as growth arrest. For example, hypoxia has been implicated to control cellular differentiation. Recent studies have found that hypoxia can induce cells to dedifferentiate and promote a stem cell-like phenotype (Gustafsson et al., 2005; Helczynska et al., 2003). The differentiation of myogenic and neuronal progenitors was inhibited under hypoxic conditions (Gustafsson et al., 2005). Differentiation could be reinstated in these cells by blocking the activity of the Notch pathway via a γ -secretase inhibitor (Gustafsson et al., 2005). Moreover, HIF1 α was found to activate the Notch pathway by stabilizing its effector, the Notch intracellular domain (NICD) (Gustafsson et al., 2005). These studies suggest that the repression of cell differentiation by hypoxia was mediated by the Notch signaling pathway in a HIF1 α -dependent manner (Gustafsson et al., 2005). Therefore, in addition to regulating the survival response to hypoxia, HIF1 α can mediate different cellular fates by interacting with other pathways.

4. Autophagy

4.1 Forms of autophagy

Degradation of cellular proteins can occur via two pathways, the proteasomal pathway and the lysosomal pathway [reviewed in (Kirkin et al., 2009)]. The proteasome, in conjunction with the ubiquitination of target proteins, degrades regulatory proteins and misfolded proteins that are harmful to the cell (Kirkin et al., 2009). Ubiquitination involves a three-step enzymatic reaction, whereby ubiquitin is covalently linked to its target protein (Kirkin et al., 2009). A ubiquitin E1 activating enzyme initiates the process by

forming a thioester bond with a glycine residue in ubiquitin's C-terminal (Kirkin et al., 2009). The ubiquitin molecule is then transferred to an E2-conjugating enzyme and is finally attached to its target protein via an E3 ubiquitin-protein ligase (Kirkin et al., 2009). The ubiquitinated target protein is then targeted for degradation by the proteasome (Kirkin et al., 2009).

On the other hand, the lysosome contains a variety of hydrolases and is comprised of a "limiting" membrane separating it from the cytosol (Komatsu et al., 2005; Sou et al., 2008). The lysosomal pathway degrades plasma membrane and extracellular proteins via endocytosis and degrades cytoplasmic components through the process of autophagy (Komatsu et al., 2005; Sou et al., 2008). Basal levels of autophagy is essential for the maintenance of cellular homeostasis and the recycling of organelles and long-lived proteins (Hara et al., 2006). Moreover, in yeast and mammalian cells, autophagy can also be induced by conditions of nutrient depletion (Sou et al., 2008). Defects in autophagy have been associated with cancer and neurodegenerative diseases such as Parkinson's disease and Huntington's disease [(Hara et al., 2006); reviewed in (Wang and Klionsky, 2003)].

The term autophagy constitutes of three processes: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy (Kirkin et al., 2009). In microautophagy, the lysosomal membrane invaginates and directly sequesters organelles and proteins for degradation without the formation of autophagosomes (reviewed in Cuervo, 2009; Kirkin et al., 2009). Chaperone-mediated autophagy (CMA) involves the movement of proteins for degradation across the lysosomal membrane via a chaperone protein, HSC70, and is distinct from the other forms for its selectivity as only soluble proteins can

be degraded by CMA [(reviewed in Cuervo, 2009); Yang et al., 2009]. HSC70 is able to recognize and bind a pentapeptide motif found in target proteins (Cuervo, 2009; Yang et al., 2009). The HSC70-substrate complex then interacts with the lysosomal membrane via the lysosome-associated membrane protein type 2A (LAMP-2A) (Cuervo, 2009). The LAMP-2A multimerizes to form a translocation complex, which allows the target protein to enter the lysosome for degradation (Cuervo, 2009).

Macroautophagy is a process by which the cell degrades its own organelles and long-lived proteins by sequestering them in double membrane vesicles/vacuoles known as autophagosomes [Betin and Lane, 2009; (reviewed in Geng and Klionsky, 2008)]. In animal cells, the autophagosome fuses with lysosomes, thereby forming autophagolysosomes, where the contents are hydrolyzed by enzymes (Geng and Klionsky, 2008). Moreover, the degradation of long-lived proteins and organelles by the lysosome via macroautophagy is more convenient than the proteasomal pathway, since the proteasome is restricted to degrade certain types of proteins to accommodate for its narrow opening and specific localization (Kirkin et al., 2009; Wang and Klionsky, 2003). The autophagosome, on the other hand, can accommodate various types of proteins or organelles and deliver them for degradation to the lysosome (Kirkin et al., 2009). The following sections will further elaborate on the process of macroautophagy and it will be referred to as autophagy from this point on.

4.2 Regulation of Autophagy

4.2.1 Pathways regulating autophagy

Autophagy is negatively regulated by the target of rapamycin (TOR) pathway in mammalian cells and yeast [reviewed in (Kondo and Kondo, 2006), (Noda and Ohsumi, 1998)]. Therefore, an inhibitor of the mTOR pathway, rapamycin, is able to induce autophagy in cells (Kondo and Kondo, 2006). mTOR is a serine/threonine kinase that phosphorylates 4E-BP1, which is a repressor of the translation initiation factor eIF4E [reviewed in (Gulati and Thomas, 2007)]. This in turn releases eIF4E to initiate mRNA translation and regulate protein synthesis (Gulati and Thomas, 2007). It also acts as a sensor for nutrient availability and activates another target, ribosomal kinase S6 kinase 1 (S6K1) in response to nutrient levels [reviewed in (Gulati and Thomas, 2007)]. In the presence of nutrients activated S6K1 phosphorylates the downstream target ribosomal protein, S6 (Gulati and Thomas, 2007). The TOR pathway in yeast regulates autophagy by inhibiting the formation of the ATG1p-ATG13p complex (Kamada et al., 2000). TOR induced the hyper-phosphorylation of ATG13p, which reduces its ability to bind ATG1p kinase, an interaction that is required for inducing autophagy (Kamada et al., 2000).

4.2.2 Two conjugation systems involved in autophagosome formation

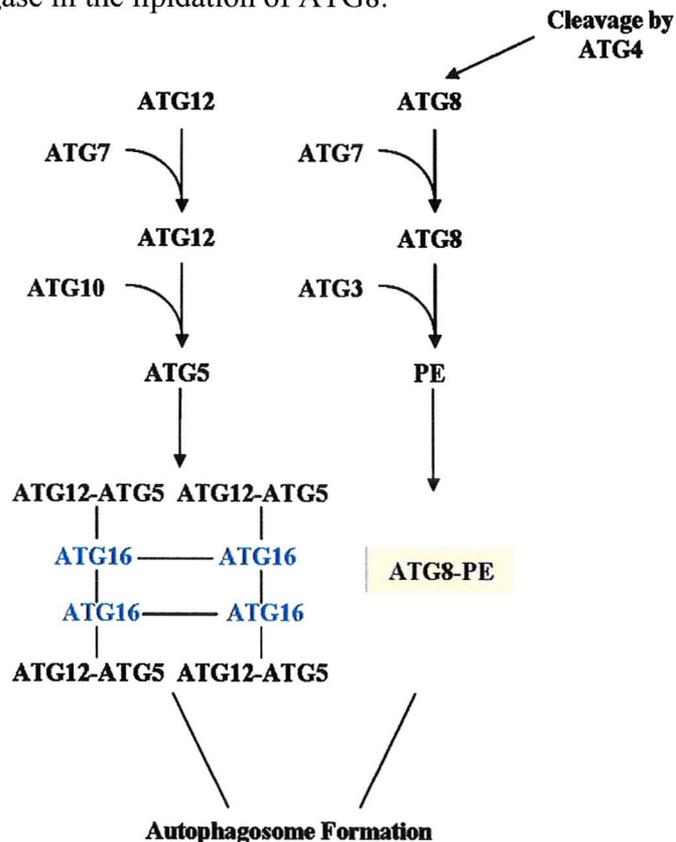
Autophagy involves three processes: nucleation, expansion, and completion [reviewed in (Geng and Klionsky, 2008)]. In yeast, the autophagic process is regulated by a group of evolutionarily conserved genes called the autophagy related (Atg) genes (Geng and Klionsky, 2008). The processes involved in autophagosome formation is similar to the steps involved in ubiquitination (Geng and Klionsky, 2008). Analogous to ubiquitina-

tion, autophagosome formation in yeast consists of two conjugation steps [Fig.7; (Mizushima et al., 1998b)]. ATG12 is activated by an E1-like enzyme, ATG7, which forms a thioester bond with the C-terminal glycine residue of ATG12 (Tanida et al., 2001). ATG12 is then transferred to an E2-like enzyme, ATG10, and is finally conjugated with its target, ATG5 (Mizushima et al., 1998a; Mizushima et al., 1998b). No E3 ligase has been identified in this process, however the ATG12-ATG5 conjugate forms a tetrameric complex with another ATG protein, ATG16 (Geng and Klionsky, 2008). Studies have found that the ATG12-ATG5 complex is essential for the elongation of the isolation membrane, which is also called the phagophore (Sou et al., 2008). The ATG12-ATG5-ATG16 complex was found to localize to the isolation membrane during its elongation, however the complex disassociates prior to the completion of the autophagosome (Fujita et al., 2008; Sou et al., 2008).

Another member of the ATG family, ATG8, is required for the formation and maintenance of autophagosomes (Sou et al., 2008). The ATG8 protein is conjugated to a lipid, phosphatidylethanolamine (PE) [Fig.7; (Kirisako et al., 1999)]. It is initially cleaved by an endopeptidase, ATG4, to expose its C-terminal glycine residue, thereby priming ATG8 for lipidation (Kirisako et al., 1999). ATG8 is linked via thioester bonds to ATG7 followed by ATG3, which serve as E1- and E2-like enzymes respectively, and is finally conjugated to PE (Kirisako et al., 1999). It has been suggested that the ATG12-ATG5-ATG16 conjugate plays the role of an E3 ligase in the lipidation of ATG8 (Fujita et al., 2008; Hanada et al., 2007). The ATG12-ATG5-ATG16 complex is also important for the proper localization of ATG8 (Fujita et al., 2008; Hanada et al., 2007). These two conju-

gation steps are essential for autophagosome formation and ATG8-PE is localized to the autophagosome membrane under conditions of starvation (Kirisako et al., 1999; Sou et al., 2008). Moreover ATG8-PE has been implicated in controlling vesicle size (Sou et al., 2008). Studies conducted on *Atg3*^{-/-} mice indicates that the ATG12-ATG5 conjugate and ATG8-PE conjugate cooperate with each other during autophagosome formation (Sou et al., 2008).

Fig.7 – Two conjugation systems involved in autophagosome formation. Step 1: ATG12 is activated by ATG7 (E1) to form a thioester bond with ATG12 C-terminal glycine residue. ATG12 is transferred to ATG10 (E2) and is finally conjugated with ATG5 via an E3 ligase that has not been identified as yet. Finally, the ATG12-ATG5 conjugate forms a tetrameric complex with another ATG12-ATG5 conjugate through ATG16. **Step 2:** ATG8 is conjugated to phosphatidylethanolamine (PE). ATG8 is first cleaved by endopeptidase, ATG4, to expose its C-terminal glycine residue, in order to prime ATG8 for lipidation. ATG8 is linked via thioester bonds to ATG7 (E1) followed by ATG3 (E2) and is finally conjugated to PE. There is evidence that the ATG12-ATG5-ATG16 conjugate may be the E3 ligase in the lipidation of ATG8.



4.3 Vertebrate homologues of yeast ATG8

ATG8 has four vertebrate homologues, which are MAP1-LC3 (Microtubule-associated protein light chain 3), GATE-16 (Golgi-associated ATPase enhancer of 16 kDa), GABARAP (γ – aminobutyric acid receptor associated protein), and ATG8-like protein (ATG8L) (Sou et al., 2006; Tanida et al., 2001; Tanida et al., 2004; Tanida et al., 2006). GABARAP is important for the trafficking of GABA_A receptors to the plasma membrane, GATE-16 functions in intra-golgi transport, and MAP1LC3 is important in mediating microtubule-cytoskeleton interactions (Kabeya et al., 2000; Paz et al., 2000; Sugawara et al., 2004; Legesse-Miller et al., 1998; Leil et al., 2004). All of these proteins were found to undergo similar processing and are localized to the autophagosome membrane under conditions of starvation (Kabeya et al., 2004; Tanida et al., 2006). Moreover, ATG4B has been shown to prime all four homologues of ATG8 and also to delipidate LC3 and GABARAP (Tanida et al., 2004).

In mammalian cells, LC3 is frequently used as a marker for autophagosome formation (Tanida et al., 2006). The mammalian counterpart of ATG4, ATG4B, cleaves the precursor form of LC3 to expose its glycine residue thus forming LC3-I (Tanida et al., 2004). LC3-I form is converted to the lipidated form, LC3-II or LC3-PE, by mammalian ATG7 and ATG3 (Sou et al., 2006; Tanida et al., 2001; Tanida et al., 2004). Similar to ATG8, LC3-II is attached to the autophagosome membrane and its formation can be induced by starvation (Tanida et al., 2004). The lipidated form of LC3 is also important for the maturation of the autophagosome in higher eukaryotes and it remains associated with the autophagosome inner and outer membranes even after autophagosome completion

(Sou et al., 2008). The inner membrane associated LC3-II is degraded by the lysosome, whereas the outer membrane bound LC3-II is cleaved by ATG4 and the unlipidated form of LC3 is recycled (Tanida et al., 2004). Therefore, LC3 appears to be the functional homologue of ATG8. Although no direct evidence has implicated GABARAP in the process of autophagy, it has been found to have roles in regulating apoptosis and it has also been characterized as a tumour suppressor gene in breast cancer cells (Klebig et al., 2005; Lee et al., 2005). Overall, more work is required to characterize the functional role of GABARAP during autophagy.

To study autophagy in model systems, accumulation of autophagosomes is confirmed by studying the localization of endogenous LC3 from the cytosol to autophagosome membrane in the form of punctate dots and the expression of LC3-II form by western blotting analysis (Vazquez-Martin et al., 2009). To determine autophagic flux, the expression of p62/sequestosome-1 is studied, a protein that links LC3 to substrates being degraded, such that a reduction in p62 levels indicates the occurrence of autophagy (Vazquez-Martin et al., 2009). The use of autophagy inhibitors is also an effective tool in dissecting its functions in different scenarios (Vazquez-Martin et al., 2009). 3-methyladenine (3-MA) is an autophagy inhibitor that restricts the formation of the pre-autophagosomal structure (PAS) (also known as the isolation membrane), whereas bafilomycin A1 (Baf A1) works by inhibiting the fusion of the autophagosome to the lysosome (Vazquez-Martin et al., 2009). The best way to characterize the occurrence of autophagy is to use electron microscopy to detect the formation of double-membraned autophagosomes.

suggesting that autophagy caused by ER stress is regulated differently than amino acid depletion-induced autophagy (Ogata et al., 2006).

In some cases autophagy is also thought to promote cell death and hence is known as Type II programmed cell death, whereas apoptosis is called Type I programmed cell death [reviewed in (Thorburn, 2008)]. Autophagic cell death is distinct from apoptosis in that the cytoskeleton remains intact for a longer period, while the organelles are degraded earlier on (Thorburn, 2008). In contrast, apoptotic cell death involves the breakdown of the cytoskeleton before the degradation of organelles, thereby allowing the organelles to remain intact (Thorburn, 2008). Overall, although there are examples of autophagy as death promoting process, autophagy predominantly presents a mechanism that can protect cells from stress-induced apoptosis.

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Objectives

G_0 or quiescence is an actively maintained state in cells that is distinct from the state of active proliferation. The C/EBP family of transcription factors have been shown to regulate many cellular processes, such as proliferation, differentiation, and growth arrest. Thus, our hypothesis is that the C/EBP family are critical regulators of growth arrest and quiescence-specific gene expression in chicken embryo fibroblasts (CEF).

The overall objective of my Masters thesis was to characterize the role of C/EBP β in regulating quiescence-specific gene expression in CEF. Chapter 1 of the results section addresses the function of C/EBP β in regulating CEF survival during conditions of serum-starvation. Chapter 2 discusses the role of C/EBP β as a regulator of quiescence-specific gene expression in CEF, particularly in response to quiescence.

Results

Chapter 1: GABARAP is a novel determinant of apoptosis in starved chicken embryo fibroblasts

In this chapter, we discuss the potential players that regulate survival of chicken embryo fibroblasts (CEF) during serum-starvation. Studies by former PhD student, Dr. Scott Maynard, showed that CEF expressing the dominant negative mutant of the transcription factor C/EBP β (designated $\Delta 184$ -C/EBP β) were able to survive through extended periods of serum-depletion. These cells were also able to resist apoptosis upon long-term starvation, thereby indirectly suggesting that C/EBP β may be involved in regulating apoptosis in starved CEF.

Cells expressing $\Delta 184$ -C/EBP β also displayed pronounced features of autophagosomes and autophagolysosomes formation. These indicate that autophagy is occurring in these cells, possibly to promote cell survival during extended period of starvation. Furthermore, $\Delta 184$ -C/EBP β expressing cells also expressed higher levels of GABARAP, a vertebrate homologue of the essential autophagy related protein, ATG8. ATG8 is essential for autophagosome formation and resistance to starvation in yeast. Therefore, we asked whether the expression of GABARAP was vital for promoting the survival of $\Delta 184$ -C/EBP β expressing CEF during starvation.

Initial experiments confirmed that over-expression of $\Delta 184$ -C/EBP β confers a survival advantage to CEF upon starvation. However, the over-expression of GABARAP did not promote autophagy or a resistance to cell death in starved CEF. Instead, knocking down GABARAP expression by shRNA enhanced CEF survival in response to prolonged

starvation, thus suggesting that GABARAP may be a determinant of apoptosis in these conditions. Thus, the mechanism by which $\Delta 184$ -C/EBP β regulates cell survival is still unknown. However, these cells are found to block the expression of another member of the C/EBP family, CHOP10. Since CHOP10 is active mediator of the response to ER stress and has pro-apoptotic functions during various stress conditions, it may negatively regulate CEF survival under starvation conditions. As $\Delta 184$ -C/EBP β expressing CEF block the expression of CHOP10, it could explain the survival advantage seen in these cells.

Preliminary work on this chapter was conducted by Dr. Scott Maynard as he contributed Figs.1(C), 2, and 4. Karen Rethoret collaborated with Dr. Scott Maynard in the analysis of autophagy by electron microscopy (Fig.2). For my part, I performed the over-expression and RNAi studies to characterize the function of GABARAP in starved CEF and my contributions to this chapter include Fig.1(A&B), Fig.3, and Figs.5-10. Shi Yang constructed the RCASBP(A)-GABARAP expression vector used in the GABARAP over-expression studies. Finally, Tetsuaki Miyake generated the CHOP10 antiserum, while Dr. Mark Gagliardi produced the antiserum for C/EBP β that was used in all studies. This manuscript was prepared for publication by Dr. Pierre-André Bédard and me.

**GABARAP is a novel determinant of apoptosis
in starved chicken embryo fibroblasts**

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Summary

We previously reported that the expression of a dominant negative mutant of C/EBP β or the disruption of the C/EBP β gene enhances the proliferation of embryonic fibroblasts. In this report, we show that chicken embryo fibroblasts expressing the dominant negative mutant of C/EBP β (designated $\Delta 184$ -C/EBP β) are also more resistant to starvation-induced apoptosis. Cells expressing $\Delta 184$ -C/EBP β progressively developed features of autophagy associated with the capacity of these cells to remain viable. In agreement with this notion, extensive processing of two members of the ATG8 family of proteins, MAPLC3 (microtubule-associated protein 1 light chain 3) and GABARAP (γ -aminobutyric-acid-type-A-receptor-associated protein), was observed in starved CEF. However, there was a clear distinction in the onset of processing of these two proteins. While processing of MAPLC3 was observed at all times, processing of GABARAP was delayed and only occurred in response to prolonged starvation. Forced expression of GABARAP had little effect on the proliferation and survival of CEF. In contrast, the inhibition of GABARAP by shRNAi partially inhibited CEF proliferation. However, the down-regulation of GABARAP enhanced considerably the survival of CEF subjected to prolonged starvation. Therefore, GABARAP was not required for CEF survival and did not impair the stimulation of autophagy upon prolonged starvation. These results suggest that members of the ATG8 family are functionally different and that processing of GABARAP is associated with the transition from reversible growth arrest to apoptosis.

Introduction

One of the first responses of adherent cells submitted to nutrient starvation or reaching confluence *in vitro* is to exit the cell cycle and to enter a state of reversible growth arrest known as the G₀ phase. A specific set of genes, known collectively as growth-arrest specific (GAS) genes, is activated during G₀ and likely contributes to the establishment of cellular quiescence and the acquisition of properties enhancing cell survival (Goruppi et al., 1997; Harding et al., 2003; Kops et al., 2002; Renaud et al., 1994; Schneider et al., 1988). However, GAS genes appear to be regulated by multiple signals since some genes, such as the p20K lipocalin gene (Gagliardi et al., 2003; Kim et al., 1999), are induced predominantly in response to contact inhibition, while others are activated in response to nutrient/serum starvation (Gustincich and Schneider, 1993; Schneider et al., 1988). We showed previously that the activation of the p20K gene depends on C/EBP β in contact-inhibited CEF (Kim et al., 1999). C/EBP β binds to two sites of the quiescence-responsive unit (QRU) of the promoter and stimulates the expression of p20K in growth arrested cells. Forced expression of C/EBP β is also sufficient to induce p20K expression in cycling cells, thereby indicating that it is a central regulator of this gene.

Little is known about the signalling pathways controlling the induction of p20K or other genes regulated by contact inhibition in fibroblasts. In contrast, the response(s) to nutrient starvation has been characterized extensively in these cells. Amino acid depletion and other stresses induce the phosphorylation of the α subunit of initiation factor 2 (eIF2 α) and the inhibition of translation initiation (Dever, 2002). The decrease in protein

synthesis, resulting from this inhibition, prevents the accumulation of misfolded proteins, reduces the production of endogenous peroxides and attenuates the stress imposed upon the endoplasmic reticulum (ER) (Brostrom and Brostrom, 1998; Harding et al., 2003). Paradoxically, the inhibition of protein synthesis also promotes the expression of ATF-4, a stress-regulated transcription factor controlling the expression of genes involved in amino acid import or metabolism and genes conferring resistance to oxidative stress (Harding et al., 2000; Harding et al., 2003). Therefore, cells in conditions of ER stress modify their pattern of gene expression to access limiting pools of amino acids in the surrounding environment and promote their survival.

Starved cells also depend on autophagy to generate amino acids from internal sources. Autophagy is a conserved process in eukaryotes in which organelles and part of the cytoplasm are sequestered into specialized structures called autophagosomes, which deliver their content to lysosomes or vacuoles for degradation and production of nutrients and energy (Klionsky and Emr, 2000; Levine and Klionsky, 2004). In model systems, where it has been characterized more extensively, autophagy promotes the survival of the cell or organism facing deleterious conditions. In yeast, mutations in a number of genes involved in the formation of autophagosomes [the Autophagy or ATG genes (Klionsky et al., 2003)] result in the quick death of the yeast cell in response to starvation (Ohsumi, 2001; Thumm et al., 1994; Tsukada and Ohsumi, 1993). Likewise, several ATG genes are required for the establishment of dauer diapause, an alternative larval stage ensuring survival of the nematode *C. elegans* under unfavourable conditions (Melendez et al., 2003). More recent studies have confirmed the role of autophagy in the survival of

mammalian cells subjected to nutrient starvation. In particular, Kroemer and collaborators reported that the down-regulation of several ATG (autophagy) genes by siRNAi triggers apoptosis in conditions of nutrient depletion (Boya et al., 2005). Autophagy is also required for survival when cells are cultured in the absence of growth factors (Lum et al., 2005).

Autophagy has been implicated in tissue remodelling during insect metamorphosis, the regression of certain tumours in mammals, and in some neurodegenerative diseases such as Alzheimers and Parkinsons (Anglade et al., 1997; Beaulaton and Lockshin, 1977; Bursch et al., 1996; Cataldo et al., 1995; Jochova et al., 1997; Migheli et al., 1997). Accordingly, autophagy has also been referred to as type II programmed cell death (Schwartz et al., 1993; Schweichel and Merker, 1973). However, the conditions and mechanisms underlying the role of autophagy in cell survival versus programmed cell death remain poorly characterized.

Prolonged starvation and ER stress can also result in the activation of programmed cell death. In these conditions, apoptosis (i.e. type I programmed cell death) is induced upon release of calcium (Ca^{2+}) ions from the ER resulting in the activation of the Ca^{2+} dependent protease Calpain and Caspases (Ferri and Kroemer, 2001; Nakagawa and Yuan, 2000; Nakagawa et al., 2000). In addition, the release of Ca^{2+} ions may cause mitochondrial dysfunction directly, inducing swelling of the mitochondria and the release of cytochrome-c and other pro-apoptotic molecules (Lemasters et al., 1998). In mouse, ER stress initiates a caspase cascade through activation of procaspase-12, which resides on the cytoplasmic side of the ER (Nakagawa et al., 2000). Therefore, like mitochondria, the

ER acts as a sensor of cell stress that governs the activation of cell death pathways.

In the course of our studies on growth-arrest specific gene expression, we observed that a dominant negative mutant of C/EBP β (Δ 184-C/EBP β) confers a survival advantage to starved chicken embryo fibroblasts by blocking apoptosis. In these conditions, CEF expressing Δ 184-C/EBP β developed pronounced features of autophagy, thereby allowing for the investigation of the autophagic process in the absence of programmed cell death. In this report, we investigate the role of a close member of the ATG8 family, γ -aminobutyric-acid-type-A-receptor-associated protein (GABARAP), and establish that it is not required for CEF survival and, in fact, reduces the viability of these cells in response to prolonged starvation. Therefore, GABARAP was associated with the transition from reversible growth arrest to apoptosis.

Materials and Methods

Cells and viruses. Early passages of chicken embryo fibroblasts (CEF) were cultured at 41.5 °C in Richter-improved minimal medium containing insulin and zinc (I⁺ medium, Irvine Scientific, Santa Ana, CA), 5% heat-inactivated bovine calf serum (Cosmic calf serum, HyClone Laboratories, Logan, Utah), 5% tryptose phosphate broth, and 1% L-glutamine, penicillin and streptomycin. For analysis of apoptotic or autophagic morphology, the cells were subjected to nutrient starvation either by allowing confluent cells to use up the nutrients in the medium (cells were not passaged and medium was not changed) or by changing their medium to serum-free medium (medium without serum or tryptose phosphate broth). CEF were infected with recombinant viruses generated with the RCASBP(A) or RCASBP(B) retroviral vectors (Petropoulos and Hughes, 1991). The

retroviral vector RCASBP(B)- Δ 184-C/EBP β was constructed as described previously (Gagliardi et al., 2001). Chicken GABARAP cDNA was obtained from the EST collection of the University of Delaware (clone pnf-b.pk0001.d10). The cDNA was excised by EcoRI/XhoI digestion and subcloned into the EcoRI/SalI site of the Cla12 adaptor plasmid (Hughes et al., 1987), to obtain convenient ClaI restriction sites at both the 5' and 3' ends of the insert. The GABARAP cDNA was subsequently excised by ClaI digestion and cloned into the ClaI site of the RCASBP(A) retroviral vector.

Construction of retroviral vectors for shRNAi expression. The 22 nucleotide target sequences of GABARAP and LC3 genes were chosen using the design tool at www.genscript.com/ssl-bin/app/rnai. The 5' base of the sense strand was altered in all cases so that it mismatched the guide strand base, in order to mimic the structure found in endogenous miRNA30. Hairpins for the first miRNA cloning site were generated by polymerase chain reactions (PCR) using 10ng of each gene-specific oligonucleotide together with 100ng of two generic flanking oligonucleotides G and H in a 50ul reaction using ProofStart polymerase (Qiagen; Table 1). Oligonucleotides A and B were used to generate the hairpin for GABARAP. Oligonucleotide sets C-D and E-F were used to generate two different hairpins for LC3. Using GeneAmp PCR system 2700 (Applied Biosystems), the PCR conditions were as follows: 5 minutes at 95°C followed by 25 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 45 seconds at 72°C. PCR products were purified by the GE Healthcare PCR purification kit, digested with *NheI* and *MluI* restriction enzymes and subcloned into pRFPRNAiC(U6-) miRNA cassette. Subsequently, the miRNA expression cassettes for GABARAP and LC3 were subcloned from

pRFPRNAiC(U6-)-GABARAP and pRFPRNAiC(U6-)-LC3 into RCASBP(A)-RNAi vector [ARK-Genomics; (Wang et al., 2009)] as a *NotI*-*ClaI* fragments.

Table 1: Oligonucleotides used in the construction of GABARAP and LC3 shRNAi vectors

A	GAGAGGTGCTGCTGAGCGACGCTCTTCTTCTTCGTCAACATAGTGAAG- CCACAGATGTA
B	ATTCACCACCACTAGGCAGCGCTCTTCTTCTTCGTCAACATACATCTG- TGGCTTCACT
C	GAGAGGTGCTGCTGAGCGATCATCATTGAACGCTATAAAGTAGT- GAAGCCACAGATGTA
D	ATTCACCACCACTAGGCAGTCATCATTGAACGCTATAAAGTACATCTG- TGGCTTCACT
E	GAGAGGTGCTGCTGAGCGTAACATGAGTGAATTGGTCAAATAGT- GAAGCCACAGATGTA
F	ATTCACCACCACTAGGCACAACATGAGTGAATTGGTCAAATACATCT- GTGGCTTCACT
G	GGCGGGGCTAGCTGGAGAAGATGCCTTCCGGAGAGGTGCTGCTGAGCG
H	GGGTGGACGCGTAAGAGGGGAAGAAAGCTTCTAACCCCGCTATTCAC- CACCACCTAGGCA

Western blotting analysis. Total cell protein extract (50 µg) was prepared in SDS sample buffer, subjected to SDS-polyacrylamide gel electrophoresis and blotted on nitrocellulose membranes (Schleicher and Schuell, BA85). The membranes were blocked in a 5% solution of milk powder dissolved in TBS (20 mM Tris pH 7.6, 140 mM NaCl). Commercial antibodies for GABARAP (M135-3) and LC3 (PM046) were purchased from MBL international (MBL International Corporation, MA) and used at dilutions of 1:200 and 1:100 respectively. Antibodies for ERK-1 (SC-94) and p53-Pab-240 (sc-99)

were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and used according to the instructions provided by the manufacturer. Antibodies for C/EBP β and CHOP-10 were generated as previously described [(Gagliardi et al., 2001), our unpublished results]. This was followed by incubation with a peroxidase-conjugated secondary antibody (Pierce, Rockford, IL) and revelation with a chemiluminescent substrate according to protocols provided by the manufacturer (ECL, Amersham).

Proliferation Assays. Proliferation was analyzed for CEF infected with RCASBP(B) and RCASBP- Δ 184-C/EBP β . Cells were split from confluent 100 mm plates into 24 well microtitre dishes, and incubated until confluent, at which point the media was changed to serum-free medium. The cells were then cultured for 9 days at 41.5°C without further change in medium. Cells were counted in quadruplicate samples, every day for 9 days. Cell numbers were determined using a Coulter counter (Beckman Coulter, model Z2, Coulter Corporation, Miami, Fl.). Simultaneously, RCASBP(B)- Δ 184-C/EBP β CEF were also seeded onto 24-well dishes at 10,000 cells per well in quadruplicates and their proliferation was monitored for 16 days without changing the medium. Proliferation was also ascertained in a similar fashion for CEF infected with RCASBP(A) control and RCASBP(A)-GABARAP retroviral vectors and CEF infected with the GFP and GABARAP shRNAi retroviral vectors. These cells were seeded from 100 mm plates to 24-well dishes at a density of 10,000 cells per well (50,000 cells/well for the GABARAP shRNAi infected cells) in quadruplicates and proliferation was quantified by counting in a Coulter counter over a period of several days. Proliferation curves and the standard error of the mean were generated from the averages of the quadruplet samples.

Northern blotting analysis. CEF were rendered quiescent either by growing them to confluence (contact-inhibited CEF) or by depleting them of serum at subconfluent levels (serum-starved CEF) or by serum-starving them after they were confluent (contact-inhibited and serum-starved CEF). Northern blotting analysis of GABARAP expression in quiescent CEF was performed as described before (Bedard et al., 1987). Briefly, 10 µg of total RNA were loaded per lane and separated on a 1.2% agarose gel containing formaldehyde. The RNA was transferred onto NYTRAN membranes and probed with a radiolabeled cDNA for GABARAP or GAPDH.

TUNEL assays. The *in situ* cell death detection kit (Roche, Catalogue no. 12156792910) was used to identify apoptotic cells according to the instructions provided by the manufacturer. Cells were fixed onto coverslips with 3.6% formaldehyde in 1x PBS for 10 minutes at room temperature and were then permeabilized with 0.1% Triton X-100 in 1x PBS for 3 minutes. After washing the cells three times in 1x PBS, the cells were incubated with Terminal deoxynucleotidyl Transferase (TdT), TMR red labeled dUTP, and 4',6-diamidino-2-phenylindole (DAPI; Sigma, Catalogue no. D9542) solution for 1 hour at 37 °C. After rinsing the slides with 1x PBS and distilled water, they were then mounted onto slides using drops of Aquapolymount (Polyscience, Cat. 18606). DAPI-stained cell nuclei and apoptotic cell nuclei were visualized using fluorescence microscopy. The apoptotic index was determined by calculating the ratio of the total number of apoptotic cell nuclei over the total number of DAPI-stained cell nuclei within a field. This experiment was carried out in triplicate and the standard error of the mean was calculated in each case. Significance between different data points was assessed using an unpaired student t-test.

Electron microscopy. Cells were fixed for 15 minutes in a solution of 3.5% glutaraldehyde dissolved in phosphate buffer saline (PBS), collected by centrifugation and again resuspended in 3.5% glutaraldehyde for 45 minutes. After a 15 minutes rinse in PBS, the cell pellet was embedded in 1.5% agar in PBS and rinsed 2 more times in PBS. Cells were post-fixed in 1% Osmium Tetroxide in PBS for 1 hour, rinsed briefly 2 times in water and then dehydrated in ethanol series up to 100% and left in 100% ethanol overnight. The next day, after a rinse in 100% ethanol, cells were placed in a 1:1 mixture of 100% Ethanol: Propylene Oxide (PO) for 1 hour and then in pure PO for 1 hour. They were then placed in 1:1 PO:Epon 812 for 2 hours and the PO was allowed to gradually evaporate overnight. Fresh EPON was then added and allowed to cure for 48 hours at 60°C. Blocks were sectioned and subsequently viewed in a Philips model 210 transmission electron microscope.

Results

The dominant negative mutant of C/EBP β blocks apoptosis in starved CEF

We previously reported that the expression of a dominant negative mutant of C/EBP β enhances the proliferation of chicken embryo fibroblasts [Fig.1A&B; (Gagliardi et al., 2003)]. In the course of these experiments, it also became clear that the dominant negative mutant, designated $\Delta 184$ -C/EBP β , conferred a survival advantage in conditions of medium/serum depletion as shown in Fig.1C. In this experiment, CEF infected with the control retrovirus RCASBP or with the virus encoding $\Delta 184$ -C/EBP β were grown to confluence and then transferred to serum-free medium. Subsequently, cell numbers were determined on a daily basis. Control cells began to die within two days after transfer to

serum-free medium. In contrast, the number of CEF expressing the dominant negative mutant of $\Delta 184\text{-C/EBP}\beta$ did not vary drastically during the course of the experiment. By day eight, most of the control cells had died, while reduction of the total number of cells was modest in CEF expressing $\Delta 184\text{-C/EBP}\beta$ (Fig.1C). The same result was obtained when CEF were maintained in serum-containing medium for extensive periods of time without replenishment. In these conditions, control CEF reached confluence, became contact inhibited and eventually died as a result of serum-starvation (Fig.1B, data not shown). This was also reflected in the cell morphology. Control CEF subjected to prolonged starvation were rounded, had detached from the plate, and depicted features of cells undergoing apoptosis. In contrast, CEF expressing $\Delta 184\text{-C/EBP}\beta$ remained flat and adherent, even nine days after reaching confluence (Fig.2). To confirm that CEF expressing $\Delta 184\text{-C/EBP}\beta$ were more resistant to apoptosis, TUNEL assays were performed on these cells following starvation. A higher incidence of apoptotic cells was indeed observed in control CEF in conditions of prolonged serum-depletion but not in $\Delta 184\text{-C/EBP}\beta$ -expressing CEF (Fig.3A&B). Therefore, the expression of the dominant negative mutant of C/EBP β reduced the incidence of apoptosis in response to prolonged starvation.

To explain the ability of $\Delta 184\text{-C/EBP}\beta$ expressing CEF to resist apoptosis, we looked into whether there was any obstruction in the activity or expression of the tumour suppressor protein, p53. p53 is a known mediator of apoptosis during cellular stress and C/EBP β has been implicated in regulating its expression and function in a cell-type and context specific manner (Boggs and Reisman, 2007; Schneider-Merck et al., 2006; Yoon

et al., 2007). In human endometrial stromal cells, C/EBP β and p53 were found to directly interact, which caused a reduction in the DNA binding capacity of p53 and inhibited p53-mediated gene expression (Schneider-Merck et al., 2006). This interaction occurred via their C-terminal regions, which includes the basic leucine zipper (bZIP) domain of C/EBP β (Schneider-Merck et al., 2006). Therefore, since the Δ 184-C/EBP β mutant contains an intact bZIP domain, it is possible that Δ 184-C/EBP β can sequester p53 away and interfere with its pro-apoptotic function. However, co-immunoprecipitation studies conducted did not show any interaction between p53 and C/EBP β in cycling Δ 184-C/EBP β CEFs (data not shown). Additionally, in mouse fibroblasts, C/EBP β was shown to bind to the p53 promoter and activate its transcription upon mitogen stimulation (Boggs and Reisman, 2007). These studies show that C/EBP β is also capable of up-regulating p53 mRNA levels prior to entry into S-phase (Boggs and Reisman, 2007). Hence, it is possible that Δ 184-C/EBP β can inhibit p53 expression and promote cell survival. However, Western blotting analysis of actively cycling control and Δ 184-C/EBP β cells showed no difference in the expression of p53 (Fig.3C). Overall, further studies are required to clarify whether C/EBP β regulates p53 expression or function in CEF during starvation conditions.

Induction of autophagy in response to the inhibition of C/EBP β and apoptosis

Δ 184-C/EBP β expressing CEF became extensively vacuolated upon prolonged starvation (Fig.2). While they were more resistant, it is clear from the slow decrease in cell number and the presence of cells positive in the TUNEL assay (Fig.3) that a certain level of apoptosis also occurred in these cells. Rounded cells, detaching from the dish,

were occasionally detected in the monolayer and accounted at least in part for the decline in the number of $\Delta 184$ -C/EBP β expressing CEF. Interestingly, adherent fibroblasts characterized by the presence of vacuoles were also detected in the population of control CEF, suggesting that the same process was occurring in the minority of control cells that did not undergo apoptosis (Fig.2). However, the size and occurrence of vacuoles were clearly increased in CEF expressing the dominant negative mutant of C/EBP β .

We sought to determine if CEF expressing $\Delta 184$ -C/EBP β were undergoing autophagy in response to prolonged starvation thus leading to the inhibition of apoptosis. Accordingly, the ultrastructure of control and $\Delta 184$ -C/EBP β expressing CEF was analyzed by transmission electron microscopy. EM micrographs of control CEF undergoing apoptosis are shown in Fig.4A&B. Condensed chromatin and ruptured mitochondria were detected in the early stages of the apoptotic process in control cells (Fig.4A). At later stages, the apoptotic cell was fully rounded and characterized by dramatic condensation of the chromatin and disorganization of the cytoplasmic content (Fig.4B). None of these features were apparent in CEF expressing $\Delta 184$ -C/EBP β . The chromatin was not condensed and ruptured mitochondria were never observed in these cells (Fig.4C-F). However, mitochondria were found in two locations of the cytoplasm of $\Delta 184$ -C/EBP β expressing CEF. Some of the mitochondria were dispersed in the cytoplasm and were not associated with any particular structure or organelle. Other more electron dense mitochondria were often clustered and closely surrounded by single membrane vacuoles that appeared to be decorated by ribosomes (Fig.4C). This dual distribution of mitochondria was generally observed at early stages i.e. before the formation of the larger vacuoles detected by phase

contrast microscopy (Fig.2D). At later stages, the $\Delta 184\text{-C/EBP}\beta$ expressing CEF were indeed characterized by the existence of large vacuoles filled with amorphous material, referred to as autophagolysosomes or autophagic vacuoles in other systems [Fig.4F & (Klionsky and Emr, 2000; Munafo and Colombo, 2001)]. Smaller double-membrane vacuoles were also observed in proximity of the autophagolysosomes and likely correspond to the autophagosomes delivering organelles and bulk proteins for degradation in the autophagolysosomes (arrowheads in Fig.4E). Taken together, these results suggest that the formation of large vacuoles in the cytoplasm of CEF refractory to apoptosis is the result of autophagy.

Processing of LC3 and GABARAP proceeds with different kinetics in nutrient starved CEF

In yeast, autophagosome formation requires the processing and covalent attachment of phosphatidylethanolamine to the ATG8 protein by a ubiquitin-like conjugation machinery (Ichimura et al., 2000; Ichimura et al., 2004; Nakatogawa et al., 2007). Four different homologues of ATG8 (MAP1-LC3, GATE-16, GABARAP and ATG8L) have been characterized in vertebrates. Thus far, lipidation and localization to autophagosomes have been reported for LC3, GATE-16, ATG8L, and GABARAP, suggesting that ATG8 function is mediated by several proteins in higher eukaryotes (Tanida et al., 2002; Tanida et al., 2003). Western blotting analyses revealed that LC3 and GABARAP are both expressed and post-translationally modified in response to nutrient depletion in CEF (Fig.5). However, the kinetics of lipid conjugation and activation was different for these proteins. While LC3 processing was detected in cycling and nutrient depleted CEF,

GABARAP was not processed until later stages, i.e. when cells displayed advanced signs of starvation (Fig.5).

Furthermore, GABARAP protein levels were elevated in CEF following the expression of the dominant negative mutant of C/EBP β (Fig.5). Induction in GABARAP expression was observed in the unprocessed form and following long-term starvation in the processed form as well (Fig.5A&D-E). These observations suggest that C/EBP β could be negative regulator of GABARAP expression. A more modest induction of LC3 was also noted at later time points in Δ 184-C/EBP β infected cells (Fig.5A-C). The induction of CHOP10, a stress-regulated member of the C/EBP family, was used in this experiment to monitor the extent of ER stress in the cell (Ron and Habener, 1992). Interestingly, the accumulation of CHOP10 was impaired by the expression of the dominant negative mutant of C/EBP β (Fig.5). This suggests that the increased survival conferred by the dominant negative mutant of C/EBP β is likely caused by a decline in the levels of ER stress in the cell.

GABARAP down-regulation promotes CEF survival in response to starvation

Northern blotting analysis showed that CEF rendered quiescent, either by contact-inhibition and/or serum-starvation, showed a modest accumulation of the GABARAP mRNA during the two starvation conditions, regardless of cellular density in comparison to actively cycling CEF (Fig.6). Moreover, GABARAP mRNA expression is diminished in contact-inhibited CEF (Fig.6). Since it was ensured that the contact-inhibited CEF were prevented from becoming starved by constant medium replenishment, these findings suggest that GABARAP transcription is modestly regulated by starvation and re-

pressed during reversible growth arrest due to high cellular density.

Moreover, since $\Delta 184\text{-C/EBP}\beta$ expression cells showed increased autophagosome formation and GABARAP expression post-starvation, we wanted to determine if GABARAP was a promoter of survival in these conditions by promoting autophagy. Hence, two experiments were performed to assess the function of GABARAP in CEF. In the first experiment, GABARAP was over-expressed with the RCASBP retroviral system and CEF proliferation was monitored for several days. As shown in Fig. 7, forced expression of GABARAP had little effect on CEF proliferation and survival following prolonged medium-depletion. In the second experiment, GABARAP expression was inhibited by shRNAi and CEF proliferation was followed for several days, as described above (Fig.8A). In order to control for any deleterious effects of inducing RNA interference, CEF were infected with a RCASBP-shRNAi vector targeting GFP (green-fluorescent protein). The GFP-shRNAi vector expresses GFP and can simultaneously repress its own GFP expression without compromising the survival of these cells (Wang et al., 2009). Therefore, inducing RNAi did not appear to affect CEF proliferation or survival.

CEF proliferation was partially inhibited by the down-regulation of GABARAP but they remained viable for longer periods of time (Fig.8B&C). This result may reflect the fact that CEF expressing the GABARAP shRNAi reached lower saturation densities and therefore were less efficient at depleting the medium of essential nutrients. To address this possibility, CEF expressing the GABARAP shRNAi were seeded at a higher density (5-fold increase) at the beginning of the experiment and compared to control CEF. In these conditions, the GABARAP depleted CEF grew at a comparable rate and

reached a similar saturation density earlier than control CEF. Nevertheless, these cells remained viable for a longer period of time, showing little sign of cell death even ten days after reaching confluence. By that time, few viable cells remained in the control samples (Fig.8B&C).

GABARAP down-regulation diminishes the level of apoptosis in starved CEF

In order to determine if GABARAP shRNAi cells were more viable by resisting apoptosis, TUNEL assays were performed. Following GABARAP knockdown, lower levels of apoptotic cells were observed even after ten days of growth without medium replenishment, whereas control cells showed a large accumulation of apoptotic cells (Fig.9). Moreover, GABARAP shRNAi expressing CEF blocked the expression of CHOP10 following prolonged starvation, thus implying that ER stress is significantly diminished in these cells (Fig.10). Therefore, GABARAP was not only dispensable for CEF survival but, in fact, reduced cell viability in conditions of nutrient depletion. These findings suggest that GABARAP plays a role in the transition from reversible growth arrest to apoptosis.

Discussion

Inhibition of apoptosis by the dominant negative mutant of C/EBP β

We previously reported that expression of the dominant negative mutant $\Delta 184$ -C/EBP β enhanced CEF proliferation, in part, by stimulating the expression and activity of proteins of the AP-1 family (Gagliardi et al., 2001). In this report, we show that $\Delta 184$ -C/EBP β enhances CEF survival by blocking apoptosis (Fig.3). A recent report described

the interaction and inhibition of p53 by C/EBP β (Schneider-Merck et al., 2006). Since p53 binds directly to the C-terminus of C/EBP β , it is possible that Δ 184-C/EBP β blocks apoptosis by interfering with the activity of this tumour suppressor. Indeed, the Δ 184-C/EBP β mutant lacks a transactivation domain but retains the basic region and leucine zipper at the C-terminus of the protein (Kowenz-Leutz et al., 1994). However, several assays failed to detect the interaction of these two proteins, suggesting that Δ 184-C/EBP β functions by a different mechanism in CEF (our unpublished results).

An alternative mechanism to explain how Δ 184-C/EBP β -expressing CEF resist apoptosis could involve Δ 184-C/EBP β -mediated repression of p53 expression. Putative C/EBP binding sites were found in the proximal promoter of p53 and C/EBP β was shown to regulate its expression upon mitogenic stimulation (Boggs and Reisman, 2007; Schneider-Merck et al., 2006; Yoon et al., 2007). However, no difference was observed in p53 levels in actively cycling control CEF versus CEF expressing the dominant negative mutant (Fig.3C). Since the effects of C/EBP β on p53 levels and activity varies depending on the cell type and/or cellular conditions (Boggs and Reisman, 2007; Schneider-Merck et al., 2006; Yoon et al., 2007), it is possible that p53 expression is only activated during conditions of cellular stress in CEF. Therefore, regulation of p53 expression needs to be studied further in Δ 184-C/EBP β expressing CEF under starvation conditions.

We then considered if GABARAP was the mediator of survival in Δ 184-C/EBP β expressing cells, as its expression was induced upon starvation in these cells (Fig 5). However, our studies indicate that the expression of GABARAP is not sufficient to impart a survival advantage in CEF (Fig. 7). Hence, GABARAP is not the effector of sur-

vival in $\Delta 184\text{-C/EBP}\beta$ expressing CEF. A more likely mechanism is that the dominant negative mutant blocks the activity and expression of CHOP10, a stress-inducible member of the C/EBP family (Fig.5A). In agreement with this notion, we found recently that the down-regulation of CHOP10 by shRNAi enhanced CEF survival in response to nutrient depletion (our unpublished results, see Chapter 2). Moreover, MEF nullizygous for this gene are more resistant to drugs that generate ER stress (Zinszner et al., 1998). Since CHOP10 is unable to homodimerize and bind DNA on its own, the dimerization with $\Delta 184\text{-C/EBP}\beta$ would also impair the activity of this protein and, as a result promote survival. However, further studies are required to define the mechanism by which $\Delta 184\text{-C/EBP}\beta$ mutant blocks apoptosis and promotes survival in serum-starved CEF.

Regulation and role of GABARAP in autophagy

CEF expressing $\Delta 184\text{-C/EBP}\beta$ develop extensive features of autophagy, as demonstrated by the presence of large autophagolysosomes and double-membrane autophagosomes (Fig.4). This is perhaps not surprising given that these cells are resistant to apoptosis and likely depend on autophagy to remain viable. To characterize the autophagic process, we looked for expression of members of the ATG8 family since this protein is critical for the assembly of autophagosomes in yeast. Two members of the ATG8 family, LC3 and GABARAP, were identified in these cells. Members of this family are activated by processing of the C-terminus followed by lipid conjugation, generating a faster migrating form on gel referred to as LC3-II and GABARAP-II, respectively. While the same machinery is involved in the modification of these proteins (Hemelaar et al., 2003; Tanida et al., 2002), Western blotting analyses indicated that LC3 and GABARAP are

activated with different kinetics. Indeed, LC3 appeared to be constitutively processed while formation of the activated form of GABARAP was only detected in response to prolonged starvation (Fig.5).

To assess the role of these proteins in autophagy, we generated RCASBP vectors expressing shRNAi for LC3 or GABARAP. Despite the fact that several constructs were generated, thus far, all attempts at inhibiting LC3 in a stable manner have failed (our unpublished results). Therefore, LC3 may perform an essential function in CEF, in agreement with the observation that the activated form of this protein is detectable in cycling and growth arrested cells (Fig.5A). In contrast, CEF with complete repression of GABARAP were easily generated with the RCASBP-shRNAi system (Fig.8). While these cells grew more slowly than normal CEF, the main feature of GABARAP repression was the prolonged survival of CEF in conditions of starvation, by resisting apoptosis as shown by TUNEL staining (Fig. 8&9). Therefore, GABARAP was not only dispensable for CEF survival but it also appeared to reduce the ability of these cells to adapt to nutrient depletion. Whether or not GABARAP functions by interfering with the action of LC3 and the assembly of the autophagosome remains to be investigated.

A role for GABARAP in mediating apoptosis has been indicated, since it can interact with two pro-apoptotic proteins, namely Nix/Bnip3L and DDX47 (Lee et al., 2005; Schwarten et al., 2009). Forced expression of GABARAP had no effect but co-transfection of GABARAP and DDX47 cDNAs induced apoptosis in tumour cell lines, suggesting that DDX47 mediates a pro-apoptotic function of GABARAP (Lee et al., 2005). GABARAP expression is reduced in invasive breast carcinoma cells. Forced ex-

pression of GABARAP suppressed the tumorigenicity of these cells in nude mice, suggesting that GABARAP functions as a tumour suppressor (Klebig et al., 2005). Delayed processing suggests a role for this protein in cells subjected to prolonged starvation. Therefore, whether or not the role of GABARAP is to mediate a switch from autophagy and reversible growth arrest to apoptosis is an interesting possibility that remains to be investigated.

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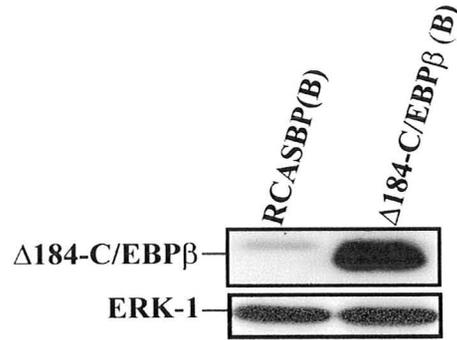
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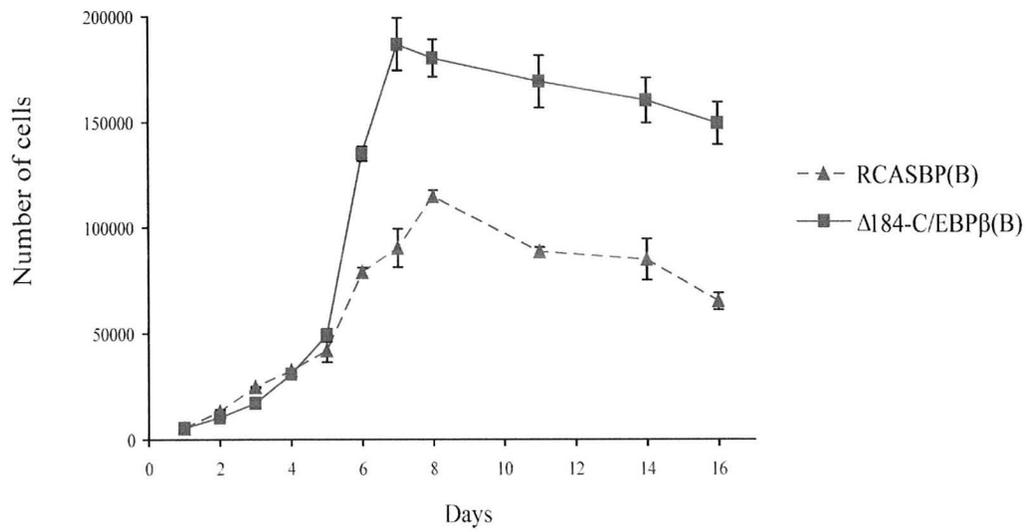
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Fig.1 – $\Delta 184$ -C/EBP β enhances proliferation and survival in starved CEF. **A)** Western blotting analysis showing the over-expression of $\Delta 184$ -C/EBP β (B) in CEF versus control CEF, RCASBP(B). Expression of ERK-1 was used as a loading control. The endogenous protein detected in control RCASBP(B)-infected CEF corresponds to the inhibitory LIP form of C/EBP β . **B)** Proliferation assay of $\Delta 184$ -C/EBP β infected CEF shows that these cells are able to reach a higher saturation density and survive for longer periods of starvation. $\Delta 184$ -C/EBP β (B)-expressing CEF were seeded onto 24-well dishes and their growth was monitored over 16 days without any media replenishment. This was done in quadruplicate and the average number of cells was used to generate the proliferation curve. The error bars shown represent the standard error of the mean. **C)** CEF over-expressing a dominant-negative mutant of C/EBP β have a survival advantage in conditions of serum-depletion. CEF infected with RCASBP- $\Delta 184$ C/EBP β or the control virus RCASBP were seeded onto 24-well dishes and grown to confluence, at which point the media was changed to serum-free media. The CEF were then kept in culture for 9 days without further change of media. Cell counts (cell number/plate) were determined at the indicated days with a Coulter counter from quadruplicate samples. The error bars represent the standard deviation.

A)



B)



C)

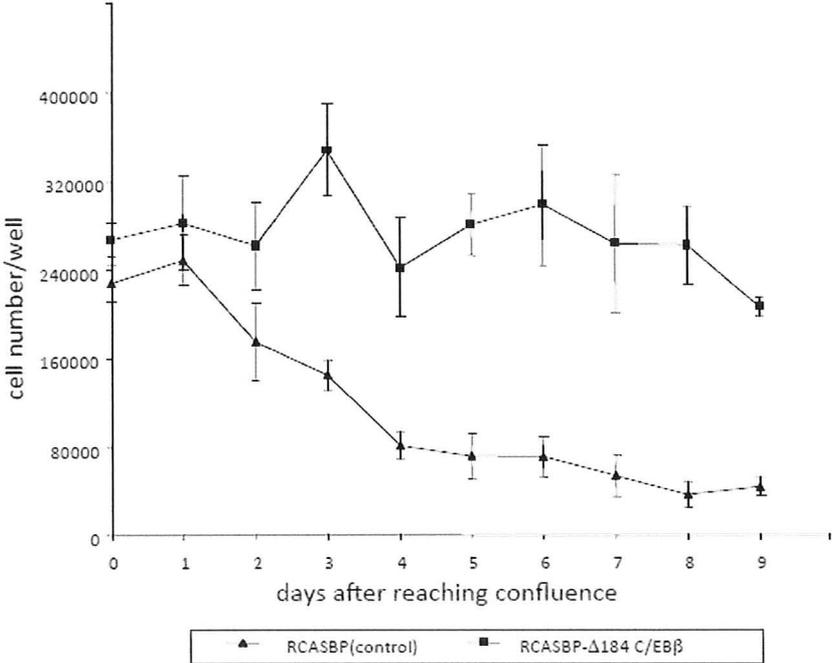


Fig.2 – $\Delta 184$ -C/EBP β CEF show signs of autophagy during starvation. CEF over-expressing the dominant-negative mutant of C/EBP β resist apoptosis and display enhanced features of autophagy in response to prolonged nutrient starvation. CEF infected with RCASBP or RCASBP- $\Delta 184$ C/EBP β were seeded onto 100 mm plates and kept in culture for an increasing number of days without medium replenishment. The term “days post-confluence” refers to the number of days spent in culture after cells have reached confluence. Control CEF infected with the RCASBP virus display signs of apoptosis while CEF infected with RCASBP- $\Delta 184$ C/EBP β remain flat and adherent at 9 days post-confluence (panels **A** and **B**). At 14 days post-confluence (panels **C** and **D**), few control cells remain in the dish while numerous RCASBP- $\Delta 184$ C/EBP β infected CEF are still present on the plate. These cells are highly vacuolated (arrowheads). Few rounded cells are also detected in the culture of CEF infected with RCASBP- $\Delta 184$ C/EBP β (arrows) while cultures of control CEF contain some adherent cells with smaller vacuoles (arrowheads). All photo-micrographs were taken at a magnification of 200X.

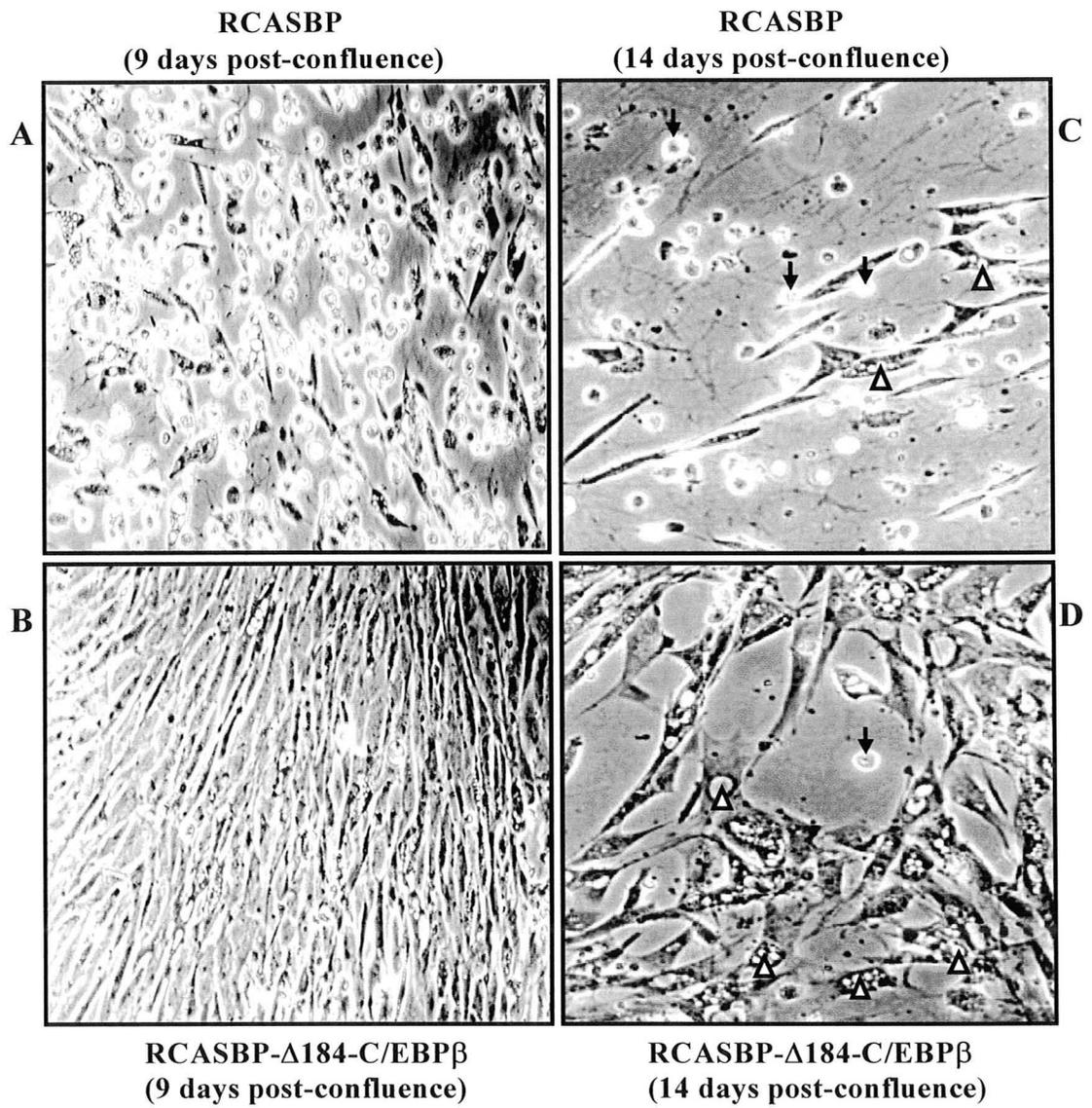
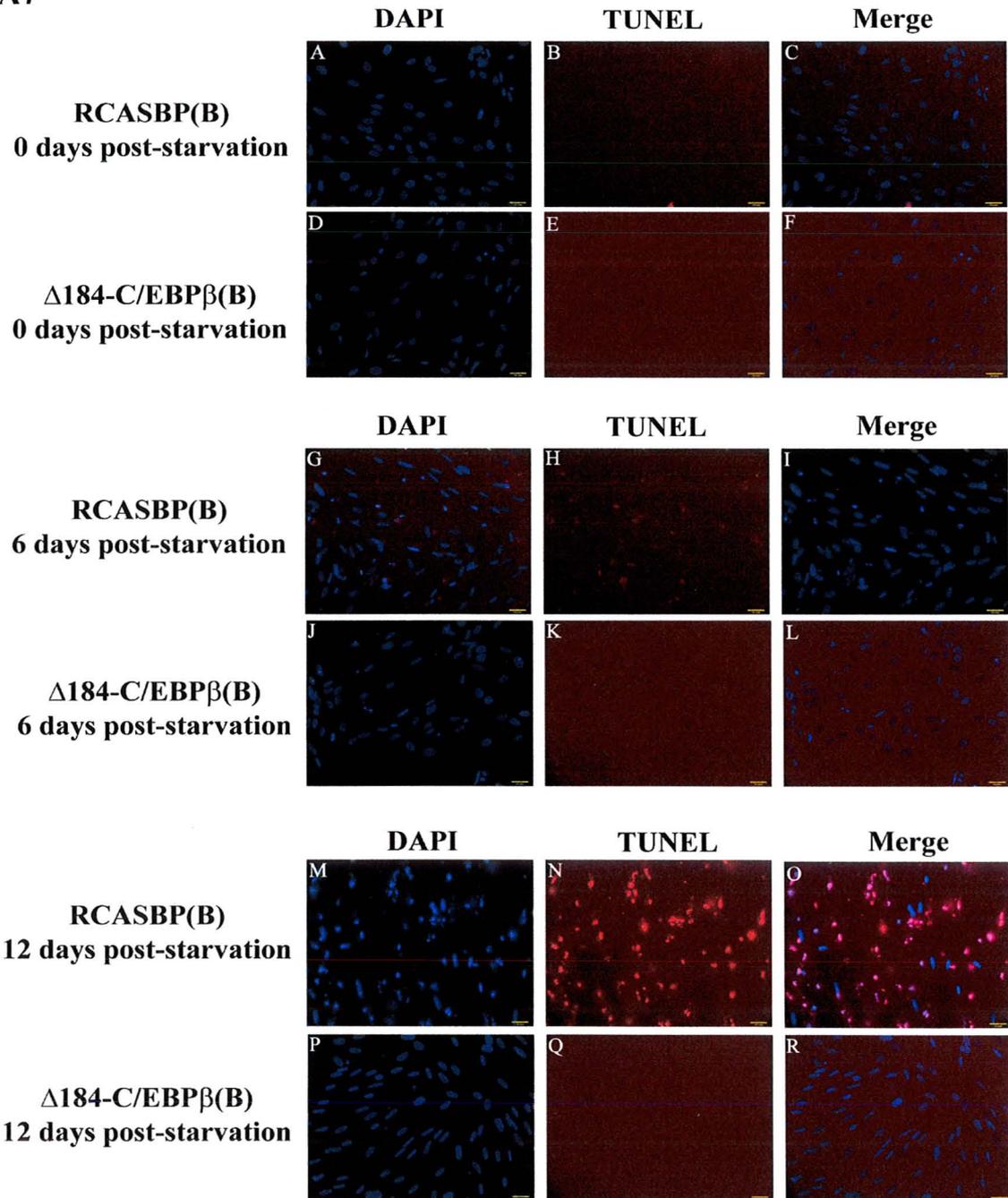
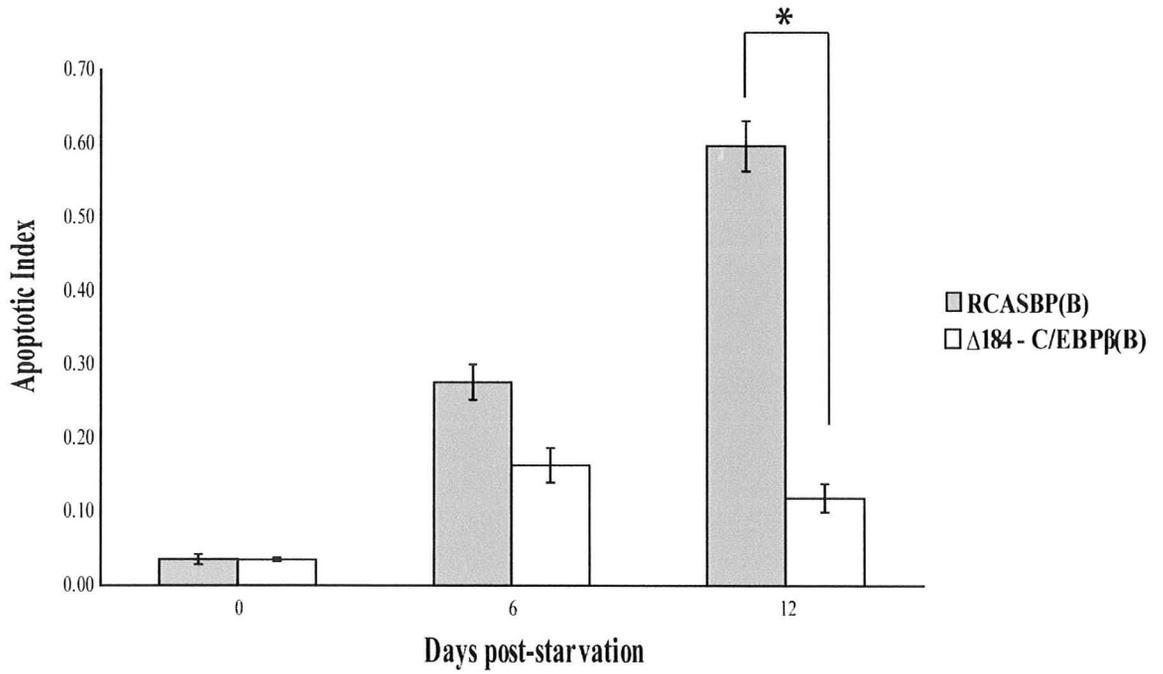


Fig.3 - CEF over-expressing $\Delta 184\text{-C/EBP}\beta$ are resistant to apoptosis. To detect the levels of apoptosis in RCASBP(B)- $\Delta 184\text{-C/EBP}\beta$ expressing CEF that were starved in serum-free media after reaching confluence, TUNEL assays were performed in triplicate on these cells, depicted as $\Delta 184\text{-C/EBP}\beta$ (B), and on control RCASBP(B) expressing cells. **A)** TUNEL assay shows that the rate of apoptosis is significantly reduced in the $\Delta 184\text{-C/EBP}\beta$ cells. Images showing TUNEL positive apoptotic cell nuclei (red) and DAPI- stained cell nuclei (blue) (400X). **B)** The apoptotic index represents the ratio of total number of TUNEL positive nuclei over the total number of DAPI-stained nuclei in control and $\Delta 184\text{-C/EBP}\beta$ cells. We observe a significant reduction in apoptosis in $\Delta 184\text{-C/EBP}\beta$ cells, especially following 12 days of starvation. The ‘*’ indicates that the comparison between the two categories are statistically significant by an unpaired student t-test ($p < 0.01$). **C)** Western blotting analysis of p53 expression in actively cycling $\Delta 184\text{-C/EBP}\beta$ (B)-expressing CEF versus control RCASBP(B).

A)



B)



C)

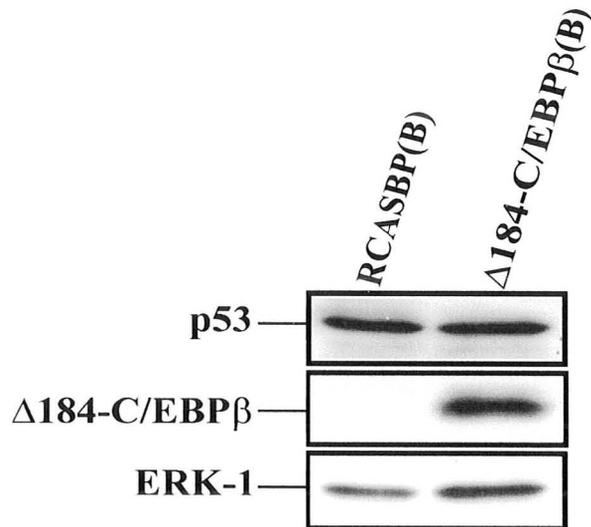
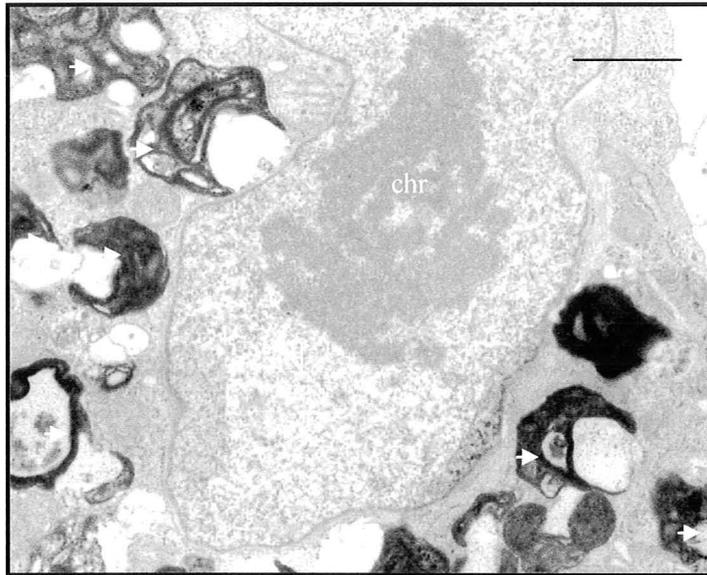


Fig.4 – Electron micrographs of $\Delta 184$ -C/EBP β CEF following prolonged starvation.

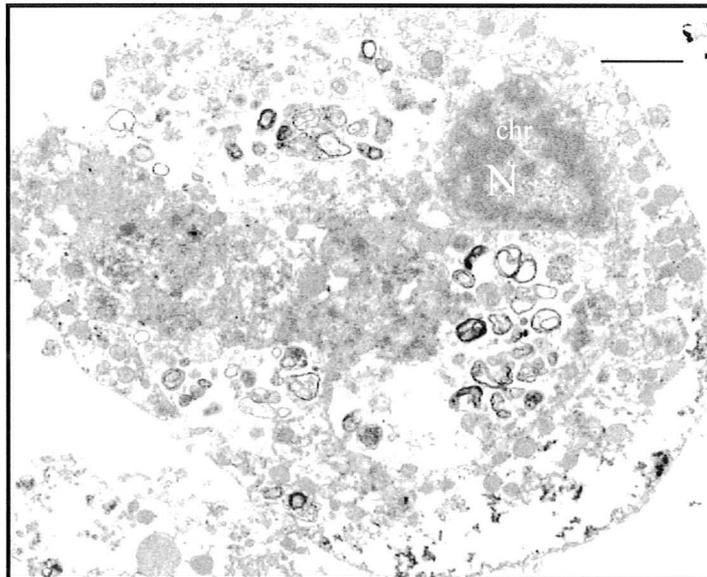
Electron micrographs of control CEF (CEF infected with the RCASBP virus) and RCASBP- $\Delta 184$ C/EBP β infected CEF in conditions of prolonged nutrient starvation. **A)** Control CEF undergoing apoptosis (at day 9 of post-confluence; magnification of 4300X) display ruptured mitochondria indicated by arrowheads. **B)** Control CEF undergoing apoptosis (day 9 of post-confluence; magnification of 2900X). Chromatin condensation (indicated by “chr”) can be seen at the periphery of the nucleus, labeled “N”. **C)** Electron micrograph of RCASBP- $\Delta 184$ C/EBP β infected CEF at day 9 of post-confluence (magnification of 2320X). Electron-dense mitochondria are clustered and surrounded by vacuoles (arrowheads). **D)** RCASBP- $\Delta 184$ C/EBP β infected CEF at day 14 of post-confluence (magnification of 7225X). Membrane-bound organelles are found within autophagolysosomes (labelled “AL”). **E)** Identification of double-membrane autophagosomes (labelled “A”; arrowheads) in RCASBP- $\Delta 184$ C/EBP β infected CEF at day 16 of post-confluence (magnification of 2465X). Larger vacuoles with a single membrane (autophagolysosomes labelled “AL”) are seen in proximity of the autophagosomes. **F)** Amorphous material is seen in autophagolysosomes in RCASBP- $\Delta 184$ C/EBP β infected CEF at day 18 of post-confluence (magnification of 2320X). The scale bars represent 2 μ m in length in panels A, B, C, E and F and 3.5 μ m in panel D.

RCASBP

A

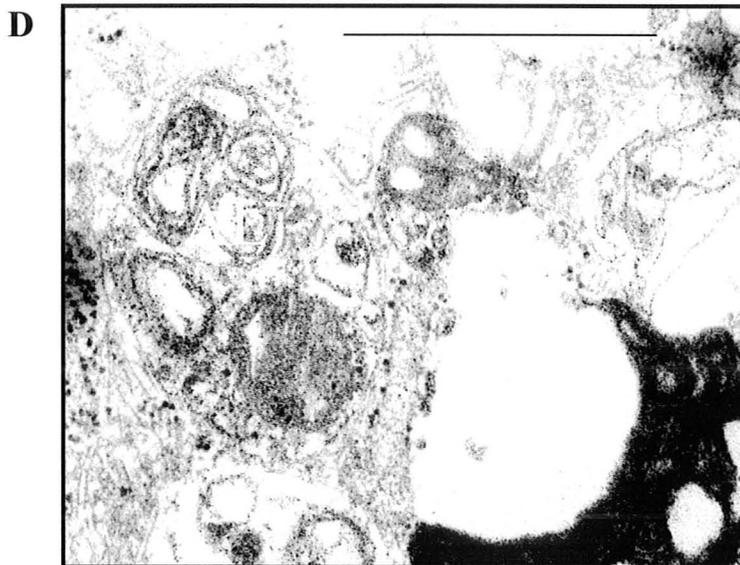
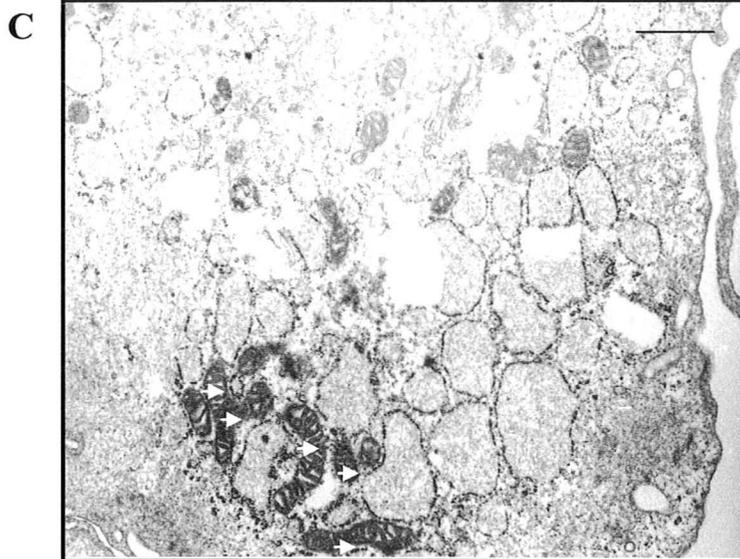


B



RCASBP

RCASBP- Δ 184-C/EBP β



RCASBP- Δ 184-C/EBP β

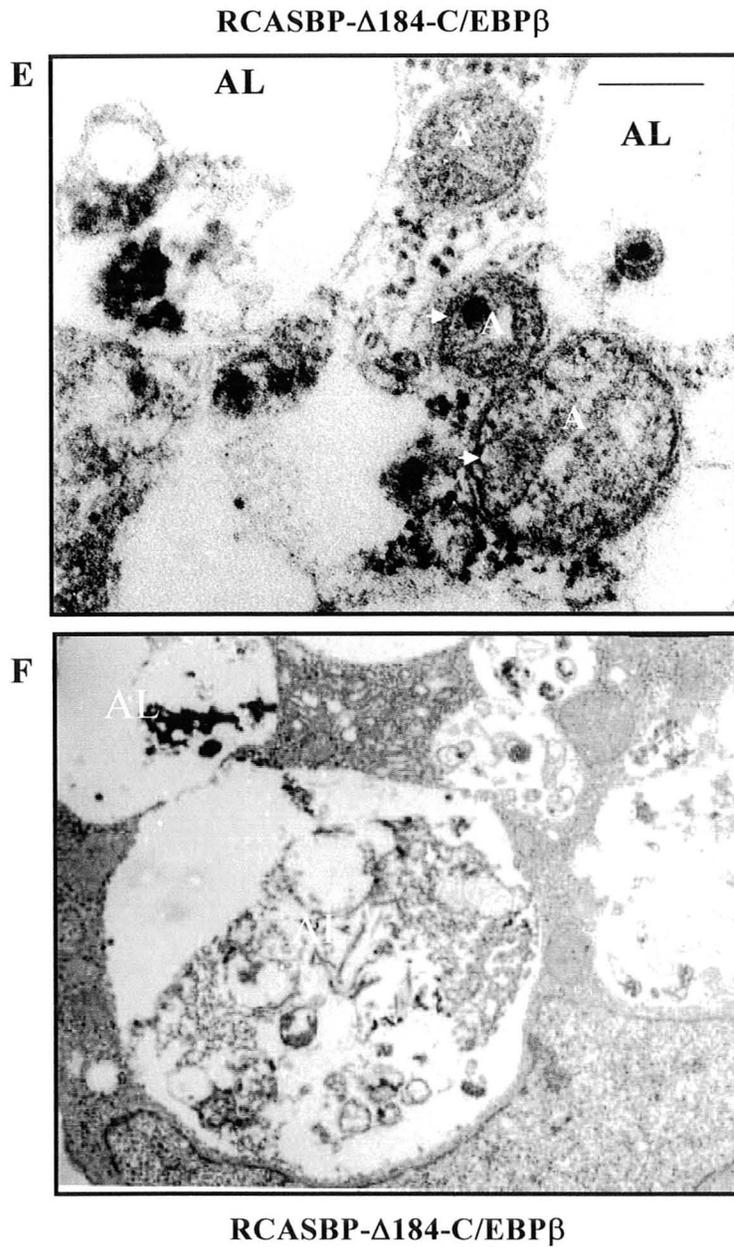
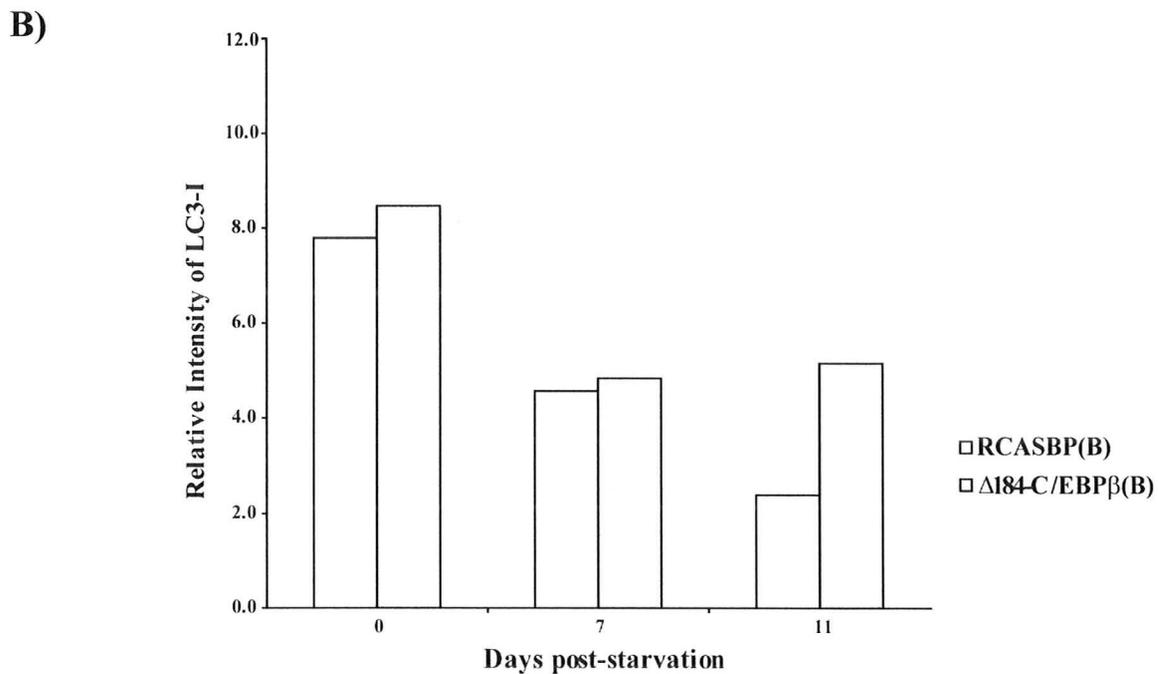
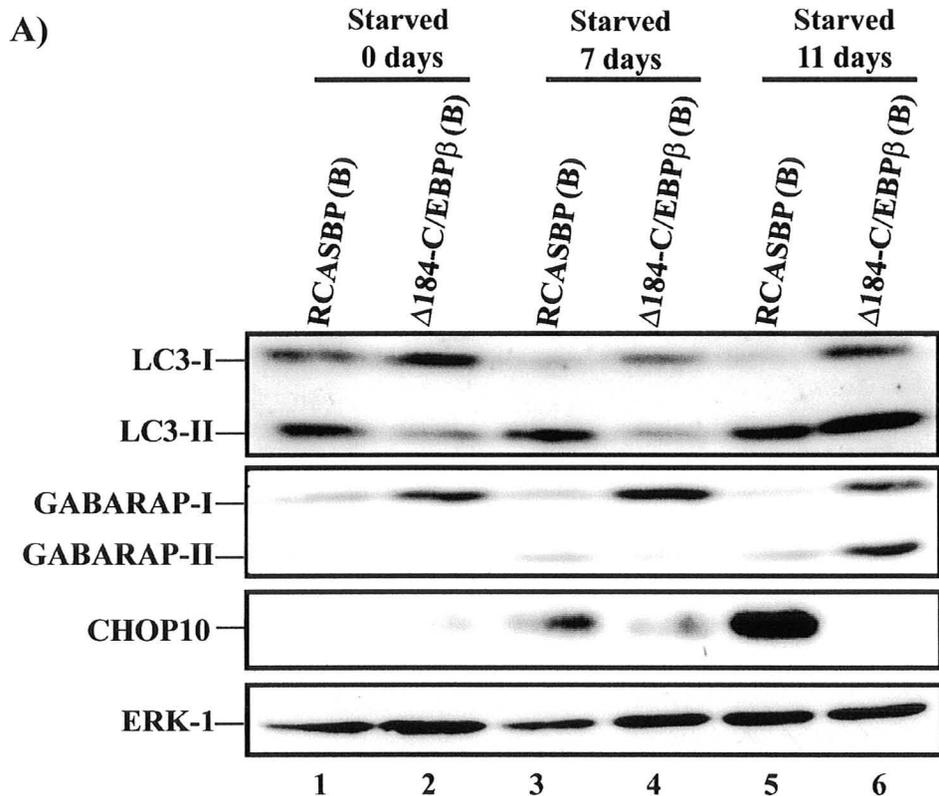
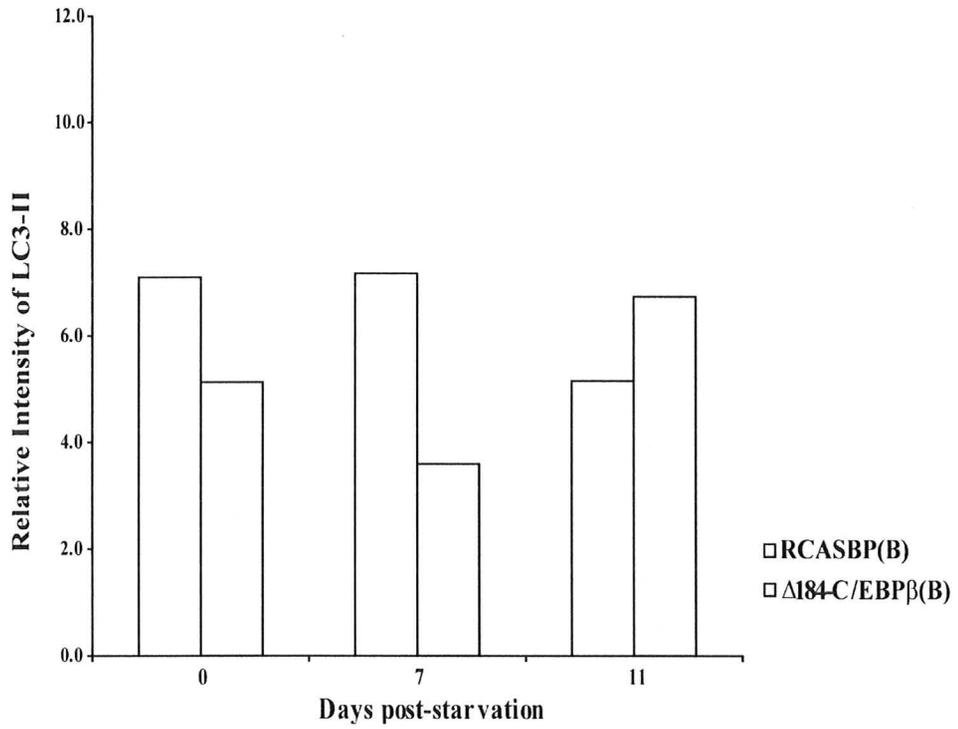


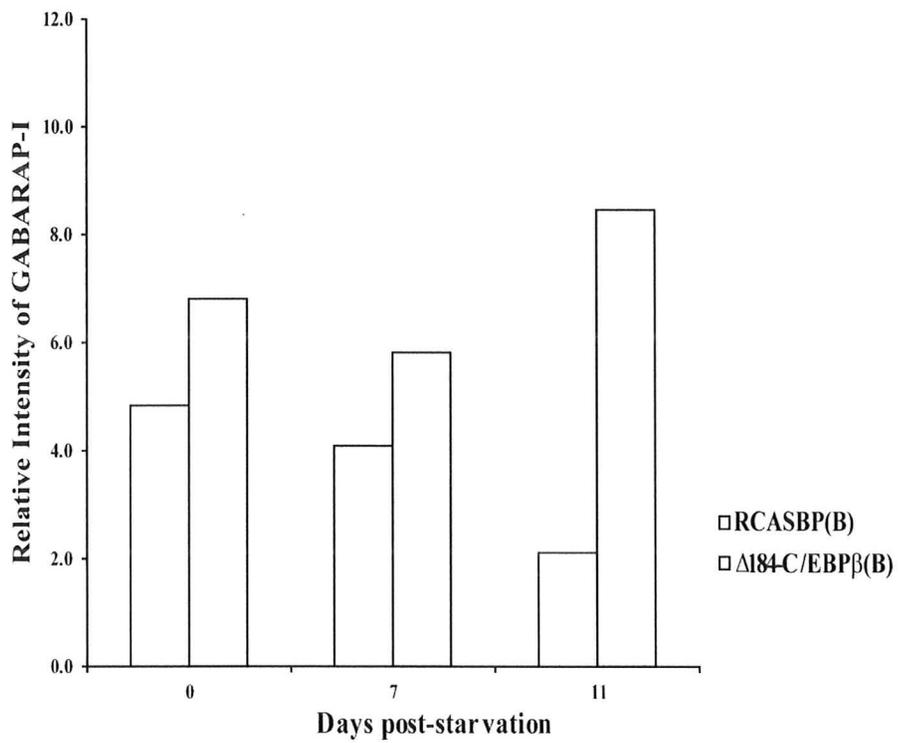
Fig.5 - Processing of two ATG8 mammalian homologues proceeds with different kinetics. **A)** Western blot analyses of RCASBP(B)- Δ 184-C/EBP β infected CEF that were serum-starved over an extended period of time. Cells were starved after reaching confluence in serum-free media for the indicated number of days and protein lysates were collected and probed for the specified anti-sera. Day 0 indicates the day cells were placed in serum-free media. Analyses indicate that the processing of MAP1-LC3 is constitutive, whereas the processing of GABARAP is inducible following starvation. CHOP-10 is used as a marker of starvation and ER stress. CHOP10 expression was inhibited in Δ 184-C/EBP β expressing cells upon prolonged starvation. Expression of ERK-1 was used as a loading control. **B)** LC3-I (unprocessed form) expression levels were quantitated after correcting for ERK-1 levels using the Image J program. **C)** Quantification of LC3-II (processed form) after correcting for ERK-1 levels using the Image J program. **D)** Quantification of GABARAP-I (unprocessed form) protein levels following correction for ERK-1 levels. **E)** Quantification of GABARAP-II (processed form) protein levels following correction for ERK-1 levels using the Image J program.



C)



D)



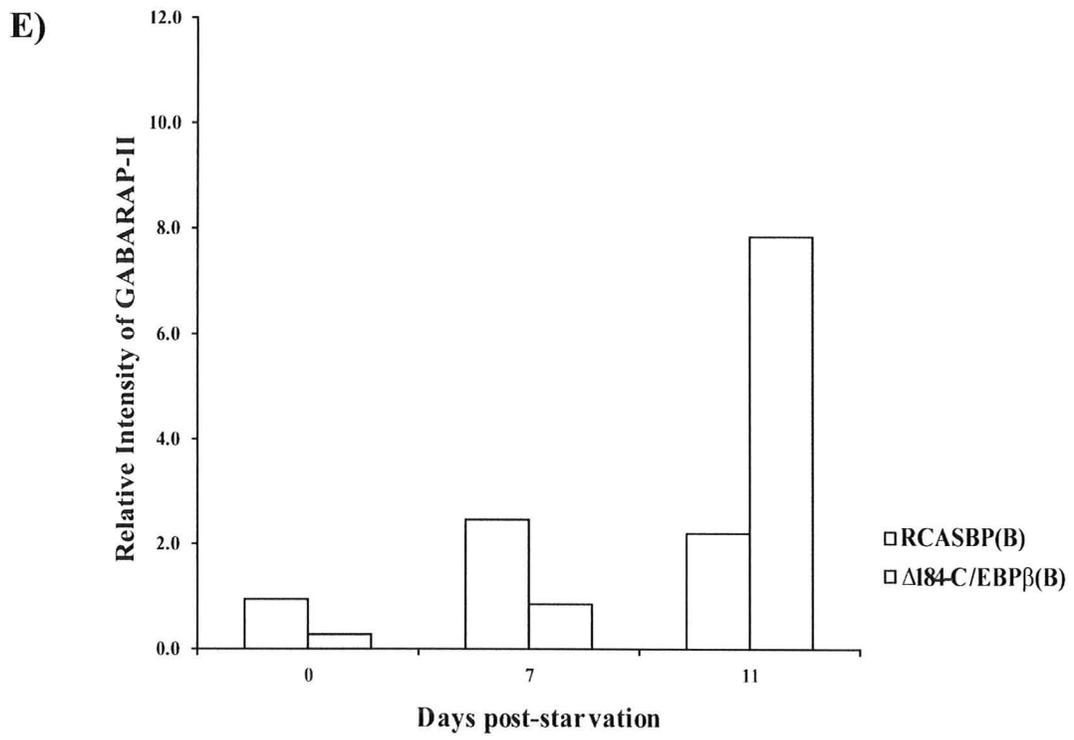
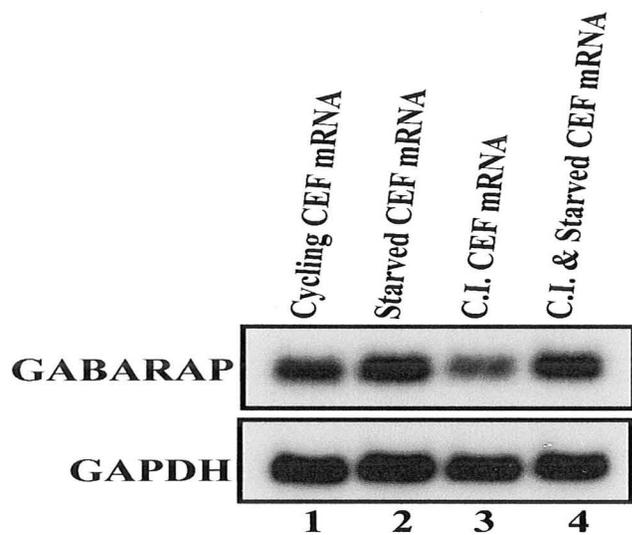


Fig.6 – GABARAP mRNA is up-regulated during starvation conditions. **A)** Northern blotting analysis of GABARAP expression in quiescent CEF shows that GABARAP expression is regulated by starvation rather than contact inhibition. CEF were made quiescent by serum-starvation and/or contact inhibition. GAPDH mRNA levels were used as a loading control. **B)** Quantification of GABARAP mRNA levels after correcting for GAPDH mRNA levels.

A)



B)

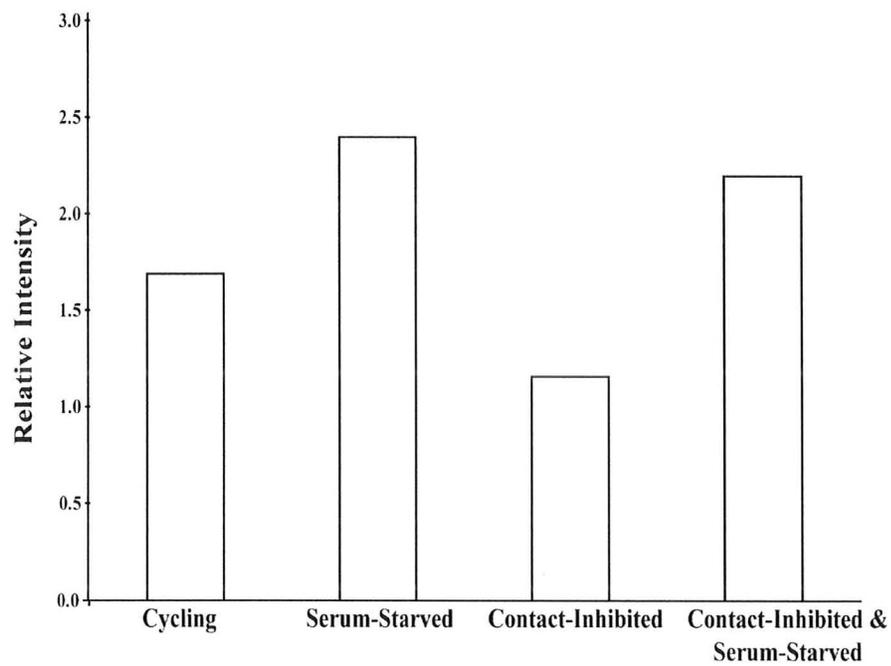
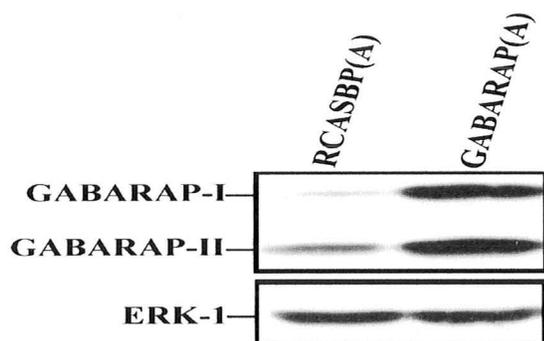
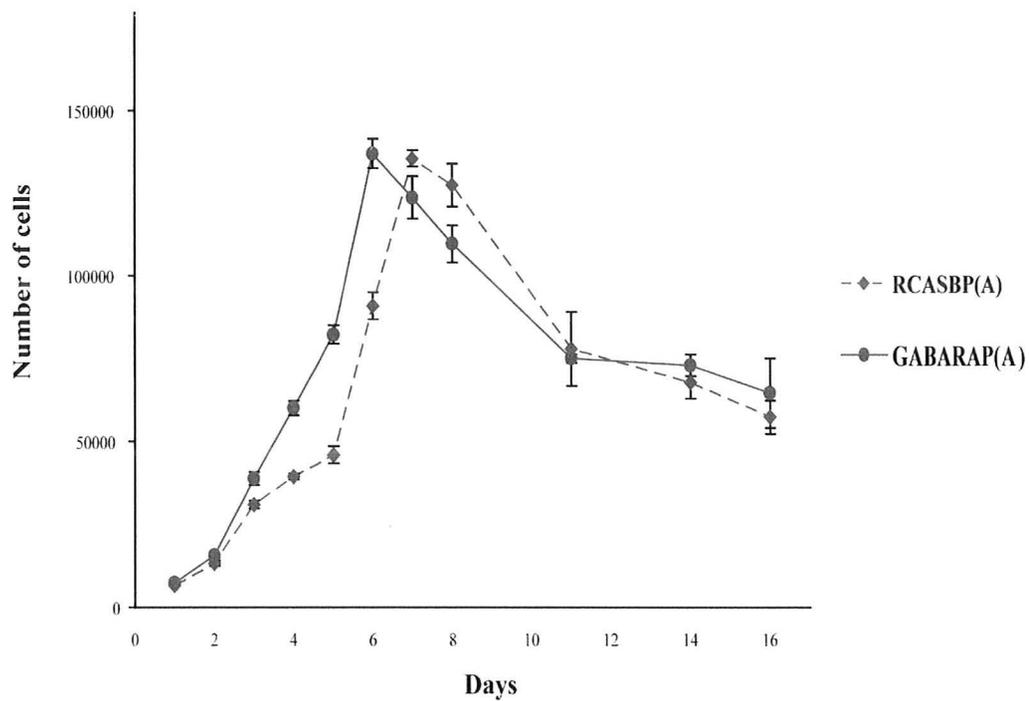


Fig.7 – Over-expression of GABARAP does not affect survival during starvation. A) Western blot analysis of CEF infected with GABARAP over-expression retroviral vector (designated GABARAP(A)) versus those infected with the control virus (RCASBP(A)). Observe the over-expression of GABARAP in GABARAP(A) infected CEF compared to control cells. **B)** Proliferation assay of GABARAP(A) infected CEF, where CEF were seeded onto 24-well dishes and their growth was monitored over 16 days without media replenishment. This was done in quadruplicate and the average number of cells was used to generate the proliferation curve. The error bars shown represent the standard error of the mean. **C)** Western blotting analysis of starved CEF following GABARAP over-expression. CEF were starved after reaching confluence in serum-free media for the indicated number of days and protein lysates were collected and probed for the specified antisera. Expression of ERK-1 was used as a loading control and CHOP10 was used as a marker of ER stress.

A)



B)



C)

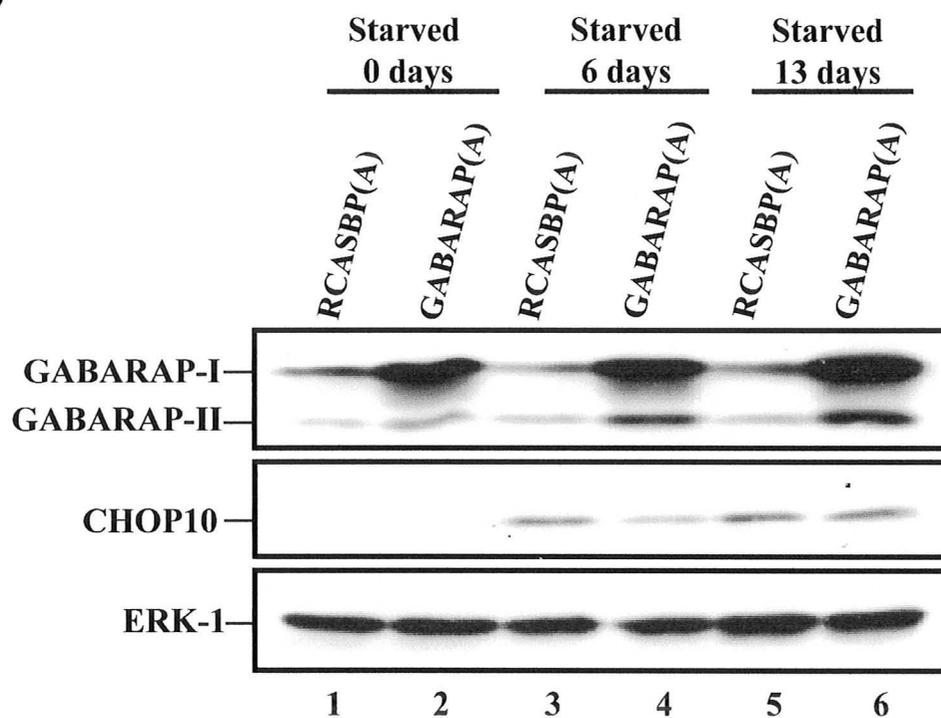
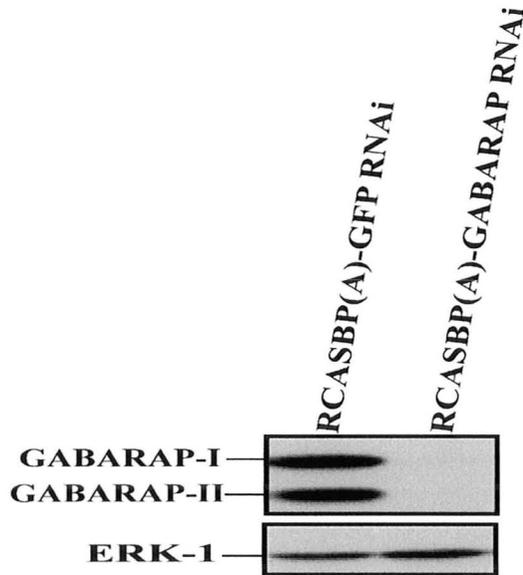
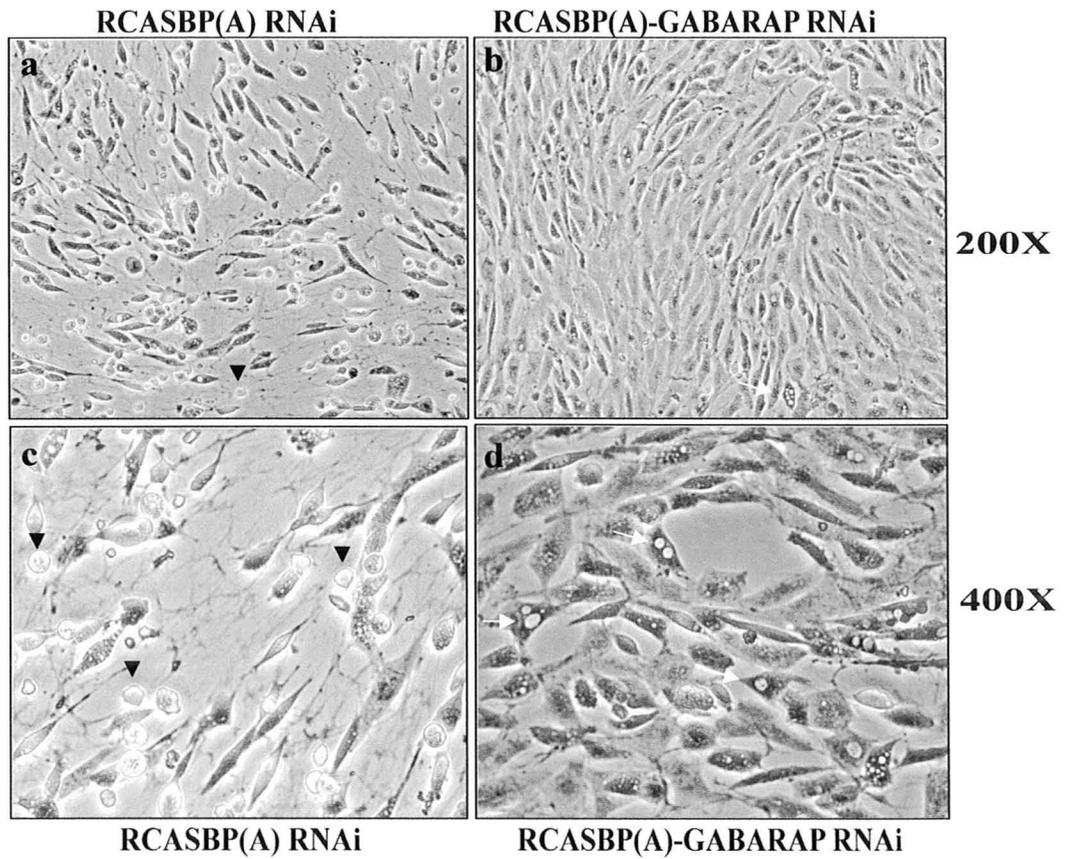


Fig.8 – GABARAP knockdown confers a survival advantage in CEF. **A)** Western blotting analysis showing the knockdown of GABARAP in CEF expressing the RCASBP(A)-GABARAP shRNAi retroviral vector versus control CEF infected with the RCASBP(A)-GFP shRNAi vector. The expression of the GFP shRNAi vector in CEF was used as a positive control for inducing RNAi in these cells. Expression of ERK-1 was used as a loading control. **B)** GABARAP knockdown confers a survival advantage in CEF starved over 9 days. Control cells (labelled RCASBP(A)-RNAi) have high levels of rounded cells (indicated by black arrows in panels a and c). However, RCASBP(A)-GABARAP RNAi infected cells are viable and show the formation of vacuoles in response to starvation (indicated by yellow arrows in panels b and d). **C)** Proliferation assay of CEF following GABARAP knockdown. GABARAP shRNAi infected CEF and control cells were seeded onto 24-well dishes and their growth was monitored over 16 days without media replenishment. This was done in quadruplicate and the average number of cells was used to generate the proliferation curve. GABARAP shRNAi cells were observed to reach a lower population density than control CEF. In order to compensate for the lower saturation density of reached by these cells, GABARAP shRNAi infected CEF were also seeded at five times the density of control cells. In both conditions, GABARAP down-regulation enhanced CEF survival upon prolonged starvation. The error bars represent the standard error of the mean.

A)



B)



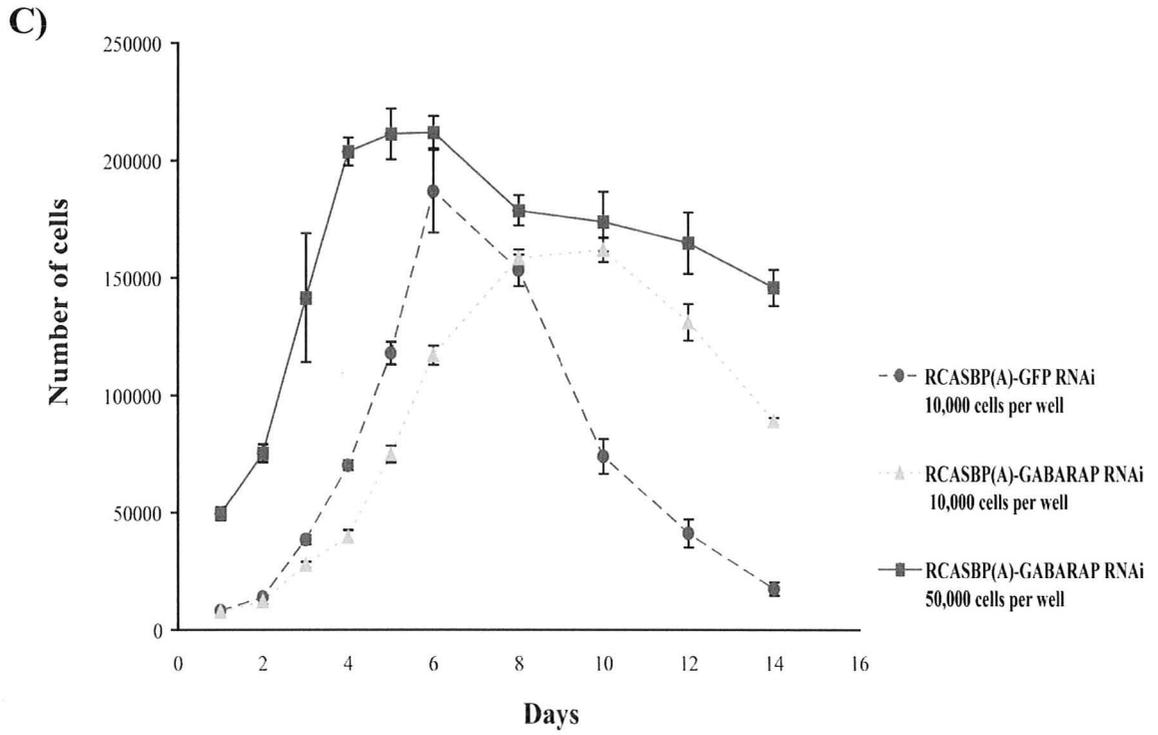
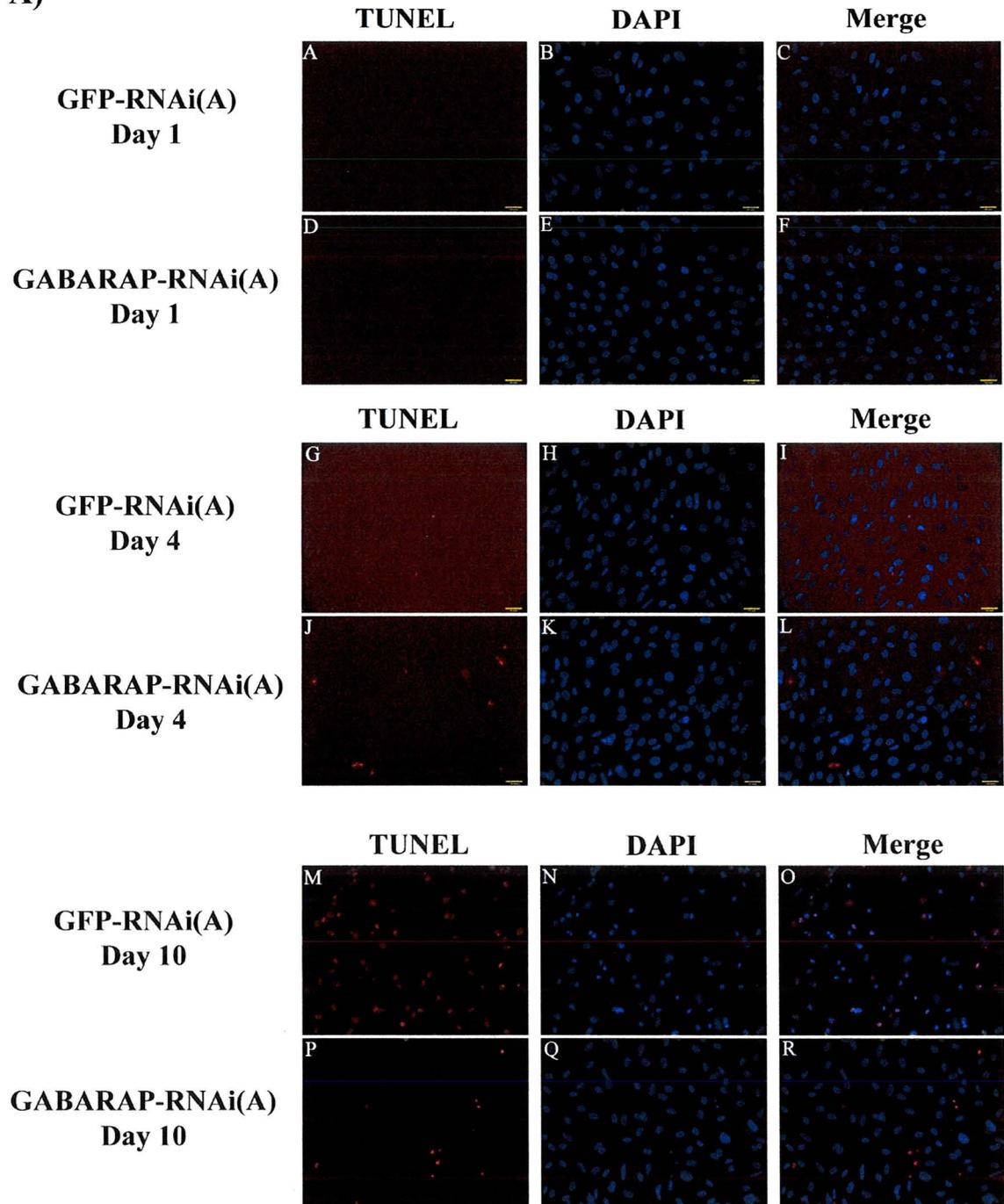


Fig.9 - GABARAP knockdown diminishes the rate of apoptosis during starvation.

To detect the levels of apoptosis in CEF starved without media replenishment after reaching confluence, TUNEL assays were performed in triplicate on GABARAP shRNAi infected CEF and control GFP shRNAi expressing cells. **A)** Images of CEF showing TUNEL positive apoptotic cell nuclei (red) and DAPI stained nuclei (blue) (magnification 400X). **B)** The apoptotic index represents the ratio of TUNEL positive nuclei over the total number of DAPI-stained nuclei in GFP-RNAi (control) and GABARAP-RNAi CEF. We observe a significant reduction in apoptosis in GABARAP-RNAi CEF as seen following ten days of growth without media replenishment. The ‘*’ indicates that the comparison between the two categories are statistically significant via an unpaired student t-test ($p < 0.01$).

A)



B)

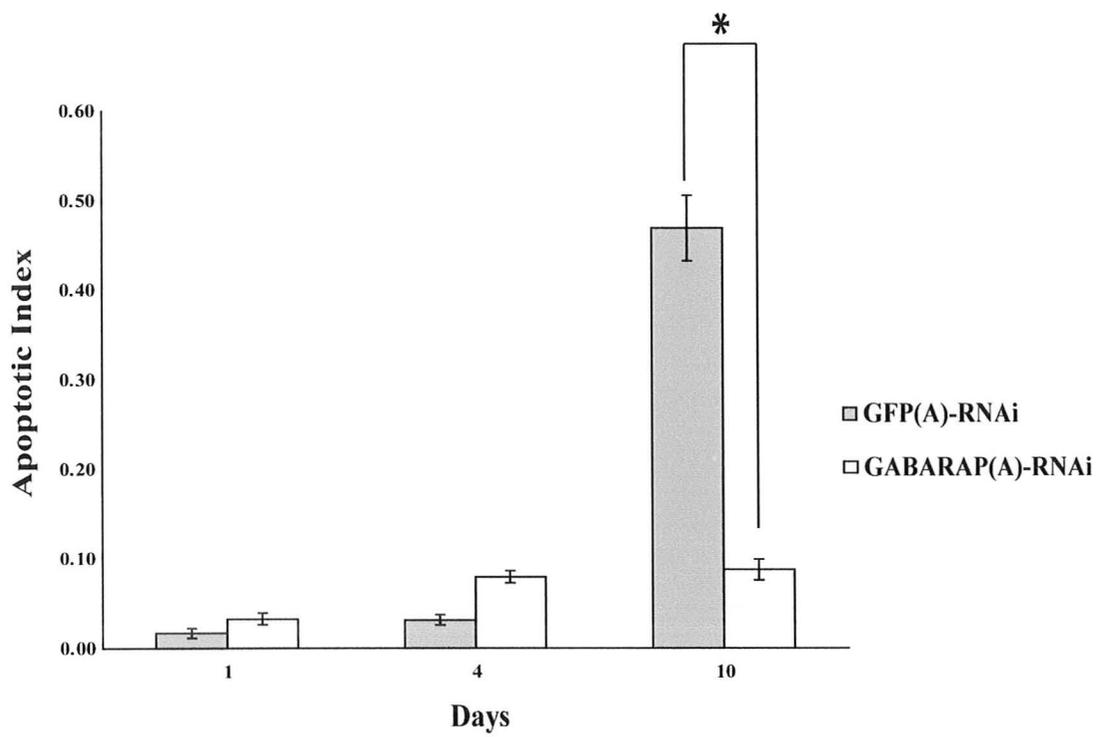
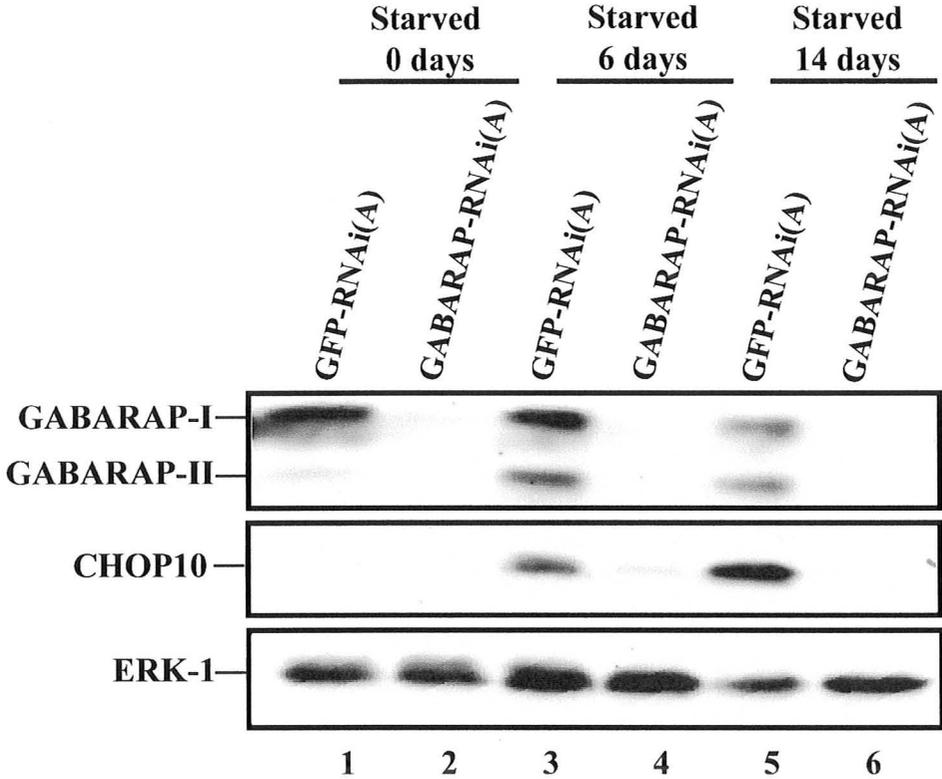


Fig.10 - GABARAP down-regulation blocks CHOP10 levels during prolonged starvation. Western blotting analysis of starved CEF following GABARAP knockdown. CEF were starved after reaching confluence in serum-free media for the indicated number of days and protein lysates were collected and probed for the specified anti-sera. Day 0 indicates the day cells were placed in serum-free media. ERK-1 expression was used as a loading control and CHOP10 was used as a marker of ER stress. CHOP10 levels are diminished upon GABARAP knockdown following prolonged starvation.



Results

Chapter 2: Control of growth arrest-specific gene expression by hypoxia and the C/EBP family

In this chapter, we address the factors involved in regulating growth arrest-specific gene (GAS) expression in CEF. Previous studies by our laboratory characterized p20K as a model GAS gene, whose promoter contains a quiescence responsive unit (QRU). The p20K QRU consists of two CCAAT/ enhancer binding protein (C/EBP) sites and its expression is activated by the transcription factor, C/EBP β , when CEF are growth-arrested by contact inhibition. Moreover, studies by a former Masters student, Tetsuaki Miyake, and our lab technician, Sam Yan, have shown that the expression of p20K is repressed by another member of the C/EBP family, CHOP10.

Additionally, gene-profiling studies conducted on contact-inhibited CEF demonstrated a signature of hypoxia in this condition, which led us to study the effects of hypoxia on p20K expression. Preliminary studies indicate that p20K is positively regulated under conditions of hypoxia. This regulation is also dependent on C/EBP β , as expression of the dominant negative mutant of C/EBP β caused a reduction in the hypoxia-induced levels of p20K. The dominant negative mutant of C/EBP β also repressed the expression of HIF1 α , thereby suggesting that the regulation of HIF1 α levels is C/EBP β -dependent in CEF.

In addition, Masters candidate Ben Fielding showed that the over-expression of CHOP10 repressed the expression of p20K during hypoxia, again reiterating that CHOP10 is a negative regulator of p20K. More studies are required to elucidate the de-

tails concerning the regulation of p20K expression by hypoxia and CHOP10. Overall, in addition to what is already known about the regulation of p20K expression, the data presented in this chapter identifies novel regulatory factors (CHOP10) and conditions (hypoxia) that regulate the expression of p20K GAS gene.

My contributions to this chapter include preparation of the RNA samples used for the gene-profiling microarray studies and I also performed the initial hypoxia studies that showed the hypoxia-induced expression of p20K, which is also C/EBP β -dependent. The tables and figures I supplied to this chapter include Tables 3 to 5, and Figs. 4B, and 5(B-E). The computer-based analyses of the gene-profiling data was performed by PhD candidate Bart Maslikowski and he generated Table 2 and Fig. 4A from these analyses. Bart also conducted the tests to determine the significance of the data presented in Fig.3. Fig. 1 was contributed by Tetsuaki Miyake; Figs. 2 and 3 supplied by Sam Yan; and Fig. 6 by Dr. André Bédard. This manuscript was prepared for publication by Dr. Pierre-André Bédard and me. This project is currently being carried on by Masters candidate, Ben Fielding who contributed Fig. 5A to this chapter.

**Control of growth arrest-specific gene expression
by hypoxia and the C/EBP family**

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Abstract

The p20K lipocalin gene is expressed in chicken embryo fibroblasts (CEF) entering G₀ as a result of contact inhibition. This expression is mediated at the transcriptional level by the interaction of C/EBP β with two elements of a 48 base pair region of its promoter, designated the Quiescence Responsive Unit or QRU. *In vitro*, when the culture media is not replenished, contact-inhibited CEF remain quiescent for a limited period of time, but eventually undergo apoptosis as a result of prolonged starvation. In these conditions, the expression of growth arrest specific (GAS) genes, such as p20K, is quickly repressed. In this report, we demonstrate that the accumulation of the ER stress effector CHOP10, a member of the C/EBP family, mediates the repression of p20K in conditions of prolonged starvation. Gene profiling analyses provided a list of candidate genes expressed preferentially in contact-inhibited but not serum-starved cells. In addition to p20K, this analysis revealed the induction of several genes of the response to hypoxia, thereby suggesting a role for a hypoxia-responsive factor in the regulation of GAS genes. We confirmed that p20K accumulates in conditions of oxygen restriction (2% O₂) and in response to chemicals capable of inducing a state of hypoxia. This induction was abrogated by the expression of CHOP10 or a dominant negative mutant of C/EBP β , thus indicating that the induction of p20K by hypoxia is controlled by the C/EBP family. The expression of HIF1 α , the critical regulator of the response to hypoxia, was also impaired by the dominant negative mutant of C/EBP β . These results suggest the existence of a C/EBP β /HIF1 α axis in the control of GAS genes and reversible growth arrest in contact-inhibited CEF.

Introduction

Cells exiting the cell cycle enter a state of quiescence known as the G_0 phase. The entry into G_0 is poorly characterized but involves the activation of a group of genes referred to as “growth arrest-specific” or GAS genes (Schneider et al., 1988). The role of GAS genes is largely undefined but may contribute directly to growth arrest (Del Sal et al., 1992; Goldstein et al., 1991), to a greater capacity of the cell to survive oxidative stress (Kops et al., 2002), to the synthesis of the extra-cellular matrix (ECM) (Casado et al., 1996; Coppock et al., 1993; Mauviel et al., 1995), the modulation of lipid metabolism (Bedard et al., 1989; Bohmer et al., 1988; Iyer et al., 1999; Provost et al., 1991) and the preparation for re-entry into the cell cycle (Lih et al., 1996; Sang et al., 2008). The regulatory mechanisms of quiescence-specific gene expression are also poorly understood. Some of the GAS genes are regulated at the post-transcriptional level, while others depend on transcriptional activation for expression in response to contact inhibition or serum starvation (Fornace et al., 1989; Krauss et al., 1990; Krauss and Weinstein, 1991; Mao et al., 1993; Mauviel et al., 1995; Smith and Steitz, 1998).

We previously characterized the activation of the p20K lipocalin gene as a model for the induction of GAS genes by contact inhibition. In chicken embryo fibroblasts (CEF), p20K is activated predominantly at confluence and, to a lesser extent, by serum/medium starvation. We identified a 48 base pair region of the promoter, termed the “Quiescence-Responsive Unit” or QRU, required for the activation of this gene at contact inhibition (Mao et al., 1993). C/EBP β binds to two elements of the QRU and, when

over-expressed, is capable of inducing the expression of p20K in cycling cells (Kim et al., 1999).

C/EBP β is activated in response to several stimuli and plays an important role in biological processes unrelated to growth arrest. For instance, we reported that the activity of C/EBP β is induced in CEF transformed by the Rous sarcoma virus (RSV) i.e. in conditions where p20K is not expressed (Gagliardi et al., 2001). We showed that mitogenic stimulation re-programs the cell to direct the activity of C/EBP β towards the expression of genes of the G₀/G₁ transition. Activator protein-1 (AP-1), a factor controlling the expression of IL8 and cyclin D1, inhibits the expression of p20K and thus plays a major role in this process (Gagliardi et al., 2003). Cells over-expressing c-Jun, JunD or Fra-2, the main components of AP-1 in cycling CEF, do not express p20K, are unable to enter G₀, and undergo apoptosis at high cell density (Gagliardi et al., 2003). Normal CEF entering G₀ down-regulate the activity of AP-1 by a number of mechanisms that include the repression of c-Jun, JunD and Fra-2 (Gagliardi et al., 2003). The expression of a dominant negative mutant of C/EBP β blocks the expression of p20K and dramatically enhances the activity and expression of AP-1 proteins in CEF (Gagliardi et al., 2003). Therefore, AP-1 and C/EBP β play opposing roles in the expression of GAS genes and the control of CEF proliferation (Gagliardi et al., 2003).

Our original analysis of the p20K promoter revealed that nucleotides adjoining the C/EBP binding sites are also important for the activity of the QRU in growth arrested CEF (Mao et al., 1993). Moreover, the QRU provides partial activation in conditions of growth arrest, suggesting that additional regulatory elements are located in other regions

of the p20K promoter. Accordingly, we sought to identify conditions and transcription factors interacting with C/EBP β in the control of p20K expression. In this report, we describe the induction of p20K by hypoxia. This induction was abolished by the expression of a dominant negative mutant of C/EBP β , thus implying a role for this factor in the response to hypoxia. Prolonged growth arrest of contact-inhibited CEF leads to starvation, the repression of p20K and, ultimately, the commitment to apoptosis. Cells with these characteristics are in a state known as post-confluence. We also describe a role for CHOP10, a stress inducible member of the C/EBP family, in the repression of p20K and repression of HIF1 α , the central regulator of the response to hypoxia, by C/EBP β . These results suggest the existence of a C/EBP β /HIF1 α axis in the control of GAS genes and reversible growth arrest in contact-inhibited cells.

Materials and Methods

Cell culture and RNA isolation. Early passages (n<10) of CEFs were cultured at 41.5°C in Richter-improved minimal medium containing insulin and zinc (I⁺ medium, Irvine Scientific, Santa Ana, CA) with 5% heat-inactivated (at 57°C for 30 minutes) cosmic calf serum or 5% new-born bovine serum (BioMedia, Cansera, Rexdale, Ontario), and 5% tryptose phosphate broth, and 1% L-glutamine, penicillin, and streptomycin solution (GIBCO BRL) (complete medium). CEFs were also starved in medium without the serum (serum-free medium) after being washed twice with the serum-free medium for varying lengths of time. Hypoxia was induced by culturing CEF in 2% O₂, or by treatment with 100 μ M cobalt chloride (CoCl₂) or 1 mM dimethyloxalylglycine (DMOG) for 24 hours. For the gene profiling studies, cycling CEF RNA was isolated from cells that

were subconfluent and actively dividing. Contact-inhibited CEF RNA was extracted from cells that were allowed to grow to 100% confluence and the medium for these cells was replenished in order to prevent them from becoming starved. Serum-starved CEF RNA was prepared from cells that were allowed to grow to subconfluent and confluent levels in normal MEM medium containing 5% CCS. The medium was then changed to serum-free medium and these cells were starved for a period of 48 hours.

Proliferation assays. CEF were seeded from 100 mm plates to 24-well dishes at 10,000 cells per well in quadruplicate samples. After 1 day of growth, the medium for the cells was changed to serum-free medium. The cells were counted using a Coulter counter (Beckman Coulter, model Z2, Coulter Corporation, Miami, FL.) for a span of 12 days following starvation. The averages of the quadruplicate samples were used to generate the proliferation curve and the standard error of the mean. The significance between certain time points was determined using an unpaired student t-test.

Cloning and plasmids construction. The chicken ApoAI cDNA sequence was amplified by PCR from a chicken quiescence specific λ cDNA library previously described (Bedard et al., 1989) with the primers (5' primer: GAGATGTGGCTGAAGGACACC and 3' primer: GATCCCGCGCCTCCTCAGC) and *Vent* thermostable polymerase (New England Biolabs) at 64°C for annealing and at 72°C for extension. The PCR products were digested by *Bam*HI and *Eco*RI restriction endonucleases, and the purified fragments were inserted into the corresponding sites of the pTrcHis-A vector (GE Healthcare). Inserted DNA was sequenced at the York University molecular biology core facility and verified by DNA alignment with the *Blast* program.

The chicken CHOP10 clone was isolated from the quiescence specific λ cDNA library using as a probe, an EST insert, encoding a partial CHOP10 cDNA (clone pat.pk0050.f8f, chicken EST project, University Delaware). To construct CHOP10 over-expression vectors, the positive cDNA was inserted in the EcoRI site of the Cla12 adaptor plasmid and sub-cloned into the ClaI site of the RCASBP vector (Hughes et al., 1987; Hughes, 2004). In addition, it was inserted into pcDNA3 vector (Invitrogen) at the EcoRI site. The dominant negative mutant of C/EBP β , RCASBP- Δ 184-C/EBP β , was generated as previously described (Gagliardi et al., 2001).

Construction of retroviral vectors for CHOP10 knockdown by shRNAi. To generate CHOP10 shRNAi retroviral vectors, the 22 nucleotide target sequences of the CHOP10 gene were chosen using the design tool at www.genscript.com/ssl-bin/app/rnai. For all targets, the 5' base of the sense strand was altered such that it mismatched the guide strand base, in order to mimic the structure found in endogenous miRNA30. Hairpins for the first miRNA cloning site were generated by PCR using 10ng of each gene-specific oligonucleotide, A and B, together with 100ng of two generic flanking oligonucleotides, C and D, in a 50 μ l reaction using Proof Start polymerase (Qiagen; see Table 1). Using GeneAmp PCR system 2700 (Applied Biosystems), PCR products were amplified under the following conditions: 5 minutes at 95°C followed by 25 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 45 seconds at 72°C. PCR products were purified by GE Health-care PCR purification kit, digested with *Nhe*I and *Mlu*I restriction enzymes, and subsequently subcloned into pRFPRNAiC (U6-) cassette. The miRNA expression cassette for CHOP10 was then subcloned from pRFPRNAiC(U6-)-CHOP10 into a modified

RCASBP(A)-RNAi vector [ARK-Genomics; (Wang *et al.*, 2009)] as a *NotI*-*ClaI* fragment.

Table 1: Sequences of oligonucleotides used to construct CHOP10 shRNAi vectors

A	GAGAGGTGCTGCTGAGCGACAGCTGAGTGCACACAACGAGTAGTGAAGC-CACAGATGTA
B	ATTCACCACCACTAGGCAGCAGCTGAGTGCACACAACGAGTACATCTGTGG-CTTCACT
C	GGCGGGGCTAGCTGGAGAAGATGCCTTCCGGAGAGGTGCTGCTGAGCG
D	GGGTGGACGCGTAAGAGGGGAAGAAAGCTTCTAACCCCGCTATTCACCAC-CACTAGGCA

Northern blotting analysis. RNA samples were extracted from cycling, contact-inhibited, and serum-starved CEF using the TRIZOL reagent. 10 µg of each RNA sample was loaded in each lane of a 1.2% agarose gel containing formaldehyde (Gagliardi *et al.*, 2003). The RNA samples were then blotted onto a Nytran membrane and the RNA was fixed onto the membrane by baking the membrane in a vacuum-sealed oven at 80°C for 2 hours. The membrane was then pre-hybridized with heat-denatured salmon sperm DNA (200µg/mL) in the hybridization solution (50% deionized formamide, 5×Denhardt's, 5×SSC, 5mM EDTA, and 0.1% SDS) at 42°C for 1 hour. The membrane was then hybridized with heat-denatured ³²P-labeled cDNA probe for 16 hours at 42°C. The blot was washed twice with 2×SSC, 5mM EDTA pH8.0, and 0.1% SDS at room temperature for 20 minutes each and then washed twice with 0.1×SSC, 5mM EDTA pH8.0, and 0.1% SDS at 55°C for 20 minutes. Film (Kodak X-OMAT) was exposed to the blot for a few days. p20K expression was used to ensure that cells were contact-inhibited and CHOP10

was used to ensure that cells were serum-starved. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was used to control for even loading.

Gene profiling analyses. Total cellular RNA was isolated and analyzed with the Affymetrix chicken GeneChip representing 32,773 transcripts and 28,418 genes. cRNA synthesis, labeling and microarray hybridization was conducted at the Centre for Functional Genomics at McMaster University (Hamilton, Canada). Probe-set data normalization and expression summaries were generated using the Affymetrix PLIER algorithm. Statistical significance of differences of expression was determined by two-way ANOVA using contact inhibition and serum starvation as factors ($\alpha = 0.05$). Two-fold or greater changes in gene expression were determined by unpaired t-test on all pair-wise comparisons between experimental conditions correcting for multiple testing (Bonferroni-corrected $\alpha = 0.05$). Probe-sets whose mean signal at a given condition did not exceed the minimum signal threshold above background in at least one such condition in the pair-wise tests, were discarded.

GST-Fusion protein purification and antibody production. CHOP10-pGEX-2T vector was transformed into BL21 *E.coli* strain. The production of GST-CHOP10 fusion protein was induced by the addition of IPTG (100mM) for 2 hours. The bacteria were collected by centrifugation and washed with ice-cold PBS. The bacteria were lysed by sonication three times for 20 seconds on ice in 1% Triton X-100 containing PBS (PBS-T). Cell debris was removed by brief centrifugation. The GST-fusion protein was purified according to the instructions provided by the supplier and used to generate rabbit polyclonal antibodies, as described before (Kim et al., 1999).

Western blotting analysis. Total cellular protein was extracted from CEFs, whereupon cells were washed twice in cold 1× PBS and collected in 1× PBS by centrifugation. The cellular pellet was then re-suspended in SDS sample buffer (2.3% SDS, 5% β-mercaptoethanol, 10% glycerol, and 62.5mM Tris-HCl pH 6.8 with 0.5mM phenyl-methyl-sulfonyl fluoride and 0.03% each of Antipain, Aprotinin and Leupeptin) and lysed for 5 minutes at 100°C. Protein concentrations were quantified by Bradford assay and 50-100µg of protein sample were run on 12% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane (Schleicher and Schuell, BA85) by blotting. The membrane was then blocked with 5% skim-milk powder in TBS (5M Tris-HCl and 0.15M NaCl) at room temperature for an hour. After which the membrane was incubated with the indicated primary antibody made in 5% milk in TBS at 4°C overnight with gentle agitation. The primary antibodies used in this study are listed below:

Chicken p20K (601Y)	Previously described (Bedard et al., 1987)
Chicken ApoAI (6531)	Kindly provided by Dr.D.Banerjee (Bhattacharyya and Banerjee, 1993)
Chicken CHOP10	This study
Chicken C/EBPβ	Previously described (Gagliardi et al., 2001)
ERK-1 (SC-94)	Santa Cruz Biotechnology (Santa Cruz, CA)
HIF1α (ab6489)	Abcam

After removing excess primary antibody by washing in TBS a few times, the blot was incubated with secondary anti-rabbit, anti-mouse, or anti-goat IgG antibody conjugated

with horseradish peroxidase in 5% milk in TBS at 4°C for 2 hours. Chemiluminescent signals generated by the enzyme of the secondary antibody in the immuno-complex were detected according to the protocol provided by the supplier (ECL, GE Healthcare) and detected on film (Kodak X-Omat).

Results

Multiple responses are controlled by C/EBP β in growth arrested CEF

CHOP10, also known as C/EBP ζ or Gadd153, is a divergent member of the C/EBP family that cannot homodimerize and must interact with a different member of the C/EBP family, such as C/EBP β , to bind DNA (Ron and Habener, 1992; Sok et al., 1999). Mammalian CHOP10 proteins are unable to homodimerize because two of the Leucine residues of the dimerization domain are located on the adjacent side of the α -helix. The same is true for the putative avian CHOP10 leucine zipper. By comparison, chicken C/EBP β contains five leucine residues perfectly aligned along the axis of the α -helix. Moreover, the charged amino acids adjoining the leucine residues and favouring the interaction with C/EBP β are also highly conserved in avian and murine CHOP10 (Ciarapica et al., 2003). C/EBP factors bind to the core sequence TGCAAT while the C/EBP β /CHOP10 heterodimer shows preference for an extended version of this element (Ubeda et al., 1996). This extended binding site resembles closely to the C/EBP element located at the 5' end of the QRU (Kim et al., 1999). In mammals, CHOP10 is induced in response to serum- starvation and ER stress (Fornace et al., 1989; Wang et al., 1996).

In order to characterize the expression of chicken CHOP10, we performed a series of Northern blotting analyses with RNA extracted from cells in different conditions of

confluence and starvation. In these analyses, starvation was obtained by replacing the complete medium with serum-free media for a period of 24 to 48 hours. The expression of CHOP10 was compared to that of p20K, a marker of contact inhibition, and apolipoprotein A1 (ApoA1), a protein whose expression is induced in response to starvation but not contact inhibition. Transferring sub-confluent, cycling CEF to serum-free media was sufficient to induce the expression of CHOP10 and ApoA1 but not p20K (Fig.1, lanes 1-3). In contrast, contact inhibition of confluent CEF caused the accumulation of the p20K mRNA but had little effect on the levels of the CHOP10 and ApoA1 transcripts in complete medium (Fig.1, lanes 4-6). The transfer to serum-free medium resulted in the induction of CHOP10 and ApoA1 expression and the transient repression of p20K. In these conditions, the addition of fresh medium, even devoid of any serum, is sufficient to stimulate the re-entry of contact-inhibited CEF into the cell cycle (Mao et al., 1993). However, this mitogenic stimulation is limited to one round of cell division and is then followed by growth arrest and the re-expression of p20K mRNA, as shown in Fig.1 (lanes 6&9).

Prolonged culture of confluent CEF without replenishing the media, leads to starvation and eventually cell death due to a lack of nutrients. Cells in this stage of cell culture are said to be in a state of post-confluence. In post-confluent CEF, the expression of CHOP10 is observed but not ApoA1 (Fig.1, lane 7), which suggests that amino acid depletion and ER stress are not significant determinants of the expression ApoA1. Interestingly, the induction of all three growth-arrest specific genes, namely p20K, CHOP10 and ApoA1, was abrogated by the over-expression of a dominant negative mutant of C/EBP β ,

designated $\Delta 184\text{-C/EBP}\beta$ (Kowenz-Leutz et al., 1994). Therefore, the induction of these genes, either as a result of contact inhibition (p20K) or serum-starvation (CHOP10 & ApoA1), was dependent on C/EBP β , thus indicating that multiple responses are controlled by this factor in growth arrested CEF. The over-expression of C/EBP β with the RCASBP retroviral vector was sufficient to induce the expression of p20K and ApoA1 in cycling CEF but not CHOP10 (Fig.1, lane 19). Therefore, C/EBP β was necessary but not sufficient to activate the expression of CHOP10 in CEF.

CHOP10 down-regulation enhances CEF survival and p20K expression

The results of Northern blotting analyses revealed that p20K and CHOP10 are often regulated in an opposite manner. In serum-containing medium, contact inhibition induced the expression of p20K but not CHOP10, while the transfer to serum-free medium enhanced CHOP10 but not p20K expression (Fig.1, lanes 4-5). To characterize the relationship between CHOP10 and p20K expression, CHOP10 was down-regulated by shRNAi and p20K levels were analyzed in cycling, confluent and starved CEF by Western blotting analysis. In the experiment described in Fig.2, CEF reached confluence 4 days after seeding but began to starve and die two days later (i.e. at day 6; data not shown). In these conditions, p20K expression was maximal four days after seeding but declined at later time points (Fig.2, lanes 1-4). In contrast, CHOP10 expression increased primarily in response to starvation i.e. after day 4. Significantly, the down-regulation of CHOP10 enhanced p20K expression at all time points, suggesting that CHOP10 functions as a negative regulator of this gene.

Control CEF and CEF expressing the shRNAi for CHOP10 were also seeded at

high density and then transferred to serum-free medium for increasing periods of time. In both cases, cell numbers began to decline upon prolonged starvation but CEF expressing the CHOP10 shRNAi remained viable for a longer period (Fig.3). Therefore, not only was CHOP10 dispensable for CEF survival but, in fact, it reduced the survival of these cells in the absence of serum. Thus, as described in other species, avian CHOP10 functioned as a mediator of ER stress, limiting the expression of p20K and the survival of serum-starved CEF.

Gene profiling analyses of quiescent cells

Gene profiling was performed to identify genes regulated by quiescence induced by either contact inhibition and/or serum-starvation in CEF. In this study, we compared the pattern of gene expression of cycling, contact inhibited, sub-confluent serum-starved, and confluent serum-starved CEF (Fig.4). A summary of these analyses is provided in Table 2. The most dramatic difference in gene expression pattern was observed between the contact-inhibited and cycling conditions, where a majority of genes was repressed and a smaller portion was activated (Table 2). However, within the subconfluent-starved and confluent-starved conditions, similar portions of genes were either up-regulated or down-regulated in comparison to the actively dividing state (Table 2). The proportion of genes expressed or repressed in confluent-starved cells was almost double the amount of changes seen in subconfluent-starved CEF (Table 2). With respect to contact inhibition, the two starvation conditions showed a similar pattern of expression in the number of activated or repressed genes. Additionally, the smallest difference in the pattern of gene expression was observed between the subconfluent-starved and high cell density-starved

conditions (Table 2). Overall, these findings imply that the two starvation conditions show comparable patterns of gene expression, while contact inhibition stimulates a more distinctive pattern of gene regulation.

However, some genes were also regulated in a similar manner by all three quiescent states. Genes repressed during all conditions include cell cycle genes like cell division cycle associated 8 (CDCA8) and the inhibitors of DNA binding genes (ID1, ID2), (Tables 3-5). Members of signaling pathways are also differentially regulated during quiescence. For example, a member of the Wnt signalling pathway, secreted frizzled related protein 2 (SFRP2) was induced in all quiescent states (Tables 4&5, data not shown), while bone morphogenetic protein 2 (Bmp2) and transforming growth factor β 3 (TGF β 3) of the TGF β pathway were repressed (Tables 3-5).

While there was some overlap in the patterns of genes regulated by different conditions of growth-arrest, several genes are regulated by a specific condition. For example, genes such as p20K and CDK inhibitors (CKI) p27 and p18 were activated primarily by contact inhibition, while others like CHOP10, MAP1LC3C, ARF, and ApoA1 were induced only during serum starvation irrespective of cellular density (Tables 3&4, Fig.4, and data not shown). The CKI p15 was activated under starvation but repressed during contact inhibition, thereby reiterating the observation that the two forms of quiescence are characterized by distinct patterns of gene expression (Tables 3& 4). Interestingly, some of the genes induced most markedly at contact inhibition are also well-characterized targets of HIF1 α and the response to hypoxia (Wenger et al., 2005). This is the case for carbonic anhydrase 9 and 12, as well as enolase 2 (Table 3). In fact, the ex-

pression of the mRNA for HIF1 α , the critical factor responsible for the activation of this class of genes, was also elevated in contact-inhibited but not serum-starved CEF. These observations suggest that contact inhibition in CEF shows a signature of hypoxia.

p20K is induced in conditions of hypoxia

Since p20K is primarily regulated by contact inhibition, we attempted to determine if its expression is also controlled by hypoxia. CEF were maintained in 2% O₂ (hypoxia) for 24 hours and compared to cells in normal laboratory conditions (normoxia). As shown in Fig.5A, CEF expressed elevated levels of p20K when cultured in conditions of hypoxia. Interestingly, this level of p20K was markedly reduced upon over-expression of CHOP10 (Fig.5A, lanes 1-2). This was true in normoxic or hypoxic conditions, and is in agreement with the notion that CHOP10 is a potent inhibitor of p20K expression.

The results of these studies were confirmed by treating CEF with chemicals that induce the hypoxic response such as the specific inhibitor of prolyl hydroxylases (PHDs), dimethylxalylglycine (DMOG) and cobalt chloride (CoCl₂). PHDs hydroxylate HIF1 α at specific residues that allows for its interaction with ubiquitin E3 ligase pVHL, which targets it for proteasomal-mediated degradation. Indeed, p20K levels were also elevated in DMOG-treated CEF while cobalt chloride had a more modest effect (Fig.5B). CEF expressing the dominant negative mutant Δ 184-C/EBP β were also studied to determine if the induction of p20K by hypoxia was C/EBP β -dependent. As shown in Fig.5B, the activation of p20K in 2% O₂ was abolished by the expression of Δ 184-C/EBP β . This suggests that in addition to regulating the expression of p20K during contact inhibition, C/EBP β activity is also required for p20K expression in response to hypoxia.

Regulation of HIF1 α by C/EBP β

Our gene profiling analyses indicated that the expression of HIF1 α is regulated by contact inhibition in CEF. However, HIF1 α is regulated primarily through protein stabilization in response to hypoxia. In these conditions, HIF1 α is no longer hydroxylated on proline residues and recognized by the von Hippel-Lindau (VHL) tumour suppressor, which targets HIF1 α for proteasomal degradation (Wenger et al., 2005). CEF were treated with DMOG to confirm the accumulation of HIF1 α in response to the inhibition of prolyl hydroxylases (Fig.5E). Significantly, the expression of HIF1 α was strongly reduced in the presence of the dominant negative mutant Δ 184-C/EBP β . This was also true in DMOG-treated cells, which suggests that the expression of HIF1 α is controlled by the C/EBP family in CEF.

Discussion

Regulation of growth arrest specific gene expression by hypoxia

The p20K lipocalin is induced rapidly in response to contact inhibition but expressed poorly when sub-confluent cells are serum-starved. This indicates that not all conditions of growth arrest promote the expression of the p20K gene. Gene profiling analyses confirmed that different sets of genes are induced in response to contact inhibition and serum starvation. Since most of the previous studies performed on growth arrest-specific genes have used serum starvation to induce quiescence, it is not surprising that few of the contact inhibition-specific genes identified in our studies have been described before (Coppock et al., 1993; Fornace et al., 1989; Iyer et al., 1999; Schneider et al.,

1988).

One of the distinguishing features of the gene signature of contact-inhibited CEF is the presence of genes induced by hypoxia, including carbonic anhydrase 9, carbonic anhydrase 12 and enolase 2 (Table 3). This result prompted the analysis of p20K in response to oxygen limitations. Western blotting analysis confirmed that p20K expression is induced in CEF cultured in 2% O₂ or treated with chemicals capable of inducing the response to hypoxia, such as the specific prolyl hydroxylase inhibitor DMOG (Fig.5). Whether or not contact inhibition leads to oxygen depletion as a result of the high cell density remains to be investigated. However, we note that only a small subset of the known hypoxia-responsive genes was induced in contact-inhibited CEF, indicating that a typical response to hypoxia was not activated in these cells (Wenger et al., 2005).

Carbonic anhydrases are regulators of intracellular pH, providing protection against reactive oxygen species (ROS). Since lipocalins function in part as lipid scavengers, the induction of p20K may represent an adaptive response to the presence of ROS and peroxidated lipids. A second lipid transport protein, fatty acid binding protein 4 (FABP4), is also part of the genes strongly activated by contact inhibition. These findings suggest that processes involved in lipid metabolism are particularly affected in these conditions of growth arrest. Whether or not the induction of p20K and FABP4 promotes the survival of contact inhibited CEF and is therefore essential for reversible growth arrest remains to be investigated.

Role of the C/EBP family in the control of p20K by hypoxia and contact inhibition

We previously identified C/EBP β as the key determinant of p20K expression in

contact-inhibited CEF (Kim et al., 1999). C/EBP β binds to two elements of a 48 base pair region of the p20K promoter designated the Quiescence-Responsive Unit (QRU). Whether or not the QRU is also required for activation by hypoxia remains to be determined. However, several lines of evidence have implicated the C/EBP family in the control of p20K expression in response to oxygen limitations. First, the induction of p20K by hypoxia was abolished by the expression of a dominant negative mutant of C/EBP β (Δ 184-C/EBP β) or by over-expressing CHOP10, a stress inducible member of the same family, functioning as an inhibitor of C/EBP β (Fig.5A&B). Secondly, the expression of HIF1 α , the central regulator of gene expression in response to hypoxia, was reduced markedly by the expression of Δ 184-C/EBP β (Fig.5C), thereby providing a mechanism which accounts for the inhibition of p20K expression by the dominant negative mutant of C/EBP β . In this scenario, the accumulation of CHOP10 would impair the expression of the hypoxia-dependent program and lead to the repression of p20K and other gas genes, promoting apoptosis.

A recent gene profiling study has provided a list of genes regulated by contact inhibition and by serum starvation (Coller et al., 2006). However, these authors relied on cell sorting to isolate a population of cells with a G1 DNA content, a lengthy procedure requiring the disruption of cell-cell contact. Therefore, it is perhaps not surprising that these authors have failed to identify genes of the hypoxia response in the signature of contact-inhibited cells. They propose that the transcriptional repressor HES1 is critical for the state of reversible growth arrest (Sang et al., 2008). In our experimental conditions, HES1 was not activated by contact inhibition and was in fact repressed modestly in

these conditions (Table 3).

Recent reports have described the interaction of HIF1 α with C/EBP α (Jiang et al., 2005; Yang et al., 2008). The effects of this interaction remain poorly characterized but one report described a cooperative as well as physical interaction between these two proteins in the induction of C/EBP α activity (Jiang et al., 2005). In myeloid leukemic cells, the effects of the C/EBP α /HIF1 α interaction were independent of the recruitment of HIF1 α to its cognate element as down-regulation of ARNT/HIF1 β (the binding partner of HIF1 α) had no effect on the activity of HIF1 α (Janardhan, 2008; Song et al., 2008).

It is possible that a similar interaction between HIF1 α and C/EBP β mediates the activation of p20K by contact inhibition and hypoxia. If so, it remains to be seen if the action of HIF1 α is mediated entirely through the QRU and independently of DNA binding. However, it is also possible that a cooperative interaction between HIF1 α and C/EBP β will require additional elements of the p20K promoter. Hypoxia response elements (HRE) are often composed of a core binding site followed by a 3' ancillary sequence located about 8 nucleotides away (Kimura et al., 2001). Interestingly, a 60 base pair region of the p20K promoter, located about 300 nucleotides upstream of the QRU, harbours two potential copies of this composite element (Fig.6A). Since the larger p20K promoter region is more active in quiescent CEF than the 48 base pair QRU alone (Mao et al., 1993), this observation raises the possibility that HIF1 α interacts with the upstream region and the QRU to mediate the activation of the p20K promoter in a C/EBP β -dependent manner. This model is depicted in Fig.6B.

In conclusion, we have described the regulation of the p20K gene by hypoxia and implicated genes induced in conditions of oxygen limitation in the response to contact inhibition. A role for HIF1 α has been described in the survival and physiology of murine stem cells. In this case, HIF1 α interacts with the intracellular domain of the Notch receptor to control the expression of Notch-responsive genes and maintain the undifferentiated state of stem cells (Gustafsson et al., 2005). Whether or not a possible interaction between HIF1 α and C/EBP β functions in an analogous manner to promote reversible growth arrest in contact-inhibited CEF remains to be investigated.

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Table 2: Summary table indicating the number of probe-sets found to be differentially expressed between any pair-wise comparison indicated. The proportion of genes repressed during contact inhibition (C.I.) is dramatically high with respect to cycling conditions, while nearly half of that proportion of genes is activated. Gene expression in the subconfluent and confluent starvation conditions are regulated in similar patterns with respect to contact inhibition. Both starvation conditions show a higher portion of activated genes in comparison to the contact-inhibited state. A similar proportion of genes are up-regulated or down-regulated by the subconfluent-starved state in comparison to the cycling state. Whereas, the confluent-starved condition has about double the number of genes that are differentially regulated by the subconfluent-starved versus cycling state. Minor number of genes are differentially regulated between the two starvation conditions.

	(C.I. & Starved)/ C.I.	Starved/ C.I.	(C.I. & Starved)/ Cycling	C.I./ Cycling	Starved/ Cycling	(C.I. & Starved)/ Starved
up-regulated	466	510	413	638	277	23
down-regulated	199	218	414	935	206	29
total	665	728	827	1573	483	52

Table 3: Pattern of gene expression during contact inhibition. Changes in gene expression in contact inhibition (C.I.) with respect to cycling conditions are expressed as Log2 difference of means (C.I./Cycling) and linear fold-change values. The negative difference of means and linear fold-change values indicate genes that are repressed during starvation, whilst positive values indicate activation of gene expression by serum-depletion. Genes highlighted in orange represent starvation-specific genes. The one highlighted in green is a marker of contact inhibition and those highlighted in yellow indicate the signature for hypoxia.

Gene	Difference of Means	Linear Fold-Change	P-value
CA9: Carbonic anhydrase IX	5.79	55.33	0.0001
FABP4: fatty acid binding protein 4, adipocyte	5.34	40.50	0.0005
p20K: quiescence-specific protein	5.33	40.22	0.0002
ANGPTL5: angiopoietin-like 5	4.82	28.25	0.0048
CA12: carbonic anhydrase XII	4.64	24.93	0.0000
ENO2: enolase 2	3.31	9.92	0.0000
PTN: pleiotrophin	2.25	4.76	0.0006
TPD52: tumor protein D52	2.01	4.03	0.0018
HIF1A: hypoxia-inducible factor 1, alpha subunit	1.75	3.36	0.0024
ERO1L: ERO1-like (S. cerevisiae)	1.74	3.34	0.0001
PDGFRL: platelet-derived growth factor receptor-like	1.62	3.07	0.0059
GOLGA4: golgi autoantigen, golgin subfamily a, 4	1.45	2.73	0.0020
Cell cycle progression 1	1.31	2.48	0.0013
CDKN2C: cyclin-dependent kinase inhibitor 2C (p18)	1.18	2.27	0.0067
ULK1: unc-51-like kinase 1 (C. elegans)	1.12	2.17	0.0005
CDKN1B: cyclin-dependent kinase inhibitor 1B (p27, Kip1)	1.06	2.08	0.0000
BIRC2: baculoviral IAP repeat-containing 2	1.01	2.01	0.0006
DKK1: dickkopf homolog 1 (Xenopus laevis)	-6.87	-116.97	0.0000
ID1: inhibitor of DNA binding 1	-5.88	-58.89	0.0004
ID2: inhibitor of DNA binding 2	-4.11	-17.27	0.0000
APOA1: apolipoprotein A-1	-4.07	-16.80	0.0002
BMP2: bone morphogenetic protein 2	-3.70	-13.00	0.0000
CDKN2B: cyclin-dependent kinase inhibitor 2B (p15)	-3.24	-9.45	0.0000
TRAIIP: TRAF interacting protein	-3.04	-8.22	0.0002
TGFB3: transforming growth factor, beta 3	-2.88	-7.36	0.0003
FZD4: frizzled homolog 4 (Drosophila)	-2.81	-7.01	0.0002
AATF: apoptosis antagonizing transcription factor	-2.27	-4.82	0.0017
EGR1: early growth response 1	-2.27	-4.82	0.0003
DDX47: DEAD (Asp-Glu-Ala-Asp) box polypeptide 47	-2.16	-4.47	0.0005
RBL1: Retinoblastoma-like 1 (p107)	-1.58	-2.99	0.0023
MAP1LC3C: microtubule-associated protein 1 light chain 3 gamma	-1.43	-2.69	0.0052
NLE1: notchless homolog 1 (Drosophila)	-1.38	-2.60	0.0008
RORA: RAR-related orphan receptor A	-1.27	-2.41	0.0069
DAPK1: death-associated protein kinase 1	-1.26	-2.39	0.0001
BCLAF1: BCL2-associated transcription factor 1	-1.18	-2.27	0.0000
HES1: hairy and enhancer of split 1, (Drosophila)	-1.09	-2.13	0.0006
CDCA8: cell division cycle associated 8	-1.04	-2.06	0.0003
CEBPZ (CHOP10): CCAAT/enhancer binding protein zeta	-1.01	-2.01	0.0010

Table 4: Pattern of gene expression in response to serum-starvation. Changes in gene expression between starvation and cycling conditions are expressed as Log₂ difference of means (Starved/Cycling) and linear fold-change values. The negative difference of means and linear fold-change values indicate genes that are repressed during starvation, whilst positive values indicate activation of gene expression by serum-depletion. Genes highlighted in orange represent starvation-specific genes and the one highlighted in green is a marker of contact inhibition.

Gene	Difference of Means	Linear Fold-Change	P-value
FGF18: fibroblast growth factor 18	6.61	97.68	0.0059
ANGPTL5: angiopoietin-like 5	4.07	16.80	0.0081
SFRP2: secreted frizzled-related protein 2	3.02	8.11	0.0007
p20K: quiescence-specific protein	2.85	7.21	0.0014
RORA: RAR-related orphan receptor A	2.37	5.17	0.0024
PTN: pleiotrophin	1.88	3.68	0.0016
DDX4: DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	1.88	3.68	0.0065
SFRP1: secreted frizzled-related protein 1	1.74	3.34	0.0006
APOA1: apolipoprotein A-I	1.69	3.23	0.0015
PDGFRL: platelet-derived growth factor receptor-like	1.65	3.14	0.0053
ARF tumor suppressor	1.65	3.14	0.0001
CDKN2B: cyclin-dependent kinase inhibitor 2B (p15)	1.47	2.77	0.0003
BIRC2: baculoviral IAP repeat-containing 2	1.39	2.62	0.0065
Cell cycle progression 1	1.38	2.60	0.0008
CAPN2: calpain 2, (mII) large subunit	1.35	2.55	0.0010
MAP1LC3C: microtubule-associated protein 1 light chain 3 gamma	1.32	2.50	0.0029
TP53INP1: tumor protein p53 inducible nuclear protein 1	1.11	2.16	0.0035
DKK1: dickkopf homolog 1 (<i>Xenopus laevis</i>)	-8.00	-256.00	0.0005
ID1: inhibitor of DNA binding 1	-5.83	-56.89	0.0000
ID2: inhibitor of DNA binding 2	-2.90	-7.46	0.0000
TGFB3: transforming growth factor, beta 3	-2.78	-6.87	0.0041
TRAIIP: TRAF interacting protein	-2.69	-6.45	0.0005
FGFR2: fibroblast growth factor receptor 2	-2.05	-4.14	0.0001
SMAD9: SMAD family member 9	-1.95	-3.86	0.0002
BMP2: bone morphogenetic protein 2	-1.94	-3.84	0.0005
SMAD6: SMAD family member 6	-1.87	-3.66	0.0018
RASL11B: RAS-like, family 11, member B	-1.66	-3.16	0.0045
BIRC5: baculoviral IAP repeat-containing 5 (survivin)	-1.56	-2.95	0.0005
CCNB2: cyclin B2	-1.40	-2.64	0.0034
BCLAF1: BCL2-associated transcription factor 1	-1.37	-2.58	0.0059
CDC48: cell division cycle associated 8	-1.01	-2.01	0.0039

Table 5: Pattern of gene expression in response to high cell density and serum-starvation. Changes in gene expression between the contact-inhibited and starved conditions versus the cycling condition (C.I &.Starved/Cycling) are expressed as Log2 difference of means and linear fold-change values. The negative difference of means and linear fold-change values indicate genes that are repressed during starvation, whilst positive values indicate activation of gene expression by serum-depletion. Genes highlighted in orange represent starvation-specific genes. The one highlighted in green is a marker of contact-inhibition and those highlighted in yellow indicate the signature for hypoxia.

Gene	Difference of Means	Linear Fold-Change	P-value
SFRP2: secreted frizzled-related protein 2	4.55	23.43	0.0000
p20K : quiescence-specific protein	4.35	20.39	0.0005
CA 12: carbonic anhydrase XII	3.83	14.22	0.0000
CAPN9: calpain 9	2.76	6.77	0.0004
RORA: RAR -related orphan receptor A	2.17	4.50	0.0062
PTN: pleiotrophin	2.12	4.35	0.0004
APOA1: apolipoprotein A-I	1.91	3.76	0.0018
PDGFRL: platelet-derived growth factor receptor-like	1.90	3.73	0.0010
MAP1LC3C: microtubule-associated protein 1 light chain 3 gamma	1.75	3.36	0.0006
SFRP1: secreted frizzled-related protein 1	1.60	3.03	0.0009
Cell cycle progression 1	1.34	2.53	0.0031
TP53INP1: tumor protein p53 inducible nuclear protein 1	1.31	2.48	0.0064
ARF tumor suppressor	1.27	2.41	0.0002
ERO1L: ERO1-like (S. cerevisiae)	1.23	2.35	0.0011
PDCD4: programmed cell death 4	1.09	2.13	0.0004
Dishevelled, dsh homolog 1 (Drosophila)	1.03	2.04	0.0004
GOLGA4: golgi autoantigen, golgin subfamily a, 4	1.02	2.03	0.0067
DKK1: dickkopf homolog 1 (Xenopus laevis)	-8.05	-265.03	0.0067
ID1: inhibitor of DNA binding 1	-6.35	-81.57	0.0000
TGFB3: transforming growth factor, beta 3	-3.41	-10.63	0.0017
TRAIIP: TRAF interacting protein	-3.33	-10.06	0.0002
ID2: inhibitor of DNA binding 2	-3.05	-8.28	0.0011
BCLAF1: BCL2-associated transcription factor 1	-1.98	-3.94	0.0016
BIRC5: baculoviral IAP repeat-containing 5 (survivin)	-1.95	-3.86	0.0069
BMP2: bone morphogenetic protein 2	-1.91	-3.76	0.0003
CCNB2: cyclin B2	-1.75	-3.36	0.0009
SMAD6: SMAD family member 6	-1.66	-3.16	0.0031
SMAD9: SMAD family member 9	-1.56	-2.95	0.0008
AATF: apoptosis antagonizing transcription factor	-1.47	-2.77	0.0015
NLE1: notchless homolog 1 (Drosophila)	-1.44	-2.71	0.0027
FGFR2: fibroblast growth factor receptor 2	-1.28	-2.43	0.0022
CDCA8: cell division cycle associated 8	-1.23	-2.35	0.0019
BCL2-antagonist/killer 1	-1.20	-2.30	0.0002
RBL1: Retinoblastoma-like 1 (p107)	-1.20	-2.30	0.0019
DDX47: DEAD (Asp-Glu-Ala-Asp) box polypeptide 47	-1.14	-2.20	0.0082

Fig.1 – CHOP10 mRNA expression in starved or/and post-confluent CEF. Total RNA was extracted from CEFs under each of the indicated conditions and analyzed by Northern blotting analysis. Expression of the p20K mRNA is indicative of contact inhibition (lanes 4 to 9). ApoAI mRNA is expressed when CEF are starved in serum-free medium. Samples were prepared from CEF infected with the control virus RCASBP or the virus encoding a dominant negative mutant of C/EBP β (Δ 184-C/EBP β). RNA loading was monitored by probing for GAPDH mRNA. S-Cnf. or SC: Sub-Confluent, Cnf.: Confluent, P-Cnf.: Post-Confluent, serum+: complete medium, serum-: serum free medium.

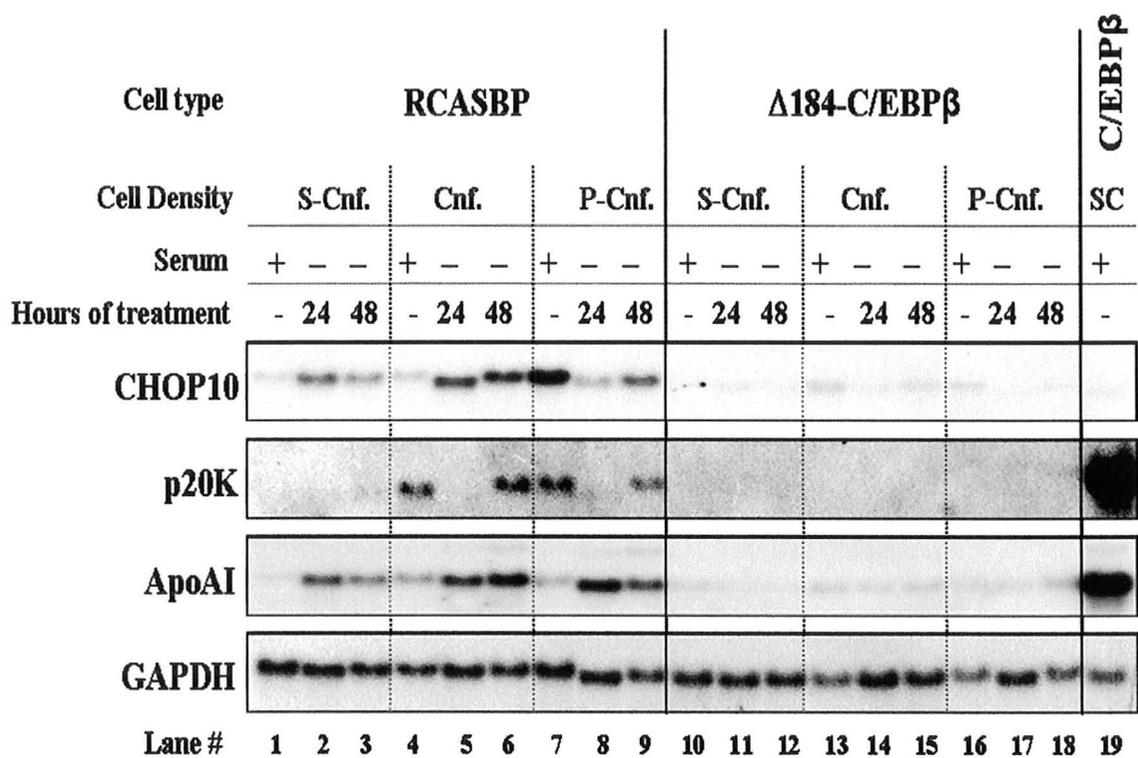


Fig.2 – p20K expression in conditions of CHOP10 inhibition by shRNAi. CEF were infected with a RCASBP-GFP shRNAi control virus or CHOP10 shRNAi-expressing virus (CHOP10-354). CEFs were seeded onto 100mm plates and harvested on the indicated day. Whole cell lysates were prepared and subjected to Western blotting analysis with the indicated anti-sera. Equal protein loading (80ug) was monitored by the level of ERK-1. The inhibition of CHOP10 enhanced p20K expression in contact-inhibited CEF (Day 4; data not shown).

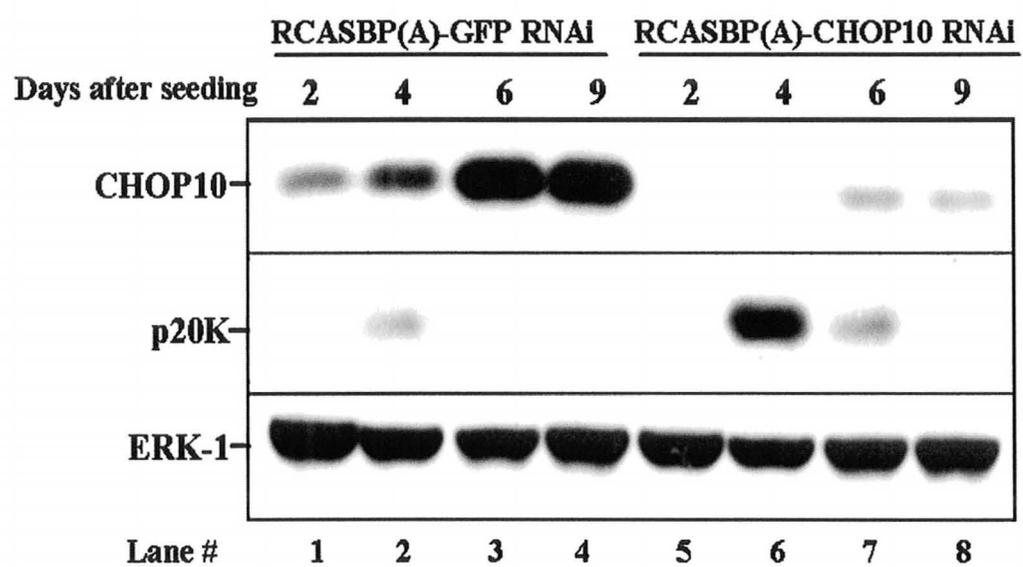


Fig.3 – Survival of CEF in conditions of CHOP10 down-regulation. Equal numbers of CEF were seeded onto 24-well dishes at day 0 and grown in complete medium. At day1, CEF were transferred to serum-free medium. Cell numbers were determined using a Coulter counter. Each time point represents the average of four separate samples; error bars represent the standard error of the mean. Down-regulation of CHOP10 improved CEF survival in conditions of serum/nutrient depletion. The difference between the two cell types in the last time point is significant ($p < 0.05$) by the student t-test and is indicated by ‘*’.

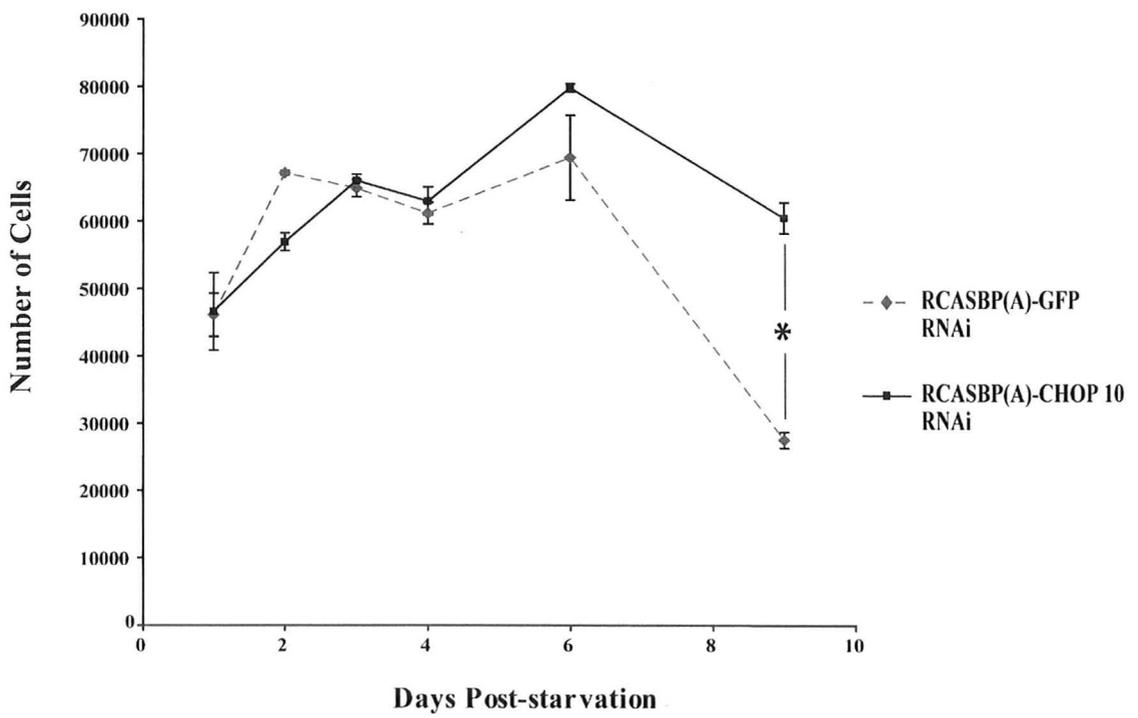


Fig.4 – Different programs of growth arrest regulate different sets of genes. **A)** Un-supervised hierarchal clustering demonstrating a minimum of two-fold change of gene expression between at least two conditions is indicated. Statistical significance of differences of expression was determined by two-way ANOVA using contact inhibition and serum starvation as factors ($\alpha = 0.05$). Two-fold or greater changes in gene expression were determined by unpaired t-test on all pair-wise comparisons between experimental conditions (Bonferroni-corrected $\alpha = 0.05$). Yellow indicates genes that are up-regulated and blue represents genes that are repressed. **B)** Northern blotting analyses of genes regulated by different modes of reversible growth arrest, contact inhibition and/or serum-starvation, versus actively cycling conditions. p20K expression was used as a marker of contact inhibition and CHOP10 was a marker for starvation.

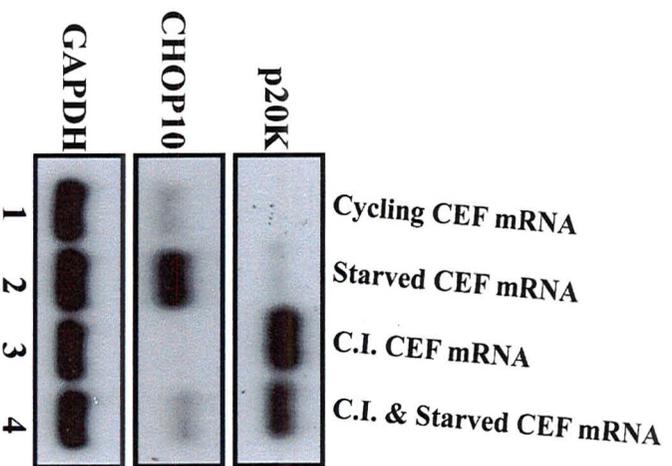
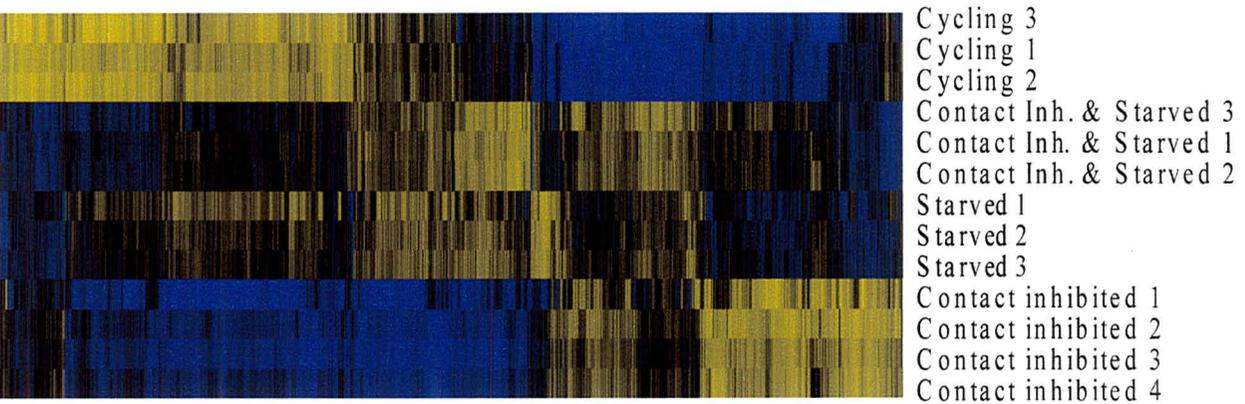
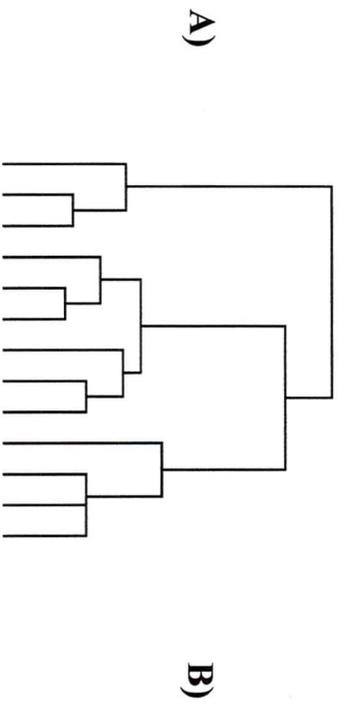
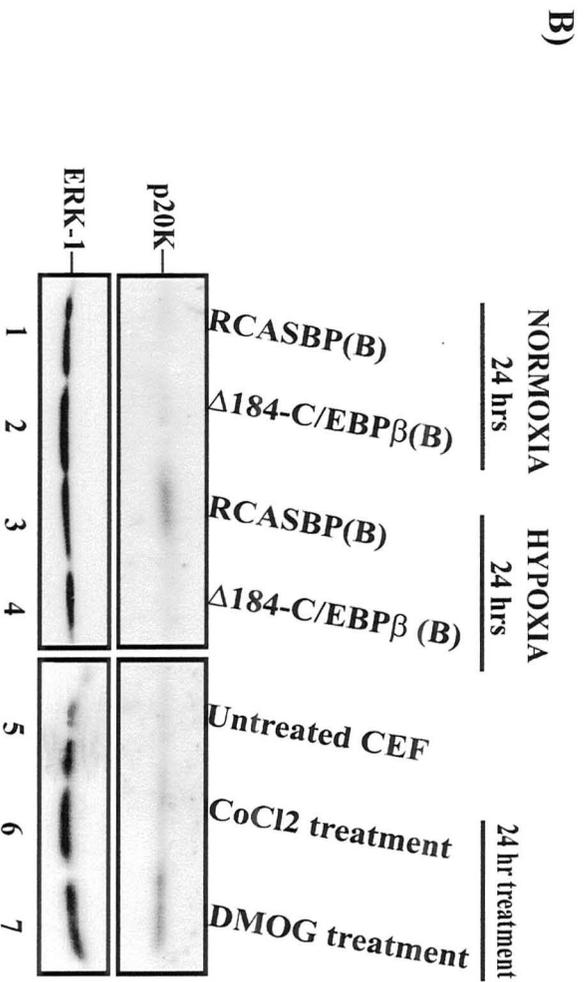
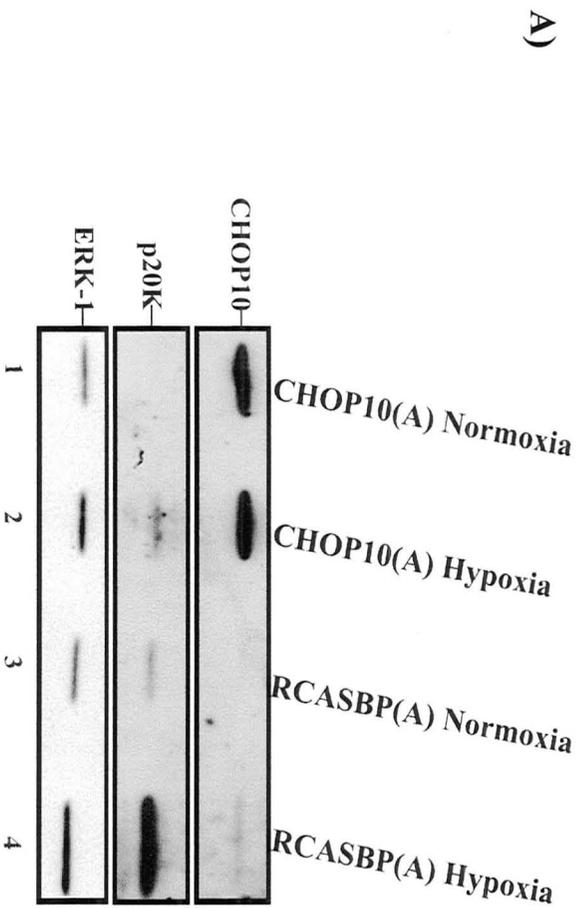
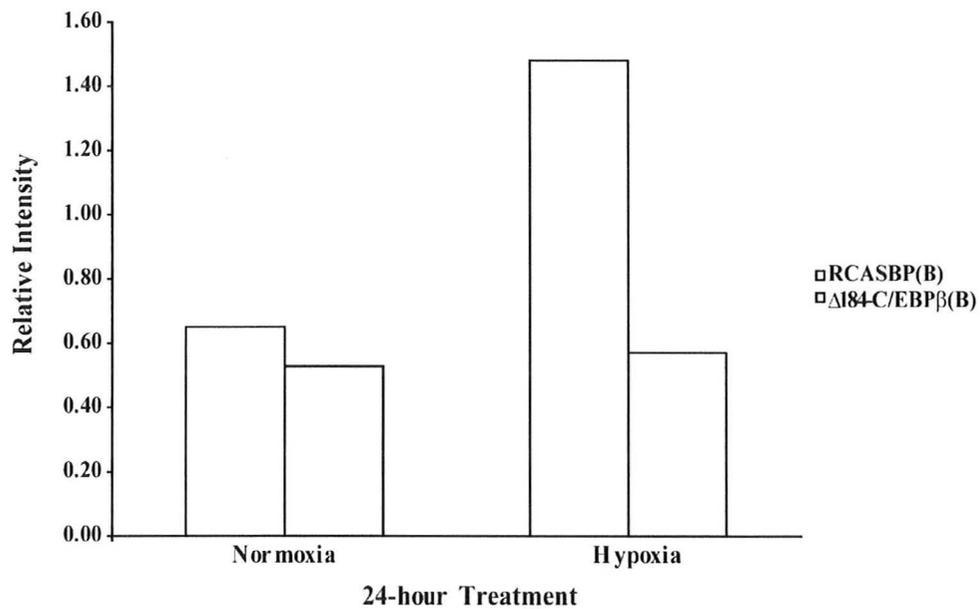


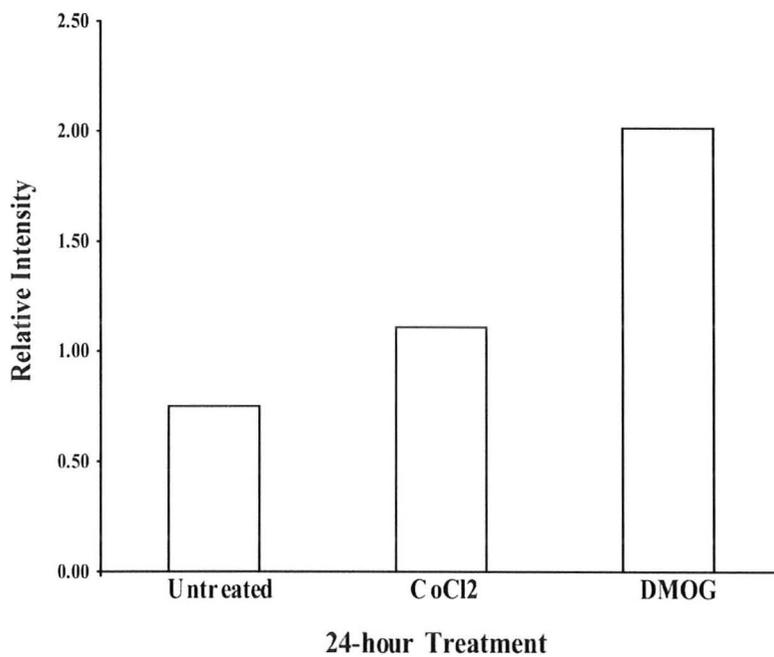
Fig.5 – Regulation of p20K by hypoxia and the C/EBP family. **A)** The expression of p20K was analyzed by Western blotting following a 24 hour-treatment in normoxia (21% O₂) or hypoxia (in 2% O₂) during conditions of CHOP10 over-expression, where CEF were infected with RCASBP(A)-CHOP10 expression vector [labelled CHOP10(A)], versus CEF infected with a control vector [labelled RCASBP(A)]. **B)** Western blotting analyses showing that the induction of p20K by hypoxia is blocked by the expression of a dominant negative mutant of C/EBP β (Δ 184-C/EBP β). p20K expression is also induced when normal CEF are treated with the hypoxia mimetics cobalt chloride (100 μ M) and dimethyloxaloylglycine (DMOG; 1 mM) for 24 hours. **C&D)** Quantitation of p20K protein levels after correcting for ERK-1 levels using the Image J program. **E)** Western analyses indicate that the expression of HIF1 α is also induced by the prolyl hydroxylase inhibitor, DMOG, and this induction is inhibited by the dominant negative mutant of C/EBP β (labelled Δ 184-C/EBP β).



C)



D)



E)

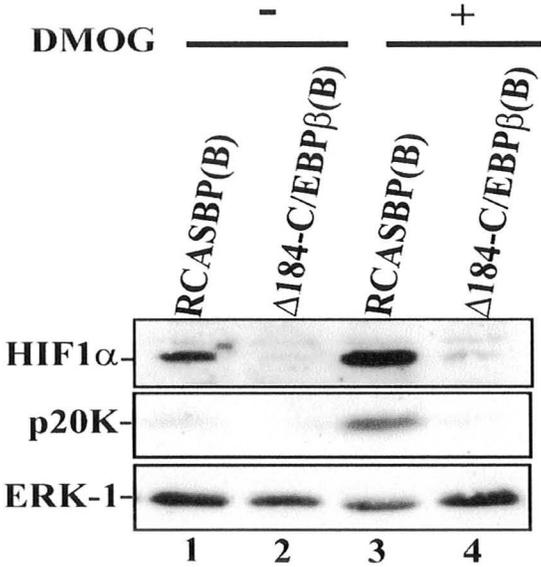
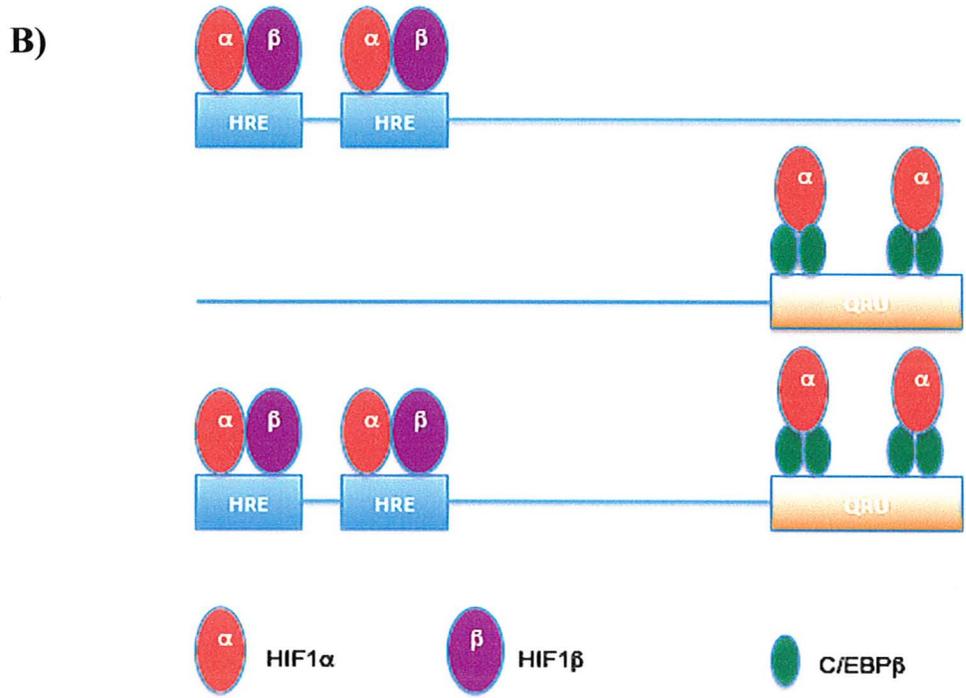
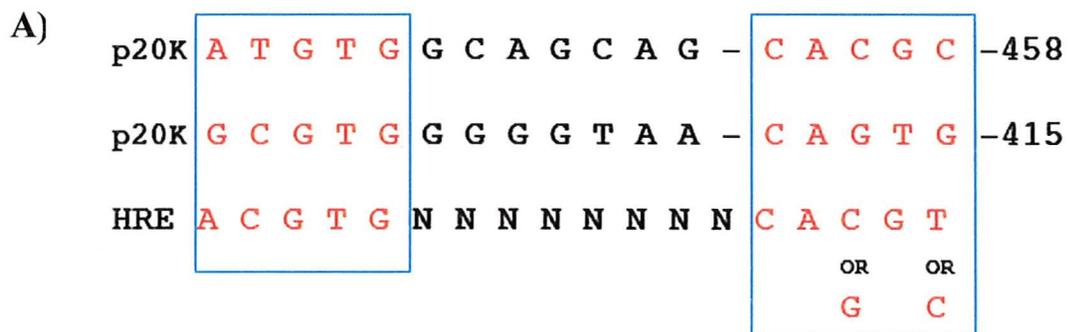


Fig.6 – Model of p20K regulation by hypoxia and contact inhibition. **A)** Two putative composite HIF1 binding sites (hypoxia response elements or HRE) are located in the p20K promoter region, upstream of the QRU, and are compared to the consensus sequence (Kimura *et al.*, 2001). **B)** The induction of the p20K promoter may involve the binding of HIF1 α to the HRE, as a heterodimer with HIF1 β , and/or to C/EBP β on the QRU without DNA binding.



Discussion and Conclusions

1. Regulation of CEF survival by C/EBP β during starvation

1.1.1 Forced expression of the dominant negative mutant of C/EBP β confers a survival advantage in starved CEF

In accordance with the findings of Gagliardi *et al.* (2003), over-expression of the dominant negative mutant of C/EBP β (Δ 184-C/EBP β) in CEF induced rapid proliferation and enhanced survival in response to prolonged nutrient-depletion (Chapter 1, Fig.1&2). TUNEL assays performed on Δ 184-C/EBP β -expressing cells, during starvation, further confirmed that the dominant negative mutant of C/EBP β promotes survival by interfering with apoptosis (Chapter 1, Fig.3A&B). C/EBP β inhibition also enhances the proliferation of CEF during logarithmic growth by increasing the activity of AP-1 (Gagliardi *et al.*, 2003). The stimulatory effects on CEF proliferation conferred by Δ 184-C/EBP β was also seen in C/EBP β ^{-/-} mouse embryo fibroblasts (MEF) (Gagliardi *et al.*, 2003). Hence, these observations suggest that C/EBP β negatively regulates proliferation in CEF in part by promoting apoptosis during cellular stress and by antagonizing the activity of AP-1.

In order to define a mechanism by which Δ 184-C/EBP β blocks apoptosis in CEF during starvation, we attempted to characterize a downstream mediator of C/EBP β -dependent apoptosis. A potential candidate was the tumour suppressor protein p53, since it is a regulator of apoptosis during cellular stress and C/EBP β has been shown to regulate the expression and activity of p53. The activator form of C/EBP β , LAP, was shown to regulate the transcription of p53 following re-entry into the cell cycle upon mitogen

stimulation (Boggs and Reisman, 2007). Furthermore, in human endometrial stromal cells, C/EBP β and p53 were shown to interact via their C-terminal domains (Schneider-Merck et al., 2006). As a result, C/EBP β was able to block the transcriptional activity of p53 by sequestering it away from its target genes (Schneider-Merck et al., 2006). Thus, we asked whether Δ 184-C/EBP β expressing CEF were able to resist apoptosis by repressing p53 expression or activity.

Since the Δ 184-C/EBP β mutant retains the C/EBP β C-terminal domain, it could also directly interact with p53 to block its pro-apoptotic functions. However, co-immunoprecipitation studies performed on cycling Δ 184-C/EBP β -CEF were not successful in showing an interaction between C/EBP β and p53 (data not shown). Additionally, we found no difference in p53 protein levels in cycling CEF expressing Δ 184-C/EBP β (Chapter 1, Fig.3C). However, as we only studied p53 expression in proliferating cells expressing Δ 184-C/EBP β but not in starved cells, we cannot rule out the possibility of an involvement of p53 in mediating apoptosis during cellular stress and quiescence in CEF. Therefore, further studies are required to determine if p53 has a role in CEF during starvation.

1.1.2 CHOP10 expression is repressed in starved Δ 184-C/EBP β CEF

Following several days of starvation, control CEF were found to express high levels of CHOP10, while no induction of CHOP10 was seen in cycling cells (Chapter 1, Fig.5A). The expression of CHOP10 was completely inhibited in CEF deficient in C/EBP β activity following prolonged starvation (Chapter 1, Fig.5A). Since CHOP10 is a marker of ER stress, it is likely that the lack of CHOP10 in starved Δ 184-C/EBP β ex-

pressing cells is indicative of the absence of ER stress in these cells. Whereas CHOP10 is highly activated in control cells, as a result of increased ER stress caused by long-term starvation. Similar to our findings in starved $\Delta 184\text{-C/EBP}\beta$ expressing CEF, apoptosis induced by ER-stress was significantly lower in $\text{C/EBP}\beta^{-/-}$ MEF than wildtype MEF (Li et al., 2008). This reduction in apoptosis was also accompanied by the repression of CHOP10 levels in the liver of $\text{C/EBP}\beta^{-/-}$ mice but not in their kidneys (Li et al., 2008). These studies imply that the loss of CHOP10 expression in $\text{C/EBP}\beta$ -deficient cells is mediated in a cell-type and context-specific manner. Alternatively, given that CHOP10 has pro-apoptotic functions during cellular stress, it is possible that $\Delta 184\text{-C/EBP}\beta$ expressing CEF are able to resist apoptosis due to a loss of CHOP10 expression and function. However, whether CHOP10 modulates apoptosis during starvation in CEF warrants further investigation.

1.2 GABARAP regulates apoptosis in CEF during starvation

CEF expressing $\Delta 184\text{-C/EBP}\beta$ developed autophagosomes and accumulated increasing amounts of γ -aminobutyric acid receptor associated protein (GABARAP) following prolonged starvation (Chapter 1, Fig.4&5). In addition to resisting apoptosis, $\Delta 184\text{-C/EBP}\beta$ cells appear to enhance their survival during starvation by undergoing autophagy. Given that GABARAP is a mammalian homologue of the yeast protein ATG8, which is an essential regulator of autophagy, and its expression was elevated in $\Delta 184\text{-C/EBP}\beta$, we wanted to determine if GABARAP has a role in mediating survival in starved CEF. However, the over-expression of GABARAP in CEF did not confer a survival advantage nor did it induce any pronounced signs of autophagy in response to star-

vation (Chapter 1, Fig.7). These observations suggest that the sole expression of GABARAP is not sufficient to promote autophagy and survival in serum-starved CEF.

We also studied the effect of stably down-regulating the expression of GABARAP by shRNAi in CEF during starvation (Das et al., 2006). Following GABARAP down-regulation, cells showed reduced proliferation rates and reached lower saturation densities with respect to control cells (Chapter 1, Fig.8). However, these cells were also able to survive better following prolonged nutrient-depletion, even when they reached an equivalent saturation density as control cells (Chapter 1, Fig.8). Therefore, knocking down GABARAP in CEF impairs proliferation but confers a survival advantage under starvation conditions. Similarly, GABARAP knockout mice were found to be fertile and viable, which reiterates that GABARAP is not essential for survival of the organism (Mohrluder et al., 2009). In neuronal cells, GABARAP is essential for transporting GABA_A receptors to the plasma membrane and for increasing the level of these receptors found on the membrane (Leil et al., 2004). It is possible that this function of GABARAP is important for cellular proliferation. Perhaps some receptor or protein trafficking critical for cell proliferation is impaired in these cells, such that proliferation is reduced following the loss of GABARAP activity. This warrants further investigation.

TUNEL assays confirmed that the enhanced survival conferred by GABARAP knockdown was the result of a reduction in the rate of apoptosis (Chapter 1, Fig.9). Additionally, starved GABARAP-RNAi cells were found to repress the expression of CHOP10 (Chapter 1, Fig.10). This suggests that these cells are able to resist apoptosis either by decreasing levels of ER stress in these cells or due to a deficiency in the pro-

apoptotic function of CHOP10. The lack of CHOP10 expression may be explained by the lower levels of ER stress found in starved GABARAP-RNAi cells, as compared to control cells (Chapter 1, Figs.8&9). Hence, our findings suggest that GABARAP is not required for cell survival in response to starvation and may actually be involved in the regulation of apoptosis in these conditions.

GABARAP was shown to interact with a Bcl-2 homology domain 3 (BH3) only protein, BNIP3L/Nix, which is known to have a role in promoting apoptosis (Schwarten et al., 2009). Moreover, a study by Lee *et al.* found that GABARAP interacts with a RNA helicase, DEAD box polypeptide 47 (DDX 47), and through this interaction promotes apoptosis in SKOV-3 ovarian cancer cells (Lee et al., 2005). The over-expression of either GABARAP or DDX47 was insufficient to induce apoptosis in these cells (Lee et al., 2005). However, when both proteins were expressed simultaneously, there was an increase in the rate of apoptosis, suggesting that the pro-apoptotic function of GABARAP is dependent on its interaction with DDX47 (Lee et al., 2005). Hence, these findings suggest that GABARAP may indirectly mediate apoptosis via its protein-protein interactions.

1.3 Differential processing of ATG8 homologues

Although GABARAP and LC3 are processed via a similar mechanism, there are differences suggesting that GABARAP and LC3 are functionally unique. In starved $\Delta 184\text{-C/EBP}\beta$ expressing CEF, the processed form of GABARAP (GABARAP-II) accumulated gradually following several days of starvation, whereas LC3 processing was constitutive, with the processed form (LC3-II) accumulating at lower levels of starvation (Chapter 1, Fig.5A). Therefore, the processing of two mammalian homologues of ATG8

followed different kinetics in starved CEF. Similarly, Tanida et al. (2006) found that GABARAP and LC3 lipidation did not occur in the same way. In different mouse tissues, the level of lipidated form of LC3 (LC3-phosphatidylethanolamine or LC3-PE or LC3-II) increased following 24hr to 48 hrs of starvation, while GABARAP-II levels were not induced during starvation (Tanida et al., 2006).

Further evidence indicating a functional difference between LC3 and GABARAP can be seen in experiments conducted by Tanida *et al.* (2004), where LC3 was localized to the punctuate spots of autophagosomes following starvation, while this was not seen with GABARAP (Tanida et al., 2004). Upon the over-expression of cysteine protease, Atg4B, the lipidated form of LC3 was diminished and its localization was more cytosolic following starvation (Tanida et al., 2004). However, Atg4B over-expression had no effect on the localization of GABARAP, thereby reinforcing the hypothesis that the homologues are functionally different and may be regulated differently (Tanida et al., 2004). Additionally, like the ATG8-phosphatidylethanolamine (ATG8-PE) conjugate in yeast, the LC3-PE form is essential for autophagosome formation in higher eukaryotes (Tanida et al., 2004). In contrast, the function of the lipidated form of GABARAP in autophagy is unknown (Tanida et al., 2004), although in neuronal cells, the processing of GABARAP was shown to be essential for its function in regulating GABA_A receptor trafficking and it also affected the localization of GABARAP within the cell (Chen et al., 2007). Hence, it appears that LC3 is the functional homologue of ATG8, whereas our studies suggest that GABARAP may function in the interface between autophagy and apoptosis.

So far, any attempts to study the role of LC3 in starved CEF have been unsuccessful-

ful, as we were unable to stably knockdown its expression. In trastuzumab-resistant breast cancer cells, LC3 knockdown caused a reduction in cell proliferation rates, implying that LC3 plays a role in regulating proliferation of these cells. Autophagy is not only important for cell survival during starvation, as it is also essential for maintaining cellular homeostasis in normal conditions by preventing the build-up of long-lived proteins and non-functional organelles (Boya et al., 2005). Therefore, as LC3 is possibly the functional homologue of ATG8, blocking its expression and activity can hinder the proliferation capacity and survival of cells. Hence, it is possible that by promoting autophagy, LC3 is an important mediator of cell survival in cells under normal and stressful conditions. However, further investigation is needed to determine the role of LC3 in regulating autophagy and survival in CEF.

1.4 Conclusions

Overall, several conclusions can be drawn from this section. Firstly, the dominant negative mutant of C/EBP β increases survival in CEF during serum-starvation, suggesting a role for C/EBP β as a regulator of survival in CEF. However, the survival effects of this mutant is not regulated by the induction of GABARAP. Instead, GABARAP appears to regulate apoptosis in CEF, since a lack of GABARAP expression enhances cell survival and reduces the level of apoptosis. More work is required to determine the process by which GABARAP promotes apoptosis in starved CEF. Since the Lee *et al.* (2005) study indicated that GABARAP-mediated apoptosis occurs via DDX47, it would be interesting to study the effects of down-regulating DDX47 levels on CEF survival. If it is a downstream effector of GABARAP, ablation of DDX47 levels should confer a survival

advantage to CEF and diminish the levels of apoptosis under starvation conditions.

Additionally, the GABARAP over-expression and RNA interference studies indicate that accumulation of GABARAP in starved $\Delta 184$ -C/EBP β expressing CEF was not responsible for promoting the survival of these cells. If GABARAP regulates apoptosis in CEF, the build up of GABARAP levels could occur as a result of the diminished levels of apoptosis present in these cells. Hence, the mechanism by which $\Delta 184$ -C/EBP β infected cells resist apoptosis is still unknown. Since the levels of CHOP10 were diminished in $\Delta 184$ -C/EBP β cells, and it has pro-apoptotic functions during ER stress, it may mediate apoptosis in starved CEF. This notion is supported by the results of CHOP10 shRNAi studies described in Chapter 2 of the results section (Further discussed in the following section).

Although the dominant negative mutant of C/EBP β is capable of impairing the activity of C/EBP β , this mutant does not solely block C/EBP β function, as it is able to homodimerize or heterodimerize with other C/EBP family members. Therefore, an RNAi construct specific for knocking down C/EBP β expression will be useful in validating the results of its dominant negative form.

2. Genes regulated by alternate forms of reversible growth-arrest and C/EBP β in chicken embryo fibroblasts.

2.1 Quiescence by contact inhibition is associated with a signature for hypoxia

The accumulation of the processed form of GABARAP in starved CEF expressing $\Delta 184$ -C/EBP β led to the hypothesis that C/EBP β possibly regulates GABARAP ex-

pression and apoptosis during serum-starvation in CEF (Results, Chapter 1). Moreover, C/EBP β negatively regulates AP-1-mediated cell proliferation in CEF and has been shown to modulate growth arrest in CEF due to contact inhibition (Gagliardi et al., 2003; Kim et al., 1999). Hence, these studies indicate that C/EBP β controls multiple responses in CEF.

2.1.1 G₀ induced by different signals activates distinct patterns of gene expression

To characterize the pattern of gene expression in CEF growth-arrested by contact inhibition and/or serum-starvation, gene-profiling analyses were conducted. CEF were induced to enter reversible growth arrest by two means. One method was to allow the cells to grow to 100% confluence and enter G₀ as a result of contact inhibition. Alternatively, cells were grown to subconfluent levels in complete media and then the media was replaced with serum-free media for a period of 48 hours to induce starvation. In addition, CEF were cultured to enter quiescence via contact inhibition and serum-starvation, such that cells were contact-inhibited and then subsequently starved for 48 hours. Samples of RNA from each condition were used for the gene-profiling studies. RNA from proliferating and subconfluent CEF was also used as a negative control. p20K was used as a marker for contact inhibition, while CHOP10 expression was studied as a marker for starvation (Chapter 2, Fig.4B). Using the Probe Logarithmic Intensity Error (PLIER) program, a list of differentially regulated genes was generated. (Chapter 2, Table 2-5).

We observed that genes upregulated in contact inhibited CEF included hypoxia-regulated genes such as carbonic anhydrase IX, enolase II, and even HIF-1 α (Chapter 2, Table 3). This suggests that contact inhibition is associated with a signature of hypoxia.

Starvation-induced genes, like MAP1LC3 and apolipoprotein A1 (apoA1) are repressed in contact-inhibited CEF (Chapter 2, Tables 4&5). Additionally, the apoA1 gene is activated during starvation regardless of cellular density, which reiterates the findings of T. Miyake (2004) that apoA1 is exclusively regulated by lipid starvation (Chapter 2, Table 3-5; Miyake T., Masters Thesis). Other genes of interest repressed by contact inhibition include pro-apoptotic gene DAPK1, autophagy-related and tumour suppressor gene ULK-1, p15, DEAD box protein DDX47, and CHOP10; whereas, genes activated include cyclin dependent kinase inhibitors (CKI) p18 and p27, where p27 is also a GAS gene known to be regulated by cell density [(Yanagisawa et al., 1999); (Chapter 2, Table 3&4)]. CKI p15 is repressed by high cellular density, but activated in the subconfluent-starvation state, which may reflect the fact that different CKIs regulate different forms of growth-arrest and that, like apoA1, p15 is a starvation-specific induced gene. These results support the notion that alternative forms of reversible growth arrest occur in response to different external signals, by activating distinct patterns of gene expression.

2.1.2 Different signaling pathways are regulated by G₀

Some signaling pathways are also differentially regulated by quiescence. The Notch signaling pathway is highly conserved and is important in controlling cellular proliferation, differentiation, apoptosis, and developmental processes [reviewed in (Iso et al., 2003)]. A modifier of this pathway, Notchless homolog 1 is down-regulated in contact-inhibited cells irrespective of the presence of serum [Tables 3&5; (Royet et al., 1998)]. Downstream effectors of the Notch pathway, the HES (hairy/enhancer-of-split) and HEY (Hes-related with YRPW motif) are transcriptional repressors that repress the expression

or activity of differentiation factors such as neurogenin [reviewed in (Iso et al., 2003)]. The expression of HES1 was also down-regulated in contact-inhibited CEF, which may imply that contact inhibition primes cells for entering a state of terminal differentiation (Chapter 2, Table 3).

Members of the Wnt pathway were also differentially regulated by G_0 . The activation of the *dishevelled* gene is exclusive to the combined condition of contact-inhibition and starvation (Chapter 2, Table 5). The *dishevelled* gene encodes a phosphoprotein that has a role promoting the Wnt signaling pathway (Lee et al., 2008). The secreted frizzled-related protein 2 (SFRP2) encoding gene is activated in all three conditions, whereas SFRP1 is induced only in starved and high density-starved conditions (Chapter 2, Table 3-5). The SFRPs are secreted glycoproteins that antagonize the Wnt signalling pathway and have roles in regulating cell survival and differentiation (Chung et al., 2009; Descamps et al., 2008). For example SFRP1 and SFRP2 have been shown to repress differentiation of mouse myoblast and satellite cells, with SFRP2 having a more prominent role (Descamps et al., 2008). Moreover, re-expression of the SFRPs, particularly SFRP2, in cervical cancer cells led to a reduction of tumorigenicity in these cells by blocking cell proliferation and tumour invasiveness, possibly through inhibiting the Wnt pathway (Chung et al., 2009), suggesting that the Wnt signaling pathway plays a role in regulating growth arrest.

2.1.3 Different forms of G_0 show a common pattern of gene expression

Although Holler *et al.* showed that in human diploid fibroblasts, different sets of genes were expressed in response to distinct methods of inducing quiescence, they also

characterized a pattern of gene expression common to all three states of quiescence (Coller et al., 2006). Similarly, in CEF all three conditions repressed the expression of genes including bone morphogenetic 2 (Bmp2), inhibitor of DNA binding proteins (ID), dickkopf (DKK1), and transforming growth factor β 3 (TGF β 3) (Tables 4, 6, 8). ID1 is a helix-loop-helix (HLH) transcriptional repressor that is down-regulated during lymphocyte differentiation (Xu et al., 2003). The repression of gene products that inhibit differentiation of cells, i.e. *Id* genes, suggests that quiescence can prime cells to enter different forms of growth arrest like differentiation (Chapter 2, Tables 3-5). Furthermore, the levels of ID2 and ID3 were up-regulated in human foreskin fibroblasts immediately following serum-stimulation from G₀, and are candidates for regulating the exit from G₀ in the presence of serum (Iyer et al., 1999). This suggests that the repression of ID2 is important for maintaining cells in a quiescent state.

Additionally, anti-apoptotic genes, like SFRP2 encoding gene and baculoviral IAP repeat-containing 2 gene (BIRC2), which is also known as cellular inhibitor of apoptosis 2 (cIAP2), are activated in all conditions [Table 3-5 (Jin and Lee, 2006; Zhang et al., 2009)]. Whereas, pro-apoptotic genes, like Bcl-2 associated transcription factor 1 (BCAF1) (Liu et al., 2007), are repressed in all quiescent conditions. This indicates that quiescence actively maintains cells in a viable state and protects the cell from undergoing apoptosis (Tables 3-5; data not shown). The activation of the pleiotrophin (*PTN*) gene is also common to all three conditions in comparison to actively proliferating cells (Tables 3-5). PTN is a heparin-binding cytokine that has a diverse array of functions and is a proto-oncogene which can promote cell proliferation [reviewed in (Deuel et al., 2002)].

Cells activated by the platelet-derived growth factor (PDGF) are also found to show elevated *PTN* gene expression, (Deuel et al., 2002). This may suggest that quiescent CEF are particularly responsive to PDGF stimulation to exit G_0 , since the PDGF α -receptor is also a GAS gene regulated by serum-starvation in CEF (Lih et al., 1996). These findings indicate that G_0 -arrested CEF can also express cytokines and transcription factors, which will enable them to re-enter the cell cycle when conditions become favourable.

2.2 p20K expression is regulated by the cellular environment and members of the C/EBP family

2.2.1 p20K expression is regulated by contact inhibition and hypoxia in a C/EBP β dependent manner

p20K expression is regulated by cell cycle stages, since its expression is induced by reversible growth arrest by C/EBP β and repressed during mitogenesis by AP-1 (Gagliardi et al., 2003; Kim et al., 1999). As genes regulated by contact inhibition showed a signature of hypoxia, we decided to study the expression of p20K in hypoxic conditions. Moreover, the p20K promoter was found to contain putative HRE sites that have not yet been characterized [(Mao et al., 1993), Fig.6], suggesting that it may be regulated in response to hypoxia. Actively cycling cells subjected to 24 hours of 2% O_2 levels or treatment with hypoxia mimetics led to the accumulation of p20K (Chapter 2, Fig.5). Hypoxic-induction of p20K was also C/EBP β -dependent, as expression of the dominant negative mutant of C/EBP β inhibited this hypoxia-stimulated expression of p20K (Chapter 2, Fig.5B-E). In addition, since p20K levels were highly up-regulated following treatment with chemicals that directly stabilize HIF1 α in normoxic conditions, it

is implied that p20K expression is directly controlled by HIF1 α during hypoxia (Chapter 2, Fig.5B&C). The modest increase in p20K expression following CoCl₂ treatment may reflect the lower specificity of CoCl₂ treatment versus the DMOG treatment (Chapter 2, Fig.5B). Therefore, we conclude that p20K is a novel hypoxia-responsive gene, whose expression is regulated by hypoxia and contact inhibition in a C/EBP β -dependent manner (Chapter 2, Figs. 2&5).

It is possible that C/EBP β may interact with HIF1 α to activate p20K expression during hypoxia. In addition to forming homodimers or heterodimers with other family members, C/EBP proteins can interact with other transcription factors as well (Ramji and Foka, 2002). Recent studies have shown the existence of a HIF1 α -C/EBP α axis [reviewed in (Janardhan, 2008)]. For example, in leukemia cells C/EBP α was found to directly interact with HIF1 α and inhibit its transcriptional activity (Yang et al., 2008). Moreover, this interaction induced the differentiation of myeloid leukemia cells during hypoxia, which was mediated by C/EBP α in a HIF1 α -dependent manner that required the transactivation domain of C/EBP α and the basic helix loop helix region of HIF1 α (Janardhan, 2008; Jiang et al., 2005; Yang et al., 2008). Hence, in leukemia cells, reporter assays showed that HIF1 α increased the transcriptional activity of C/EBP α , whereas C/EBP α was found to inhibit HIF1 α DNA binding ability, compete with the HIF1 α binding partner HIF1 β , and also repress HIF1 α regulated gene expression (Jiang et al., 2005). However, in breast cancer cells hypoxia caused a reduction in C/EBP α transcription in a HIF1 α -dependent manner (Seifeddine et al., 2008). Hence, it appears that the effects of HIF1 α on the regulation of C/EBP α vary in a cell-type specific manner.

In our studies, the expression of HIF1 α was also inhibited by the dominant negative mutant of C/EBP β following hypoxic treatment (Chapter 2, Fig.5C). This suggests that C/EBP β may play a role in regulating HIF1 α expression during hypoxia and since it is upregulated during contact inhibition, it may be a new target of C/EBP β during G₀ (Chapter 2, Table 3). Therefore, similar to the C/EBP α –HIF1 α axis, our findings may represent the existence of a C/EBP β -HIF1 α axis in CEF that can regulate p20K expression. A model depicting how C/EBP β can interact with HIF1 α to activate p20K is shown in Fig.6 (Chapter 2). p20K expression could be activated either by a HIF1 α -HIF1 β heterodimer that can bind to its putative HRE sites located in the QRU and activate its expression. Alternatively, HIF1 α may directly interact with C/EBP β dimer without binding to the QRU and enhance their ability to induce p20K expression. This may occur independently or cooperatively. However, as this is a new field of investigation, more work is required to dissect the C/EBP β /HIF1 α interaction.

2.2.2 CHOP10 negatively regulates CEF survival and p20K expression

During cellular stress, CHOP10 has also been known to have pro-apoptotic roles (Marciniak et al., 2004). In agreement with this notion, knocking down CHOP10 expression in CEF was shown to confer a survival advantage in response to long-term starvation (Chapter 2, Fig.3). Therefore, our findings suggest that during starvation, CHOP10 may promote the switch from G₀ to apoptosis in CEF. These findings also suggest that in the case of Δ 184-C/EBP β expressing cells, down regulation of CHOP10 levels could potentially explain why the cells are able to resist cell death during long-term starvation.

Furthermore, under conditions of serum-starvation, p20K was highly expressed in

CEF following the down-regulation of CHOP10 as compared to control cells (Chapter 2, Fig.2). This implies that p20K expression is inhibited by CHOP10 under these conditions, since repressing CHOP10 expression was able to alleviate the inhibition of p20K in response to starvation. Additionally, in response to hypoxia, the over-expression of CHOP10 also repressed p20K protein levels (Chapter 2, Figs.5A). In contrast, control cells showed a massive induction in p20K expression (Chapter 2, Figs.5A). Overall, these observations suggest that CHOP10 is a negative regulator of p20K during cellular stresses like starvation.

It is plausible that during starvation, CHOP10 blocks p20K expression by binding to C/EBP β . Transcription of p20K is regulated via a quiescence responsive unit (QRU) located within its promoter. This region contains two C/EBP sites (sites A and B) and the activation of both sites was required to induce p20K expression (Kim et al., 1999). Additionally, the sequence of site A is highly similar to that of the CHOP10 consensus binding sequence. Therefore, we hypothesize that the elevation of CHOP10 levels during starvation can increase the proportion of CHOP10/C/EBP β heterodimers formed, and these dimers can preferentially bind to the A site of the p20K QRU. As a result, CHOP10 can sequester C/EBP β away from the B site of the QRU. Since the induction of both C/EBP β sites is required for p20K expression, accumulation of CHOP10/C/EBP β dimers during starvation would repress p20K. Similarly, in the response to hypoxia, CHOP10 may interfere with p20K expression by either sequestering C/EBP β away from HIF1 α or by directly inhibiting HIF1 α expression. Therefore, additional studies are needed to dissect this model.

2.3 Conclusions

C/EBP β activates p20K under conditions of reversible growth arrest induced by contact inhibition (Kim et al., 1999). However, p20K levels are repressed in cycling conditions, which is mediated by AP-1 as it can sequester C/EBP β away from the p20K promoter (Gagliardi et al., 2003). This indicates that the regulation of gene expression by C/EBP β can vary depending on its interacting factor. Hence, our studies have uncovered new conditions of p20K expression (hypoxia) and implicated new C/EBP β interacting proteins (CHOP10 and HIF1 α) in the regulation of this quiescence-specific gene.

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