CHOP IS A KEY REGULATOR OF p20K IN HYPOXIA AND CONTACT INHIBITED CHICKEN EMBRYONIC FIBROBLASTS

CHOP IS A KEY REGULATOR OF p20K IN HYPOXIA AND CONTACT INHIBITED CHICKEN EMBRYONIC FIBROBLASTS

By DORA Y. CAMACHO, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (2013) McMaster University

(BIOLOGY) Hamilton, Ontario

TITLE: CHOP is a key regulator of p20K in hypoxia and contact inhibited Chicken Embryonic Fibroblasts

AUTHOR: Dora Yudi Camacho, B.Sc. (Montclair State University)

SUPERVISOR: Dr. Andre Bédard

NUMBER OF PAGES: CIII, 103

Abstract:

Growth arrest specific (GAS) genes are responsible for regulating cell proliferation, gene expression, and apoptosis (Fleming et al., 1997). Given these roles, it is important to study the mechanisms of action and regulation of Gas genes in quiescence (G_0). If altered, quiescence can lead to abnormal development, degenerative diseases, and cancer (Liu et al., 2007). The p20K lipocalin is a GAS gene expressed in contact inhibited cells (Kim et al., 1999). Recent studies have also demonstrated that p20K is highly inducible in hypoxia, and its activation in hypoxia and contact inhibition is dependent upon a 48bp promoter region referred to as the *Quiescence Responsive Unit* (QRU) (Mao et al. 1993; S. Kim et al. 1999; Fielding, MSc. Thesis, 2011). Furthermore, p20K is highly regulated by *CAAT/enhancer binding protein* (C/EBPβ) as C/EBPβ crucial interaction with different QRU binding sites are essential for the activation of p20K (Kim et al., 1999). CHOP, a C/EBP homologous protein belonging to the C/EBP family, has been identified as an inhibitor of C/EBPβ activity (Sok et al., 1999). Co-immunoprecipitation experiments showed that CHOP induction either by ER stress or starvation promoted the formation of C/EBPβ heterodimers inhibiting p20K expression in Chicken Embryonic Fibroblasts (CEFs). Experiments using a CHOP knockdown by shRNA demonstrated that CHOP repression induced robust expression of p20K in cycling CEF cells after 24 hours in hypoxia, but not at earlier time points. In addition, as documented by RT-qPCR and Western blotting analysis, both p20K and CHOP mRNA and protein expression levels show to have an inverse relationship in hypoxia and contact inhibition. Since CHOP expression is greatly induced during ER stress and starvation, two ER stress inducible agents (tunicamycin and thapsigargin) were used to stimulate high levels of CHOP in CEF, leading to the induction of CHOP and repression of p20K. CEF were also treated with DMOG, a hypoxia mimetic that can

stabilize Hypoxia Inducible Factor (HIF) under normoxic conditions (Barnucz et al., 2013). Surprisingly, in DMOG treated cells CHOP levels did not decrease, while p20K was highly induced. At later time points, while DMOG induces modest induction of CHOP, it decreases phospho-ERK levels (a transcriptional repressor of p20K) demonstrating a separate signal/pathway controlling p20K induction in these conditions that requires follow up studies. Collectively, these results demonstrate that the down-regulation of the p20K GAS gene in response to ER stress is mediated by the induction of CHOP and segregation of C/EBPβ as heterodimers.

Acknowledgments

I am very thankful to my supervisor, Andre Bédard, for granting me the opportunity to join his lab. His immense knowledge and continued guidance helped me during my research and thesis writing. I would also like to thank all of my past and present lab members for their valuable advice and insight on this project.

Lastly, I would like to thank my family, my parents, and friends, and especially Edwin Ortiz, Jr. for all their undying support and understanding during my studies away from home.

Table of Contents

List of Tables and Figures

List of Abbreviations:

AP-1: Activator Protein-1
ASNS: asparagine synthetase
ATF-2: Activating transcription factor-2
ATF-4: Activating transcription factor -4
Bcl-2: B-cell leukemia/lymphoma 2
BR: Basic Region
bZIP: Basic leucine zipper domain
C/EBP: CAAT/enhancer binding protein
CDK: cyclin dependent kinase
CEF: Chicken Embryonic Fibroblast
CHM: Chicken Heart mesenchymal cell
CHO: Chinese-hamster ovary
CHOP: CAAT/enhancer binding protein (C/EBP) homologous protein
CKI: Cyclin dependent kinase inhibitors
Co-IP: Co-Immunoprecipitation
DM or DMSO: Dimethyl sulfoxide
DMEM: Dulbecco's modified Eagle's medium
DMOG: Dimethyloxaloylglycine
DNA: Deoxyribonucleic acid
DOC: Downstream of CHOP
EDTA: ethylene dinitriol tetra-acetic acid
elF2: eukaryotic initiation factor 2
elF4: eukaryotic initiation factor 4
EMSA: Electrophoretic mobility shift assay
ERK: Extracellular signal-regulated kinase
Ex-FABP: Extracellular Fatty Acid Binding Protein
FIH-1: Factor inhibiting HIF-1
G_0: Reversible Growth Arrest
G1: Gap phase 1
G2: Gap phase 2
GADD: Growth Arrested and DNA damage
GAPDH: glyceraldehydes phosphate dehyrogenase
GAS: growth-arrest specific
GATE: Gamma activated transcription element
HIF: Hypoxia Inducible Factor
HRE: Hypoxic Responsive Element
IL: Interleukin
LAP*: full-length C/EBPβ protein

LAP: Liver-enriched transcriptional-activator protein
LIP: Liver-enriched transcriptional-inhibitory protein
LZ: Leucine Zipper
M: Mitosis
MAPK: mitogen activated protein kinase
MEF: Mouse embryonic fibroblast
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PHD: prolyl-hydroxylase
QRU: Quiescence Responsive unit
Rb: retinoblastoma protein
RCAS: Replication Competent ALV LTR with a Splice acceptor
RCASBP: Replication competent avian sarcoma virus LTR
RD: Regulatory Domain
REFs: Rat Embryonic Fibroblasts
RNA: Ribonucleic acid
RSV: Raos sarcoma virus
RT: reverse transcriptase
RT-qPCR: reverse transcription (RT) quantitative polymerase chain reaction (PCR).
SDS: sodium dodecyl-sulfate
shRNA: short hairpin RNA
S-phase: DNA synthesis phase
TAD: trans-activation domain
TBS: Tris buffered saline
TBS-T: Tris buffered saline 0.1% Tween-20
TG: thapsigargin
TNF: Tumor Necrosis Factor
TU: tunicamycin
uORF: upstream Open reading frame
UPR: unfolded protein resonse

Chapter 1: Introduction

1.1 Cell Cycle

The cell cycle is composed of four distinct phases which are important for cell renewal and growth. The first phase is known as Gap 1 phase (G1), which is where cells grow and generate important components for DNA replication (Bedolla et al., 2013). Following G1, the Synthesis phase (S) begins, where DNA replication takes place (Bedolla et al., 2013). The secondary growth phase, the Gap 2 phase (G2) is where the cell continues to grow and prepares for mitosis (Bedolla et al., 2013).. During Mitosis (M phase) cells divide giving rise to new daughter cells (Figure 1.1) (Bedolla et al., 2013).

The cell can exit the cell cycle during G1 phase as the cell enters a state of *quiescence (G_0)*. Quiescence (G_0) is a reversible non-dividing state, where cells can withdraw from the cell cycle when favorable conditions are not met, such as high cell density or nutrient deprivation (Reviewed by Williams & Stoeber 2012). When extracellular conditions become favorable, cells can resume with the cell cycle, and proceed through G1 phase (Reviewed by Williams & Stoeber 2012). During late G1 phase, cells reach the restriction point, which in yeasts, is referred to as the START point (Reviewed by Gelman & Sudol 2010). Progression of G1 to restriction point is dependent on mitogenic signals (growth factors) and high rate of protein synthesis (Reviewed by Zetterberg, A., Larsson O., Wiman, 1995). After the cells pass through the restriction point, the cell is now fully committed to divide, and becomes independent of growth factor stimulation (Reviewed by Foijer & Te Riele, 2006).

The G2 phase also contains cellular checkpoints that can detect inappropriate extracellular signals that can interrupt the continuation of cell cycle. G2 cellular checkpoint for example

ensures that a cell does not begin mitosis if DNA damage is present (Stark and Taylor, 2004). In normal cells, when the cell detects DNA damage, it triggers a mechanism that induces cell cycle arrest by the activation of p53 transcription factor or retinoblastoma proteins (Reviewed by Foijer & Te Riele, 2006). p53 can mediate both late G1 and G2/M cell cycle arrest (Agarwal et al., 1995). Cell cycle checkpoints are triggered to allow time for DNA repair to take place (Liu and Kulesz-Martin, 2001). However, p53 will induce cellular apoptosis in cells with irreparable DNA damage. In cancer cells, these growth inhibitory checkpoints during the G1 or G2 phase often fail to be activated leading to uncontrolled cell proliferation.

1.2 Cyclins and CDKs that regulate G_0-G_1 transition

Cyclin proteins and **C**yclin **D**ependent **K**inases (CDK) are important regulators of cell cycle progression. Cyclin proteins play a major role by creating a complex with their corresponding CDK proteins counterparts. Different Cyclin-CDK complexes regulate different steps of the cell cycle. For instance cyclin D interacts with CDK4 or CDK6 controlling early G1 progression (As reviewed by Black and Black, 2012; Gelman and Sudol, 2010). Cyclin D- CDK4/6 complexes also regulate entrance of quiescent cells into cell cycle (As reviewed by Black and Black, 2012). Alternatively, Cyclin E creates a complex with CDK2 that regulates G1 to S progression (Hwang and Clurman, 2005). Cyclin A/CDK2 and cyclin A/CDK1 complexes control the S phase and early G2 transition (Reviewed by Malumbres and Barbacid, 2005). During G2, type B cyclins begin to predominate through M phase, while at the same time A-type cylins are degraded by ubiquitin-mediated proteolysis (Reviewed by Malumbres and Barbacid, 2005). As a result, Cyclin B pairs with CDK1 to regulate entrance into mitosis (Figure 1.1) (Reviewed by Malumbres and Barbacid, 2005).

The retinoblastoma protein (pRb) and related pocket proteins, p107 and p130 play a significant role in cell cycle by interacting with different cyclin/CDK complexes (Cobrinik, 2005). These proteins are most commonly known for their role in S phase transition, but are also important during quiescence. Rb can exist in its hypophosphorylated (active) or hyperphosphorylated (inactive) form, and its phosphorylated state is important during G1. Rb and related pocket proteins inactivation is crucial for cell cycle progression because they bind to and repress E2F transcription factors (Reviewed by Foijer & Te Riele, 2006). E2F factors are essential for the transcription of several genes involved in G1/S transition, including Cyclin E (Reviewed by Foijer & Te Riele, 2006). Rb also regulates quiescence by inhibiting E2F transcription factors in absence of mitogen stimulation which halts cell cycle progression (Chim et al., 2006). For cell cycle to proceed, Rb needs to be inactivated. Rb is therefore initially targeted to be inactivated by Cyclin D-CDK 4/6 during mitogen stimulation. Thus, elevated levels of Cyclin D-CDK 4/6 results in partial inactivation of Rb and release of E2F (Reviewed by Foijer and Te Riele, 2006; Gelman and Sudol, 2010). The release of E2F thus activates Cyclin E/Cdk 2, which further phosphorylates Rb to its hyperphosphorylated state, resulting in full release of E2F activity and beginning of S phase (Reviewed by Foijer & Te Riele, 2006).

Cyclin dependent kinase inhibitors (CKI) also coordinate important activities during the cell cycle. There are two families of CKIs: Cdk-interacting protein/ kinase inhibitory protein (CIP/KIP) and INK4. The CIP/KIP family ($p21^{CIP}$, $p27^{KIP1}$and $p57^{KIP2}$) of CKIs inhibits Cyclin E/CDK2 and cyclin/CDK1 activity, but may enhance the association and activation of Cyclin D-CDK 4/6 (Chim et al., 2006). The cyclin dependent kinase inhibitor (CKI) $p27^{KIP1}$, for example functions as a strong inhibitor of Cyclin E/CDK2 kinase activity in conditions lacking growth factor stimulation (Reviewed by Foijer & Te Riele, 2006). Thus, $p27^{KIP1}$ prevents the functional

inactivation of Rb, and suppresses E2F factors (Reviewed by Foijer & Te Riele, 2006). $p27^{KIP1}$ accumulates during quiescence, but is rapidly degraded as cells re-enter the cell cycle from this quiescent state (As reviewed by Starostina and Kipreos, 2012). Another CIP/KIP family cyclin dependent kinase inhibitor is $p21^{CIP}$, which induces G1and G2 cell cycle arrest in response to DNA damage (As reviewed by Starostina and Kipreos, 2012). $p21^{CIP}$ is transcriptionally regulated by p53 in conditions of DNA damage or cellular senescence (Dulic et al., 1998). Lastly, p57 is another CIP/KIP family CDK inhibitor that is mainly involved in development. p57 regulates decisions to exit the cell cycle during embryogenesis (Matsuoka et al., 1995).

Additionally, INK4 family is also involved in cyclin–CDKs regulation. INK4 family ($p15^{INK4B/MST2}$, $p16^{INK4A/MST1}$, and $p18^{INK4C}$) is known to specifically inhibit Cyclin D-cdk4/6 complexes (Graña and Reddy, 1995; Sherr and Roberts, 1995; Tvrdik et al., 2002). p16 for example is a member of INK4 family that has been widely studied in several cancers because its loss signifies a pivotal point in tumor progression (Rocco and Sidransky, 2001). p16 is a tumor suppressor that is an essential regulator of cdk4 and cdk6 activity (Rocco and Sidransky, 2001). Therefore elevated levels of p16 halt cell cycle progression by inhibiting Cyclin D-cdk4/6 complexes that are necessary for Rb phosphorylation. Loss of cyclin D also results in increased levels of p27 and Cyclin E/CDK2 inhibition. The end result is cell cycle arrest at G1. The main difference between INK4 and CIP/KIP family is that the INK family only regulates Cyclin D-cdk4/6, while the CIP/KIP family is broad in regulating all G1 phase cdk-cyclin complexes (Graña and Reddy, 1995; Sherr and Roberts, 1995; Tvrdik et al., 2002). The sequential order of events that govern cell cycle are therefore important for proper cell division, as loss or over expression can lead to aberrant events that can lead to cancer progression or developmental defects.

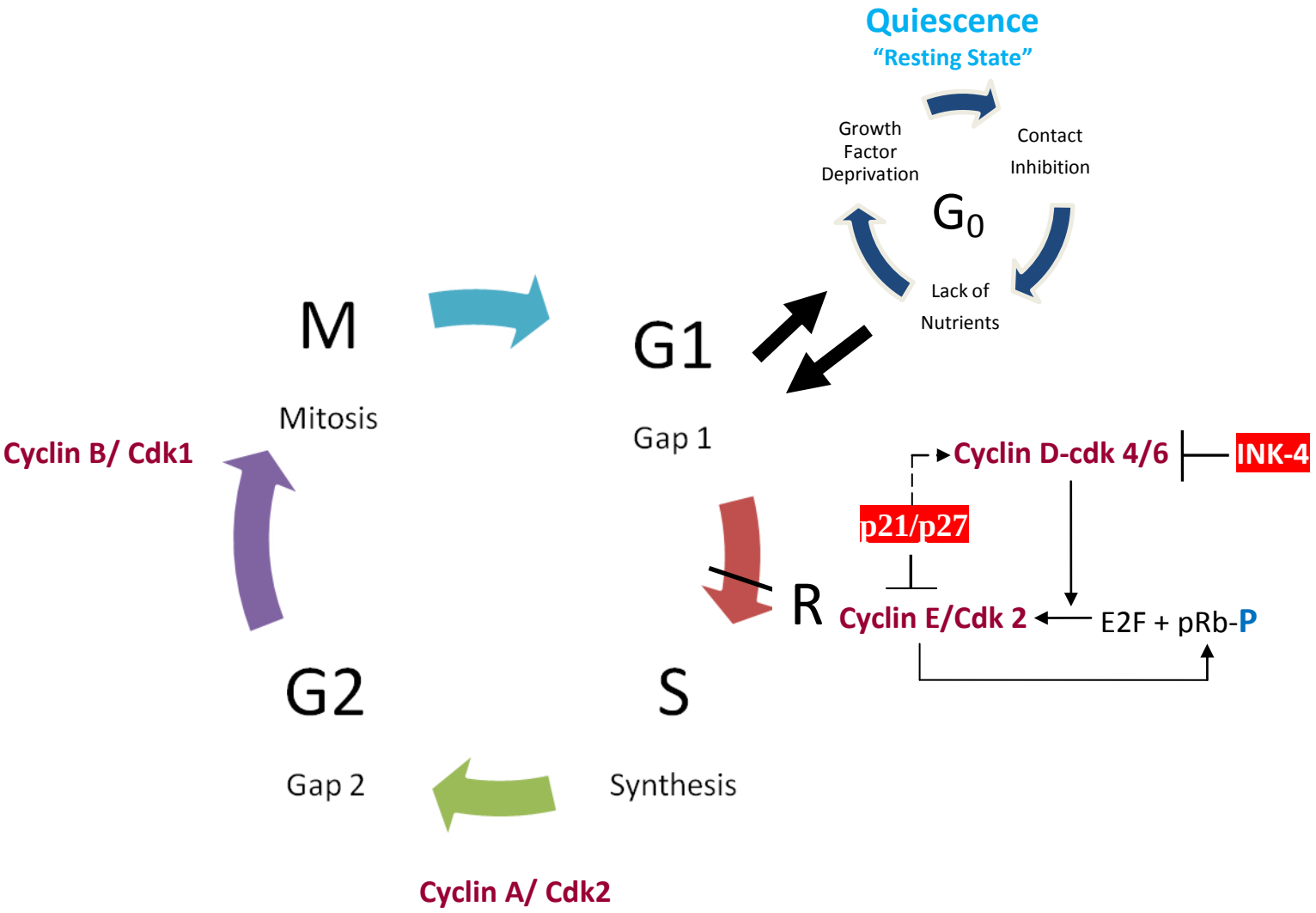


Figure 1.1: Cell cycle overview. The cell cycle is composed of Interphase (G1, S, and G2) and Mitosis. Gap phase 1 (G1) is where cell grows, leading to Synthesis (S) Phase, where DNA replication takes place. Gap phase 2 (G2) is the secondary growth phases that prepares the cell to enter mitosis. During mitosis (M) phase chromosome segregation and cytokinesis occur, giving rise to new daughter cells. During G1, the absence of nutrients or growth factors and high cell density (contact inhibition) can lead to quiescence (G0), which is a reversible cell cycle arrest. When cells exit the cell cycle during quiescence, the cells can return to cell cycle when conditions become favorable. This diagram also summarizes cyclin/cdk interactions that govern G1 progression.

1.3 Quiescence

All living cells can exit the normal cell cycle and enter quiescence (G_0), also known as a "resting" state (Gray et al., 2004). Cellular quiescence is a reversible, non-dividing state that occurs as cells exit the cell cycle (Coller et al., 2006). Many cells in the human body are terminally differentiated or are not constantly proliferating, and remain in a state of quiescence (Reviewed by Williams and Stoeber, 2012). Only a small amount of cells are actively cycling. They are located in self-renewing tissues such as epithelia and bone marrow (Potten and Loeffler, 1990; Reviewed by Williams and Stoeber, 2012). Examples of cells that have entered into a quiescent state are glial cells, thyroid follicular cells, or hepatocytes (Reviewed by Williams and Stoeber, 2012).

Cellular quiescence (G_0) is a process of regulating cell proliferation and homeostasis (Komarova and Wodarz, 2007). The decision to progress through the cell cycle or enter quiescence occurs during G1 phase (Gray et al., 2004). Nutrient and growth factor availability, and high cell density (contact inhibition) are the main factors that can induce cells to enter quiescence (Gos et al., 2005). The lack of nutrients leads cells to exit the cell cycle, however once the availability of nutrients is restored, the cell can exit the quiescence state and re-enter the cell cycle (Gray et al., 2004). In addition, quiescence can be stimulated when cells become contact inhibited (density arrested) (Gos et al., 2005). However unlike starving cells, contact inhibited cells cannot exit quiescence with the addition of fresh medium (Gos et al., 2005). Cells would have to be subcultured at lower density in order for cells to return to the cell cycle (Gos et al., 2005).

Many somatic cells, including stem cells, are often found in the common state of quiescence (Coller et al., 2006). It is hypothesized that several common cancers are sustained by "cancer

stem cells" (Komarova and Wodarz, 2007). Chronic myeloid leukemia cancer cells are an example of cancer cells that largely remain in quiescence (Dean et al., 2005). Certain cancer drug therapies can even induce quiescence in primitive cancer cells (Komarova and Wodarz, 2007). Chemotherapy agents usually have S-phase specificity, therefore making primitive quiescent cancer cells drug-resistant (Graham et al., 2002). These quiescent cancer cells do not proliferate or become apoptotic and are not sensitive to any drug activity (Komarova and Wodarz, 2007). For this reason, recent leukemia cancer research has focused on isolating quiescent cell gene markers (Graham et al., 2002). Although quiescence has been extensively studied, many questions still remain unanswered regarding the pathways that regulate this cellular state.

It is important to note that unlike quiescence, where cells can re-enter the cell cycle, senescence is an irreversible arrest in cell division (Cho and Hwang, 2012). As cells age their replication rate gradually slows down, and eventually the cells stop dividing and enter replicative senescence (Erdmann, 2005). As a result, senescent cells undergo morphological changes with an increase in lysosome levels, cell volume, mitochondria, and reactive oxygen species (ROS) (Cho and Hwang, 2012). Senescence may be triggered by several mechanisms such as telomere shortening or DNA damage, therefore it has been associated with aging and tumor suppression (Collado et al., 2007).

1.3 GAS and Gadd genes

G*rowth arrest specific* (GAS) and *growth arrest and DNA damage* (GADD) genes are two classes of genes up-regulated during quiescence. GAS and GADD genes are essential for the regulation of cell proliferation, gene expression, and cell death (Fleming et al., 1997). In addition, GAS and GADD genes are down-regulated when cells undergo terminal differentiation

or senescence (Fleming et al., 1997; Shugart et al., 1995). This indicates that these two classes of genes that are involved in growth arrest gene expression are specific to cells in quiescence, where cells can re-enter the cell cycle.

Six GAS genes (GAS 1-6) were first identified in 1988 in order to further understand the roles of genes that are expressed during cellular growth arrest (Schneider et al., 1988). These genes were up-regulated in growth arrested NIH3T3 cells, but were down-regulated after stimulation by serum (Schneider et al., 1988). GAS genes expression has been found in various tissues, fibroblast cell lines, hematopoietic cells, and during embryo development (Ciccarelli et al., 1990).

The Gadd gene family is also important in studies that regulate quiescence. Gadd genes are induced by a variety of cellular stress conditions such as nutrient deprivation in culture and in response to DNA damaging agents (Carlson et al., 1993; Jackman et al., 1994). A group of five Gadd genes were first isolated from UV radiation-treated Chinese-hamster ovary (CHO) cells (Fornace et al., 1989). The members of this family of GADD genes consist of GADD34, GADD45α, GADD45β, GADD45γ, and GADD153. Studies by Fornace et al. (1989) determined that Gadd genes are induced during growth arrest after DNA damage, and are also important for the regulation of growth arrest in quiescence. Additionally, Gadd genes play significant roles in cell cycle arrest, apoptosis, and DNA repair (Saletta et al., 2011). The *gadd153* (CHOP) and *gadd45* genes have been intently studied because their expression has been observed in every mammalian cell line (Zhan et al., 1994). GAS and GADD gene levels of expression may also fluctuate depending on stress and growth arrest conditions. For instance, mRNA levels of CHOP, Gas2, and Gas 5 levels increases in response to the depletion of essential amino acids (Fleming et al., 1997). Alternatively, in conditions of low serum, Gas 3 and Gas 6 mRNA levels

are induced (Fleming et al., 1997). Thus, not all conditions of growth arrest induce similar Gas and Gadd genes expression.

1.5 C/EBP Family and C/EBPβ

CAAT/**E**nhancer **B**inding **P**rotein (C/EBP) family is a group of leucine zipper transcription factors that participate in the regulation of cellular growth and differentiation in multiple tissues, as well as during inflammation (As reviewed by Ramji and Foka, 2002; Wei et al., 2006). By the early 1990's six members of the C/EBP family had been uncovered (C/EBPα, -β, -γ, -δ, -ε and -ζ) (Wei et al., 2006). All C/EBP family members display a conserved C-terminal basic domain (bZIP) important for DNA binding (Ron and Habener, 1992). Adjacent to the basic domain, is a leucine zipper helix structure responsible for dimerization (Ron and Habener, 1992). All family members are also able to form disulfide bonds between paired zipper helices (Williams et al., 1991). Apart from C/EBP ζ, also referred to as CHOP, all members of C/EBP family are able to homodimerize or heterodimerize with other family members to activate target genes (Ron & Habener, 1992). However, CHOP is only able to heterodimerize with other C/EBP family members (Ron and Habener, 1992). Dimerization is a must in order for C/EBP members to bind to DNA through the basic domain (As reviewed by Ramji and Foca, 2002). Any disruptions that prevent C/EBP family members from dimerizing will hinder them from binding to promoter regions of target genes.

From all the C/EBP members, the gene of interest in our studies on GAS gene regulation is **C**AAT/**E**nhancer **B**inding **P**rotein **B**eta (C/EBPβ). C/EBPβ is ubiquitously expressed, and is associated with cellular growth, differentiation, apoptosis, and inflammatory responses in different tissues (Cortés-Canteli et al., 2002; Degagné et al., 2012; Didon et al., 2011; Pal et al., 2009; Takiguchi, 1998). At first C/EBPβ was described as a transcriptional regulator of the

interleukin-6 (IL-6) gene promoter involved in immune and inflammatory responses (Akira et al., 1990). Additionally, C/EBPβ has also been extensively studied as a liver specific DNA-binding protein involved in liver and adipocyte differentiation (Darlington et al., 1998; Ferrini et al., 2001). It was also determined that C/EBPβ is a direct target of ERK-1/2, which is important during ovulation and female fertility (Fan et al., 2010). C/EBPβ has even been classified as a pro-survival factor by protecting fibroblasts cells from Tumor Necrosis Factor α (TNF) mediated apoptosis (Ranjan and Boss, 2006). These studies demonstrate that C/EBPβ has versatile and tissue specific roles, hence the importance of studying this transcription factor.

C/EBPβ mRNA generates three important isoforms: LAP*, LAP, and LIP. Liver-enriched Activating Protein (38 kDa), also referred to as C/EBPβ-1, LAP* or LAP-1, is the full length isoform of C/EBPβ. It is predominantly expressed in all normal cells (Figure 1.2) (Eaton et al., 2001). The second C/EBPβ isoform is a 34-kDa transactivator protein, known as C/EBPβ-2, LAP-2, or LAP. This isoform differs from LAP* by 23 N-terminal amino acids (Descombes et al., 1990; Eaton et al., 2001). The third isoform, 21-kDa Liver-enriched Inhibitory Protein (LIP) can act as an inhibitor of LAP* and LAP. It can act in this manner because it is missing the transactivation domain (Descombes et al., 1990). While LAP stimulates C/EBP activity, LIP acts as a competitive inhibitor. LAP* and LAP have different expression profiles demonstrating differences in gene activation and cell proliferation (Eaton et al., 2001; Kowenz-Leutz and Leutz, 1999). Studies by Eaton et al. (2001) demonstrated that only LAP was able to activate Cyclin D1 promoter, indicating that it has a role in promoting cell growth in mammary epithelial cells. Interestingly, only LAP expression was observed in mammary tumor cell lines, while LAP* was distinctively expressed in normal mammary epithelial cells (Eaton et al., 2001). Moreover, LAP* exhibits unique functions, including the interaction with SWI/SNF complex, a

chromatin remodeling complex (Kowenz-Leutz and Leutz, 1999). LIP and LAP expression ratio is also regulated by translation initiation factors elF-2 and elF-4 (Calkhoven et al., 2000). During high levels of elF-2 and elF-4, LIP and LAP are highly generated, however at low levels of elF-2 and elF-4, LAP* predominates (Calkhoven et al., 2000). These studies indicate the different functions and methods of regulation that produce C/EBPβ isoforms.

C/EBPβ isoform ratio is also under the translational control by uORFs, which encode evolutionary conserved upstream peptides in key regulatory genes (Wethmar et al., 2010). C/EBPβ generates isoforms as a result of leaky ribosome scanning mechanism (Descombes et al., 1990). This mechanism of translation is mediated through different START sites within the same reading frame. The small (40s) ribosomal subunit scans for potential AUG start sites from 5'-3' direction (Descombes et al., 1990). Depending on external stimuli or control by other cytoplasmic proteins, the first AUG encountered might not always be used for translation of protein synthesis, as in the case for C/EBPβ (Descombes et al., 1990; Jover et al., 2002). As a result, translation may begin at a second or third translation initiation sites generating different C/EBPβ isoforms (Calkhoven et al., 2000; Descombes et al., 1990).

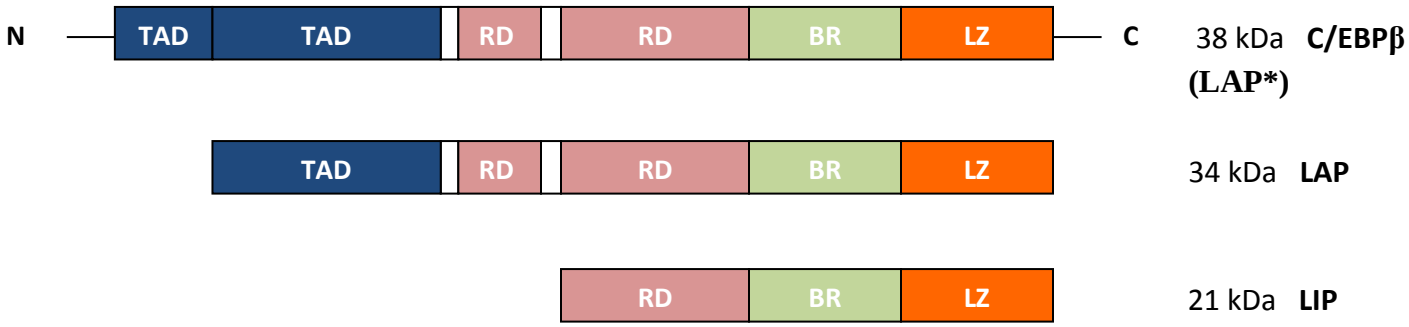


Figure 1.2. C/EBPβ Structure. C/EBPβ shares a common bZIP structure with other C/EBP family members. The full-length C/EBPβ (38kDa) structure is shown with the Transactivation Domain (TAD), Regulatory Domain (RD), Basic Region (BR) (DNA Binding), and Leucine Zipper (LZ) (Dimerization Domain). The other C/EBPβ isoforms structures are also displayed: Liver-enriched Activating Protein (LAP) (34-kDa) and Liver-enriched Inhibitory Protein (LIP) (21-kDa). LAP is a transcriptional activator that contains an N-terminal truncated version of full length C/EBPβ. LIP is transcriptional repressor lacking the Transactivation Domain. *(Adapted from Wethmar et al., 2010).*

1.6 p20K

p20K is a GAS gene product that has been well studied in Chicken Embryonic Fibroblasts (CEFs). Like other GAS genes, p20K is highly activated during quiescence (Bedard et al., 1987). p20K is part of the lipocalin family of lipid-binding proteins that is expressed in CEFs and chicken heart mesenchymal (CHM) cells (Bedard et al., 1987). As a lipocalin protein p20K has high affinity for long chain unsaturated fatty acids (Descalzi Cancedda et al., 1996). This quiescent specific protein is expressed in conditions of high cell density (contact inhibition) and hypoxia (Fielding, MSc. Thesis, 2011; Kim et al., 1999). Although p20K is highly expressed in contact inhibited CEF, its expression is rapidly inhibited in actively proliferating CEFs (Bedard et al., 1987). In addition, p20K is also repressed in RSV transformed CEF, during growth factor stimulation, or addition of insulin (Bedard et al., 1989).

In studies by Cancedda et al. (1990), p20K also referred to as the Extracellular Fatty Acid protein (Ex-FABP), which is characterized as a stress protein induced by inflammatory agents. Ex-FABP/p20K is stimulated by active tissue remodeling during chicken embryo development (Cancedda et al., 1990). Its expression was observed in dyschondroplastic and osteoarthritic diseases in adult chicken cartilage, and also in differentiated chrondrocytes (Cancedda et al., 1990; Descalzi Cancedda et al., 2002). Studies have also shown that it is associated with promoting cell survival in chondrocytes, myoblast, and cardiomyocytes (Gentili et al., 2005). Thus this protein is very important in heart development, bone, and muscle formation (Descalzi Cancedda et al., 2002).

The transcriptional activation of p20K in quiescent CEF is dependent on a 48-bp promoter region refer to as the *Quiescence Responsive Unit* (QRU) (Gagliardi et al., 2003). In addition, C/EBPβ has also been identified as an essential regulator of p20K activation (Kim et al., 1999).

The QRU contains two C/EBPβ binding sites, Regions A and B that play a major role in the activation of p20K expression (Kim et al, 1999).

In studies by Gagliardi et al. (2003), expression of p20K in contact inhibited CEF was inhibited by a C/EBPβ dominant negative mutant (RCASBP-Δ184-C/EBPβ). Moreover, expression of Δ184-C/EBPβ mutant also stimulated higher rate of proliferation. Studies by Gagliardi et al. (2003) showed that C/EBPβ controlled proliferation by acting as a negative regulator of activator protein -1 (AP-1). AP-1 is a transcription factor that can regulate proliferation, differentiation, apoptosis and transformation (Hess et al., 2004). When inhibiting C/EBPβ expression with the Δ184-C/EBPβ mutant, AP-1 expression was up-regulated in cycling CEF cells. In contrast, over expressing C/EBPβ reduced levels of AP-1 activity. Studies have also shown that AP-1 inhibits p20K expression in contact inhibited CEF. Thus, in density arrested CEF, the over expression of c-Jun, a component of AP-1, stimulated cell proliferation and blocked p20K expression (Gagliardi et al., 2003). These studies reveal that C/EBPβ and AP-1 have contrasting roles in p20K regulation and cell proliferation (Gagliardi et al., 2003).

1.7 Hypoxia and Quiescence

Cellular hypoxia (low oxygen) is a common feature occurring during development, normal cell physiology, and cancer (Chi et al., 2006). Hypoxia induces the accumulation of **H**ypoxia **I**nducible **F**actors (HIFs), which have an essential role in regulating tissue homeostasis during hypoxic conditions (Ercan et al., 2012). HIF consists of α-subunits (HIF-1 α and HIF-2 α) and HIF1β that can heterodimerize within the nucleus to activate target genes in low oxygen conditions (Pietras et al., 2011). The HIFα/HIF1β complex binds to a sequence in the promoter region of its target genes known as the **H**ypoxic **R**esponsive **E**lement (HRE) in order to activate gene expression during hypoxia (Pietras et al., 2011).

One way cells may respond to the environmental stress induced by hypoxia is by slowing down their proliferation rates. Studies by Gardner et al. (2001) showed that hypoxia induces p27, a CDK inhibitor, thus resulting in hypoxia-induced G1 arrest in primary murine embryo fibroblasts. In addition, retinoblastoma protein hypophosphorylation is also necessary to trigger hypoxia induced quiescence (Gardner et al., 2001). Other studies have also shown that hypoxia induces quiescence in tumor cells as a way to compensate and increase survival in a hypoxic environment (Gao et al., 2011). Hypoxia induced quiescence can also provide an advantage for cancer cells to evade radiotherapy and chemotherapy effects (Brown and Wilson, 2004). However, not all transformed cells lines can survive in a hypoxic environment. For instance, studies by Schmaltz et al. (1998) demonstrated that hypoxia induced quiescence in non-transformed primary fibroblast cells, but triggered apoptosis in oncogene-transformed **R**at **E**mbryonic **F**ibroblasts (REFs). This state of hypoxia induced apoptosis in transformed REFs was triggered as a result of enhanced acidosis (low medium pH) (Schmaltz et al., 1998).

Hypoxia can also contribute to the quiescent state of stem cells. This has been observed in Satellite cells which are skeletal muscle stem cells that usually remain in a state of quiescence. These cells only undergo differentiation during muscle growth, exercise, or injury (Hawke and Garry, 2001). Studies by Liu et al. (2012) demonstrated that hypoxia is largely responsible for the quiescent state of satellite cell-derived primary myoblasts. This is also demonstrated in studies where hypoxia hinders myogenic differentiation through the activation of Notch or P13K pathways (Gustafsson et al., 2005; Majmundar et al., 2012). Hypoxia can also induce myoblasts to undergo quiescence during conditions of nutrient deprivation (Liu et al., 2012). These studies demonstrate the strong correlation between hypoxia and quiescence that are important for maintenance of stem cell properties and to increase cell survival.

1.8 Induction of p20K in hypoxia

A microarray analysis demonstrated that RNA from contact inhibited CEF shared a similar gene expression pattern with cells in hypoxia. Therefore, several experiments by B. Fielding (MSc. Thesis, 2011) were conducted to confirm the expression of Gas gene protein, p20K, in hypoxic conditions (1-2% O_2). Results demonstrated a strong activation of p20K in cycling CEFs after the cells were incubated for 24 hours in hypoxia. Prior to this, p20K expression had only been observed in contact inhibited CEFs or to a lesser extent in conditions of serum-free medium. In addition, p20K was super induced when contact inhibited cells were incubated in hypoxic conditions. Transient expression assays were used to dissect different areas of p20K promoter, and results demonstrated that p20K expression in hypoxia was also mediated by the QRU. The induction of p20K in hypoxia was also C/EBPβ dependent, since experiments using a C/EBPβ dominant negative mutant (RCASBP-Δ184-C/EBPβ) displayed no p20K expression in hypoxia or contact inhibition (Fielding, MSc. Thesis, 2011; Kim et al, 1999). Recent experiments by undergraduate students Samantha Garnett (2012) and Melanie Fox (2013) showed that CEF proliferation rate slows down in hypoxia, demonstrating the inhibitory effect of hypoxia on proliferation. The results of these experiments provided further evidence that hypoxia triggers the induction of Gas genes expression, such as p20K, in quiescence.

1.9 ERK-2 binding to QRU

Extracellular signal-regulated kinase 2 (ERK-2) belongs to the mitogen-activated protein kinase (MAPK) family known to regulate proliferation, differentiation, and development (Hu et al., 2009; Mebratu and Tesfaigzi, 2009). ERK-2 activation is dependent on growth factor stimulation (Mebratu and Tesfaigzi, 2009). Although ERK-2 has been well characterized for its role in several biological processes; recent studies provided insight into its novel role as

transcriptional repressor (Hu et al., 2009). ERK-2 can bind to a DNA consensus sequence (G/CAAAG/CN G/CAAAG/C) and block access to transcription factors (Hu et al., 2009). Studies by Hu et al. (2009) showed that ERK-2 can bind to a consensus sequence of *Gamma activated transcription element* (GATE) promoter region to block transcriptional activity of interferon gamma-induced genes. Interestingly, although ERK-2 acts as a transcriptional repressor of GATE, C/EBPβ activates the GATE promoter. Paradoxically, C/EBPβ is also downstream of ERK-2 within the MAPK pathway, as studies have shown that ERK-2 can phosphorylate and activate C/EBPβ activity (Park et al., 2004). Therefore, studies by Hu et al. (2009) proposed that expression of the GATE-driven genes is controlled by competitive binding of C/EBPβ and ERK-2 to GATE element. When C/EBPβ is activated it can compete for binding to GATE promoter, which results in decline of ERK-2 binding at this site (Hu et al., 2009).

Recent studies by M. Athar (MSc. Thesis, 2011) revealed that ERK-2 acts as an inhibitor of p20K activity. The QRU promoter region of p20K revealed to have a similar consensus ERK-2 binding sequence (GAAGGAGAAAG). This ERK-2 DNA binding motif is located between and overlapping the two C/EBPβ binding sites of the QRU (Chapter 4 Figure 4.2). This provided evidence of the mechanism that represses p20K in proliferating CEFs. It is hypothesized that ERK-2 sits of on the QRU promoter of cycling CEFs, and inhibits C/EBPβ from binding and activating p20K (Athar, MSc. Thesis, 2011). Interestingly, ChIP experiments showed that ERK-2 binds to p20K QRU promoter of cycling CEF in normoxia, but not in conditions of hypoxia or contact inhibition (Athar, MSc. Thesis, 2011). These findings established the existence of a new mechanism by which ERK-2 represses p20K gene expression.

1.10 CHOP structure

CHOP, also referred to as GADD153, is another member of the C/EBP family. CHOP is a transcription factor most notably known for its high expression in response to cellular stress (Zinszner et al., 1998). CHOP is also important for its direct role in apoptosis (Maytin et al., 2001). Although CHOP expression is low during normal cell proliferation, its mechanism of expression in normal cycling cells is not well known (Fawcett et al., 1996).

The leucine zipper (bZIP) basic domain is conserved among C/EBP family members, thus allowing them to dimerize with each other (Williams et al., 1991). Unlike other C/EBP family members, CHOP contains two proline residue substitutions that disrupt its helical structure (Oyadomari and Mori, 2004; Ron and Habener, 1992). These proline residues replace basic conserve amino acids. Thus, this mutation prevents CHOP homodimers from binding to DNA, but allows it to heterodimerize with other C/EBP family members and bind to an alternative C/EBPβ element (Ron and Habener, 1992; Ubeda et al., 1996). CHOP also participates in an autoregulatory loop with C/EBPβ. Studies by Fawcett et al. (1996) examined the interaction between CHOP and other members of the C/EBP family. During Arsenite treatment, only C/EBPβ was up-regulated, while other members of C/EBP family were not (Fawcett et al., 1996). The CHOP promoter also contains a C/EBP binding site that is essential for the activation of CHOP. During Arsenite treatment, forced expression of C/EBPβ resulted in the transcriptional activation of CHOP promoter. Paradoxically, CHOP can also antagonize C/EBPβ activity, however during modest levels of CHOP expression, C/EBPβ can still activate CHOP promoter. Only elevated levels of CHOP inhibited C/EBPβ and transactivation of CHOP promoter as an autoregulatory mechanism to attenuate CHOP expression during severe cellular stress (Fawcett et al., 1996). CHOP also actively dimerizes with C/EBPα, and inhibits

adipogenesis (Batchvarova et al., 1995). C/EBPα is a member of C/EBP family that is mainly involved in development, maintenance of adipocytic differentiation, and during acute phase response (Batchvarova et al., 1995). CHOP can also inhibit C/EBP€ during myeloid differentiation by sequestering C/EBP€ from binding to it consensus DNA sequence to activate myeloid target genes (Gery et al., 2004).

Chicken CHOP is homologous to its mammalian CHOP counterpart (Figure 1.3). Chicken CHOP was cloned by former MSc. thesis student Tetsuaki Miyake (2004). Chicken CHOP (25kDa) shares similar structural features with its mammalian equivalent containing Basic Region (BR) (DNA Binding domain), and Leucine Zipper (LZ) (Dimerization domain). Chicken CHOP and mammalian CHOP are very similar in size, and their basic domain contains a conserved proline (112) residue that is also shared among humans and hamsters (Miyake, MSc. Thesis, 2004). This proline residue sets CHOP apart from other C/EBP members, since it prevents CHOP from homodimerizing. Thus, CHOP can only form heterodimers with other members of the C/EBP family. In this way, CHOP generally antagonizes C/EBP family members, and prevents them from binding to consensus DNA target sequences (Ubeda and Habener, 2003). Chicken CHOP also contains a 5' untranslated upstream open reading frame region (uORF) that is highly conserved among species, including human, mouse, and hamster (Jousse et al., 2001). Chicken CHOP uORF gene is very similar in size and 33% identical at the nucleotide level to its mouse counterpart (Miyake, MSc. Thesis, 2004). Chicken CHOP also shares structural similarity with other leucine zipper transcription factors in which five leucine residues are aligned on the same side of α-helix on its LZ domain, also referred to as the heptad repeat (Alberini, 2006; Miyake, MSc. Thesis, 2004). This is important since the basic domain

forms the α-helix during DNA binding, and the helix structure is held together by hydrophobic interaction between leucine residues (Alberini, 2006).

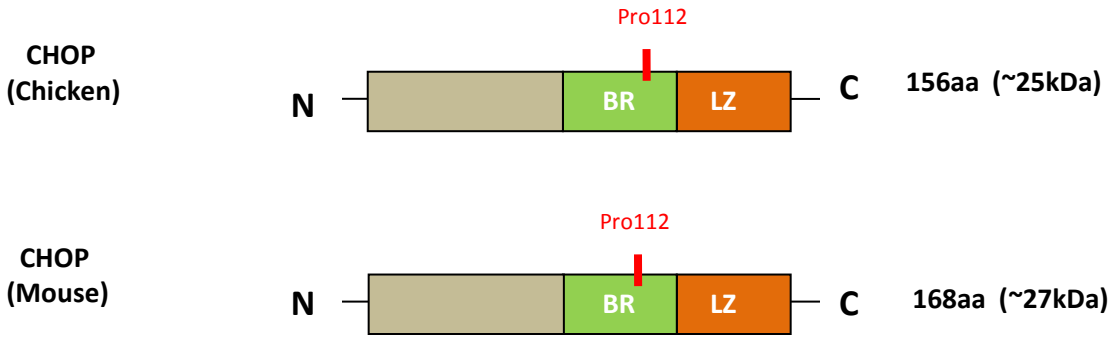


Figure 1.3 . Chicken CHOP Structure. Chicken CHOP (25kDa) structure is shown with the Basic Region (BR) (DNA Binding Domain), and Leucine Zipper (LZ) (Dimerization Domain). Chicken CHOP and mammalian CHOP are very similar in size, and they both contain a conserved proline residue in its basic domain. This proline residue prevents CHOP from homodimerizing, thus it can only heterodimerize with other C/EBP members. Chicken CHOP also has five leucine residues aligned on the same side of α-helix on its LZ domain, which is a typical structure of other leucine zipper transcription factors.

1.11 Transcriptional activation and different roles of CHOP

During high levels of ER stress, CHOP is over-expressed and accumulates in the cytoplasm (Chiribau et al., 2010). Thus, the nuclear translocation of CHOP from cytoplasm is a necessary step during prolonged ER stress. This is mediated by CHOP interaction with C/EBPβ isoform LIP. Thus CHOP stabilizes LIP from degradation, and alongside CHOP uses the LIP-CHOP complex for nuclear import (Chiribau et al., 2010).

CHOP actively participates in the induction of growth arrest or apoptosis in fibroblast cells during prolonged periods of Endoplasmic Reticulum (ER) stress or starvation (Friedman, 1996). After constant stress stimuli, CHOP is also phosphorylated on two adjacent serine residues (78 and 81) by p38-type MAP kinase (Wang and Ron, 1996). CHOP phosphorylation is required to augment its transcriptional activity. During ER stress conditions, the cellular response to unfolded proteins (UPR) is activated triggering a plethora of events involved in inhibition of protein synthesis (Harding et al., 2000). However, if the cell cannot compensate with prolonged ER-stress, other pathways will be activated that lead to cell death. As a result pro-apoptotic responsive genes are activated by UPR, including the activation of transcription factor 4 (ATF-4) (Harding et al., 2000). ATF-4 is one of the crucial protein effectors of UPR that regulate CHOP expression in conditions of persistent ER stress (Chiribau et al., 2010). The transcriptional up-regulation of CHOP is also dependant on an ATF-C/EBP composite site during cellular stress (Ma et al., 2002). The binding of ATF-4 and C/EBPβ at this particular site is crucial for CHOP promoter activation (Ma et al., 2002). In addition, during ER stress CHOP promoter activation is also dependent on an ERSE site, which is where other agents from UPR response bind. However, a mutation to ERSE site did not have a strong effect on basal levels of expression of CHOP (Ma et al., 2002). In oxidative stress conditions, the binding of AP-1 to the AP-1 binding

element within the CHOP promoter resulted in transcriptional activation of CHOP (Guyton et al., 1996). A single point mutation in the AP-1 element binding region of CHOP interfered with the binding of Fos and Jun (AP-1 family members) to CHOP promoter, reducing its transcriptional activity (Guyton et al., 1996). Alternatively, during starvation, ATF-2 binding to CHOP promoter results in the transcriptional activation of CHOP.

CHOP also plays a direct role in apoptosis. Constitutive expression of CHOP induces the activation of downstream genes of CHOP (DOC) during ER stress to initiate apoptotic mechanisms (Wang et al., 1998). The induction of DOC genes is mediated through a unique site where CHOP-C/EBPβ heterodimers can bind (Figure 1.4) (Ubeda et al., 1996; Wang et al., 1998). In arsenite-treated cells, CHOP appears to dimerize preferentially with C/EBPβ over C/EBPδ, indicating a preference in binding partners (Fawcett et al., 1996). Alternatively, as ATF4 stimulates CHOP expression, anti-apoptotic protein B-cell leukemia/lymphoma 2 (Bcl-2) is also inhibited (Cullough et al., 2001). At the same time, other pro-apoptotic members of Bcl-2 family, such as BH-3 only (BIM), are activated which are essential for initiation of apoptosis (Puthalakath et al., 1999). Consequently, CHOP is responsible for the direct transcriptional induction of BH-3 only (BIM), activation of DOC genes, and repression of Bcl-2.

Since CHOP is unable to form homodimers, it must heterodimerize with other C/EBP proteins, specifically C/EBPβ (Sok et al., 1999). Thus, CHOP has been identified as an inhibitor of C/EBP activity because expression of CHOP blocks the binding ability of C/EBP family members to their consensus DNA target sequences (Ubeda and Habener, 2003). CHOP can also form heterodimers with ATF-4, and repress ATF-4 activity in activating the asparagine synthetase (*ASNS*) gene during nutrient deprivation (Su and Kilberg, 2008). However in other

studies, CHOP can enhance C/EBP target genes. For instance, CHOP can sequester LIP from inhibiting C/EBPβ, and augments the activation of the IL-6 gene (target gene of C/EBPβ) (Hattori et al., 2003). IL-6 gene plays a role in diseases such as chronic Rheumatoid arthritis, and regulation of cytokines and acute phase proteins (Akira et al., 1990). During stress conditions when CHOP levels are induced, it can sequester LIP, but when CHOP accumulates at very high levels it represses C/EBPβ activity (Hattori et al., 2003).

CHOP can also function as a co-activator of other transcriptional proteins through a tethering mechanism (Figure 1.4). For instance, following a stressful stimulation, CHOP can interact with members of AP-1 family such as JunD, c-Jun, and c-Fos (Ubeda et al., 1999). AP-1 is known to mediate gene regulation in response to cytokines, growth factors, stress signals, infections, and oncogenic stimuli (Hess et al., 2004). As a result of CHOP interaction with AP-1 family members, AP-1 target genes are activated such as somatostatin, JunD, and collagenase genes (Ubeda et al., 1999). CHOP regulates gene expression, not through direct binding to DNA, but through protein-protein interactions through its bZIP domain with AP-1 family members that are DNA-bound (Ubeda et al., 1999).

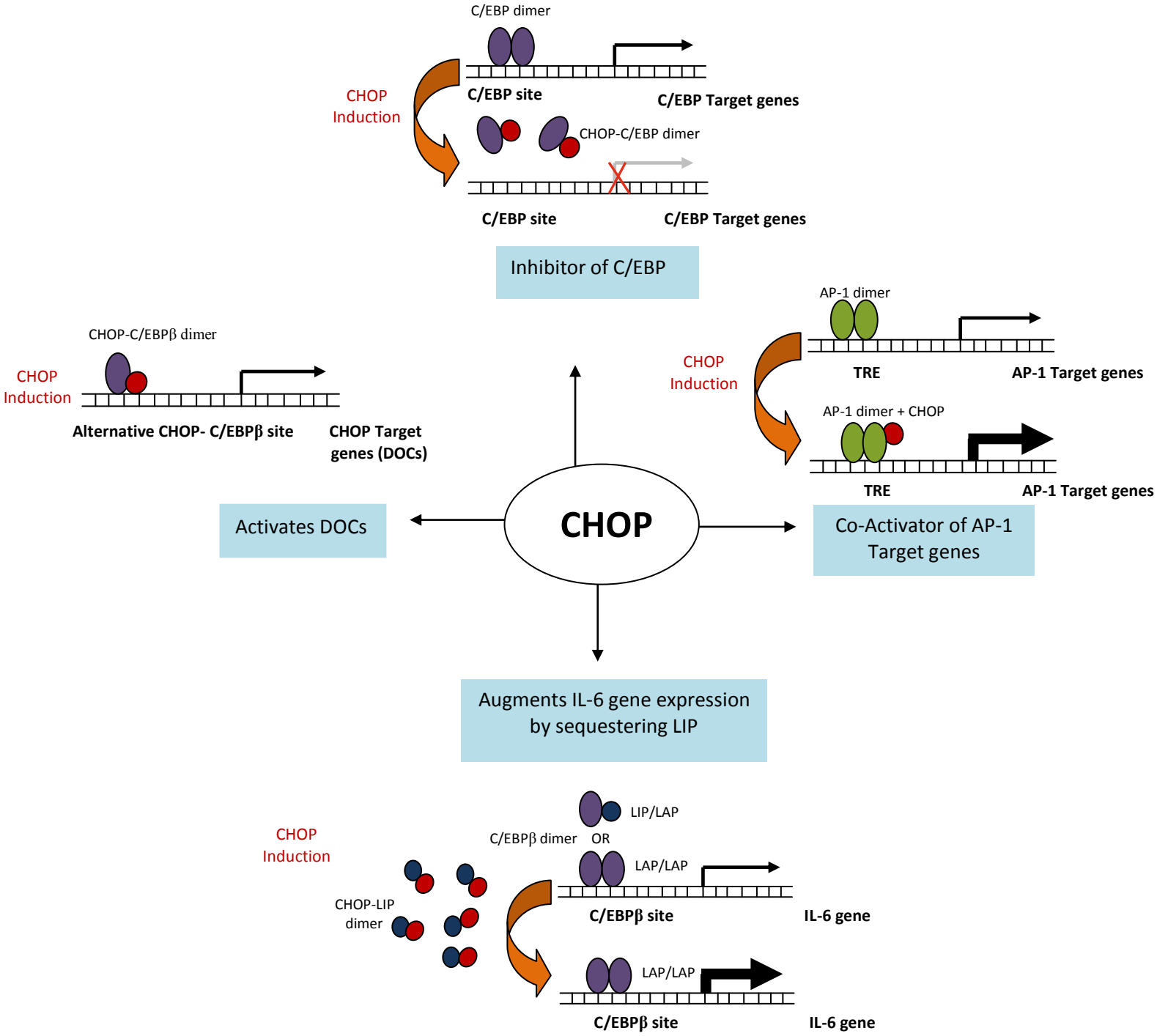


Figure 1.4. Functions of CHOP. CHOP is a transcription factor that can act as an inhibitor of C/EBPs and activator of other genes A) CHOP heterodimerizes with C/EBP family members, and prevents them from binding to consensus C/EBP target sequences. B) CHOP acts a co-activator of AP-1 target genes. C) CHOP activates downstream genes involved in apoptosis. D) CHOP can sequester LIP (Isoform of C/EBPβ), and increase IL-6 gene expression. *(Adapted from Dr. Miyake, MSc. Thesis, 2004)*

1.12 Research Rationale:

Preliminary studies by Ben Fielding (MSc. Thesis, 2011) showed that induction of p20K in cycling CEF resulted in CHOP down-regulation at 24 hours hypoxia. In addition, other experiments by B. Fielding showed that over-expression of CHOP through RCASB system repressed p20K activity in hypoxia. Therefore, the primary goal of my project is to further investigate the effect of CHOP expression on p20K. It is hypothesized that CHOP plays a role in the control of p20K expression; hence CHOP has been extensively studied during the research process.

1.13 Hypothesis:

CHOP is a key determinant of p20K expression in hypoxia or contact inhibited chicken embryonic fibroblasts (CEFs).

1.14 Main Objectives:

The main objective of my project was to study the effect of CHOP on p20K induction in various conditions such as hypoxia, contact inhibition, and ER stress in CEFs. Western blotting analysis and RT-qPCR were used to validate CHOP and p20K protein and mRNA levels in normoxia, hypoxia, contact inhibition, and conditions of ER stress. Proliferation assays and Western blotting analysis were performed using a CHOP knockdown by shRNA to investigate the effect of CHOP down-regulation on p20K and cell survival. Since CHOP is a pro-apoptotic transcription factor, it was hypothesized that its down-regulation may enhance the survival of cells as they begin to starve. In addition, it was likely that attenuating CHOP levels will have a positive effect on p20K induction. Moreover, my studies also focused on studying the

expression of p20K during prolonged periods of ER stress when CHOP is over–expressed. Preliminary data by B. Fielding suggests that CHOP may be acting as an inhibitor of p20K activity, possibly because of its interaction with C/EBPβ. It is hypothesized that CHOP induction will promote the formation of inactive CHOP-C/EBPβ heterodimers, sequestering C/EBPβ from binding to QRU promoter, hence inhibiting p20K expression. Therefore, co-immunoprecipitation experiments were used to analyze C/EBPβ and CHOP interaction.

The second objective of my project was to investigate the effect of a hypoxia mimicking agent on p20K expression. Since p20K is highly induced in hypoxia conditions (2% O_2), it was of interest to observe the expression of p20K in cells treated with DMOG, a prolyl-hydroxylase inhibitor drug that stabilizes Hypoxia Inducible Factors (HIF) under normoxic conditions mimicking a hypoxia response (Barnucz et al., 2013). Since DMOG chemically mimics a hypoxia response, it was of interest to study p20K induction in these conditions.

Chapter 2: Materials and Methods

2.1 Cell Culture

Chicken Embryonic Fibroblasts (CEFs) in early passages (n<13) were cultured in 8 ml of Dulbecco's Modified Eagle Medium (Gibco #11995). Media was supplemented with 5% heat inactivated cosmic calf serum (57ºC for 30 min) (Thermo Scientific HyClone #SH30087.03), 5% tryptose phosphate broth (Sigma, #T9157), L-Glutamine (Gibco 25030), and penicillin/streptomycin (Gibco #15140). Cells were grown at 41.5ºC in a humidified, 5% CO_2 atmosphere. Cells were split 1:3 every 2-3 days with 0.05% Trypsin-EDTA (Gibco # 25300), and seeded in 100mm culture dishes (BD Falcon #353003).

2.2 Cell Culture Conditions

2.2.1 Hypoxia Stimulation

The experiments were conducted using a conventional incubator under either normoxic conditions set to 21% O_2 or hypoxic conditions by incubating the cells in a hypoxia chamber set to 1.8-2% O_2. Cycling cells were split 1:3, left overnight (12-16 hours) to re-attach and recover, and placed in hypoxic or normoxic conditions for 24 hours, after which cell lysates were collected. For contact inhibited cells, cells were split 1:3, and once cells reached confluency, media was changed 24 hours prior to placing in hypoxia chamber to avoid any nutrient depletion bias. Cycling or contact inhibited cells in normoxia were subjected to parallel conditions, but were kept in a 21% O_2 incubator.

2.2.2 ER Stress

CEF cells were subjected to ER stress stimuli by treating confluent cells with either 1μg/ml tunicamycin (Sigma #T7765), 1μg/ml thapsigargin (SigmaT9033) or 0.1 % DMSO (diluent control) (Santa Cruz # K1011) for different times ranging from 12-36 hours. Cells were split

1:3. Once cells reached contact inhibition, media was changed 24 hours prior drug treatments. Medium was replenished to prevent any nutrient deficiency effects.

2.2.3 DMOG Treatment

A concentration of 1mM of dimethyloxaloylglycine (DMOG) was added to the media of cycling CEF, and lysates from DMOG treated and untreated cells were collected at 24 and 36 hours post-treatment. Prior to DMOG treatment, cells were split 1:4 one day before, and left overnight to recover.

2.3 Proliferation Assays

Cell Seeding

Cells were seeded in 60 mm tissue culture plates (BD Falcon # 353002), and left overnight to re-attach and recover. The following day, cells were trypsinized and resuspended in 2ml of 0.05% trypsin. A 500µl trypsin mixture with floating cells aliquot was taken and resuspended in 9.5ml of IsoFlow™ Sheath Fluid counting solution (Fisher Scientific). Cells were counted in triplicate for a period of 4 consecutive days using a Beckman Coulter Particle Counter. Alternatively, cells were seeded in 24 well plates (BD Falcon #353047), and left overnight to re-attach and recover. Cells lines were seeded in quadruple at equal numbers, and only the eight center wells of each 24 well dish was used. Next day, cells were re-suspended in 500µl of 0.05% Trypsin. The entire 500µl trypsin mixture with floating cells was transferred and resuspended in 9.5ml of IsoFlow™ counting solution. Cells were then counted using a Beckman Coulter Particle Counter. Each suspension was counted twice. Cells were counted every day for a period of 7 consecutive days, and every other day after day 7.

2.4 Transfection Protocol:

2.4.1 DNA Precipitation for Transfection

Ten micrograms of DNA construct supplemented with twenty micrograms of Salmon sperm DNA was used for each DNA precipitation per 100mm plate. For ethanol-precipitation, DNA was stored overnight at -20ºC, or 1-2 hours in -80ºC. To recover the precipitated DNA, the tube was centrifuged at 13,000 x g for 10 minutes at 4ºC. The supernatant was discarded, and the DNA pellet was rinsed by adding 70% cold ethanol followed by a second centrifugation at 13,000rpm for 5 minutes. The supernatant again was discarded, and the DNA pellet was air dried for 3 minutes. The DNA was resuspended in 500 µl of 250mM $CaCl_2$.

2.4.2 Calcium Phosphate Transfection:

CEF were split 1:3 one day prior to transfection. The following day, 4 hours prior to transfection, medium was changed and 6 ml of complete medium was added per 100mm tissue culture plate. At the time of transfection, cells were about 50-70% confluent. The DNA was precipitated and resuspended as described above, and slowly (drop-wise) 500 µl 2x HBSP pH 7.12 (1.5mM Na_2HPO_4, 10mM KCl, 280mM NaCl, 12mM glucose, and 50mM Hepes) was added to the DNA while vortexing. The DNA precipitate was allowed to sit at room temperature for 20 minutes. One milliliter of final DNA precipitate mix solution was added to CEF culture dropwise, and mixed gently by swirling the plate. Plates were incubated with the DNA precipitate for 5 hours. After incubation, the medium was gently aspirated, and cells were glycerol shocked (15% glycerol in HBSP buffer) for 1 minute. Plates were then washed twice with serum-free medium, and 9 ml of complete medium was added to each plate (Figure 2.2).

RCAS vectors used for transfection experiments are listed in Table 2.1. The CHOP RCAS vectors were made by former lab technician, Sam Yan. RCAS retroviral vector able to express a

short-hairpin RNAi (shRNA) was used during transfection for CHOP down-regulation. RCAS is an acronym for **R**eplication **C**ompetent **A**LV LTR with a **S**plice acceptor (Figure 2.1) (Hughes, 2004). RCAS is an avian RSV derived replication-competent retrovirus lacking a *v-src* gene (Hughes, 2004). This vector generated an RNA interference system that can efficiently target specific genes within CEF. The RCASBP vector system results in complete infection of CEF cultures. Therefore after transfection, the CEF cells were passaged three times to allow complete infection of the entire cell population. However a better down-regulation of CHOP was observed when CEF transfected with a RCAS(A) CHOP shRNA vector were passaged at least four times.

In addition, the RSV derived RCAS vector can be used to efficiently over-express proteins in developing avian embryos or CEF (Hughes, 2004). Therefore, CEF cells were infected with a RCAS(B) viral vector over-expressing CHOP. RCAS (A) or (B) refers to viral envelope glycoproteins specific to each virus. Each retroviral envelope interacts with specific receptors on host cells surface (Hughes, 2004). Thus RCAS (A) contains the A envelope that recognizes the TVA cellular receptor, and RCAS (B) expresses the B envelope that binds to the TVB receptor (Hughes, 2004).

Table 2.1- DNA viral vectors used for Calcium Phosphate Transfection

RCAS viral vectors	
Control (Empty Vector)	RCAS (A)
CHOP downregulation	RCAS (A) 354-CHOP-10 shRNA
Control (Empty Vector)	RCAS (B)
CHOP upregulation	RCAS (B) CHOP
C/EBPβ Dominant Negative Mutant (Gagliardi et al., 2001)	RCASBP Δ184-C/EBPβ

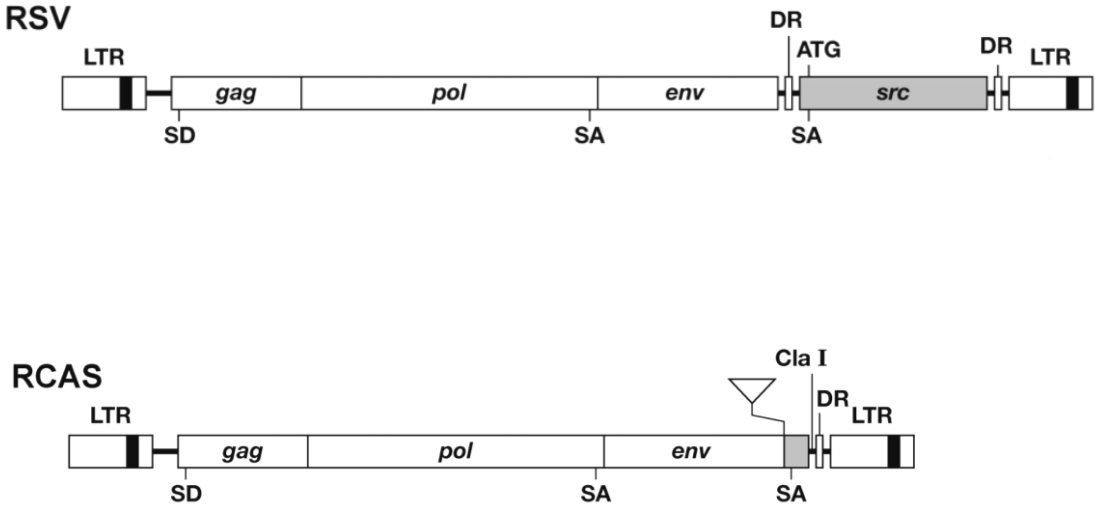


Figure 2.1- RCAS structure. The RCAS virus is missing the src oncogene that is replaced by a unique restriction site (ClaI). However, RCAS retained a small segment of src gene, which carries the src splice acceptor. The structure of RCAS is composed of long terminal repeat (LTR), *gag, pol, and env* genes, direct repeats (DR), splice donor (SD), and splice acceptor (SA). DR is crucial for viral replication and assembly *(Adapted from Hughes, 2004)*.

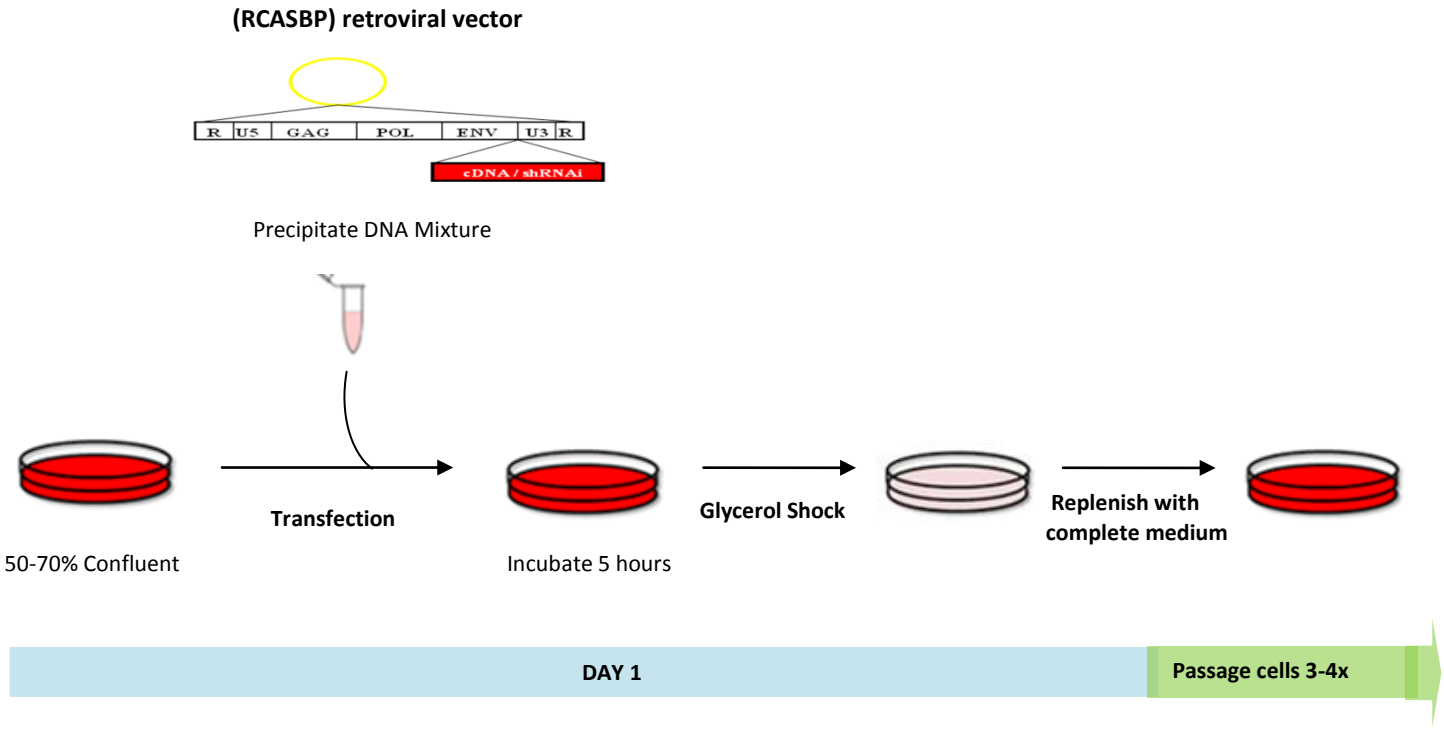


Figure 2.2- Calcium Phosphate transfection of CEF- The first step of transfection is to incubate CEF for 4-5 hours with DNA precipitate mixture containing RCASBP retroviral vector. Cells were glycerol shocked (15% glycerol in HBSP buffer) (1 minute). Then cells were washed twice with serum-free medium, and replenished with 9 ml of complete medium. After transfection, the CEF cells were passaged three to four times to allow complete infection of the entire cell population.

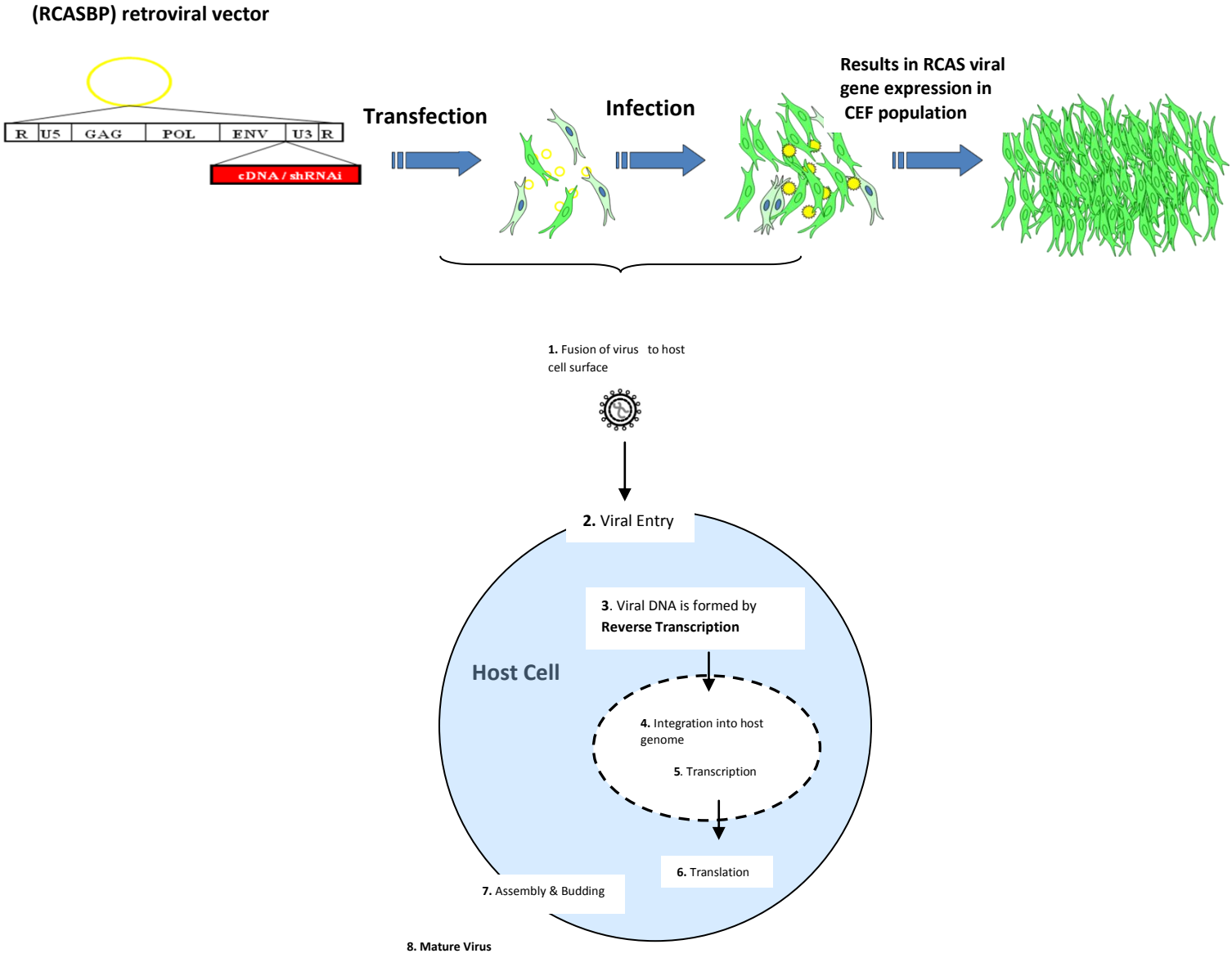


Figure 2.3- RCAS retrovirus lifecycle overview- The first step of retroviral life cycle is *fusion* of retrovirus with host cell surface. Second is *viral entry*, where RCAS viruses require receptor binding and low pH. The viral RNA is transcribed into viral DNA by *reverse transcriptase*. Then RCAS viral DNA is transported into the nucleoplasm, where *integration* into the host DNA takes place. Two copies of the genomic viral transcript associates with capsid proteins to generate viral particles released into the medium by *budding*. Successful assembly and release of the viral particles leads to infection of the CEF population. *(Adapted from Dr. Tetsuaki Miyake, MSc. Thesis, 2004).*

2.5 Cell Lysate Preparation

Cell lysates were prepared by first aspirating medium, and placing cell culture dish on ice. Plates were washed twice with 10ml of cold 1 x PBS pH 7.4 (137mM NaCl, 2.7mM KCl, 4.3mM Na2HPO4, 1.47 mM KH2PO4). Cells were collected in 1ml of 1 x PBS with a scraper, and pelleted gently by centrifugation. Supernatant was aspirated carefully, and discarded. Pelleted cells were lysed with 100-200µl of 1X SDS sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethantol, 60mM Tris pH 6.8) with Protease and Phosphatase inhibitor cocktail (Thermo Scientific 78441). Samples were heated for 3 minutes at 100ºC, vortexed for 10 seconds, and then centrifuged at 13,000 rpm for 15 minutes at 4 °C. Supernatant was transferred to a new microcentrifuge tube, and samples were stored at -80°C. Bradford assay was used to quantify protein concentration.

2.6 Western Blotting

The amount of protein used for Western blotting was 80µg per sample**.** Lysates were run on a 12 % SDS-polyacrylamide gel (SDS-PAGE) night. The following day, proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Bioscience) for 3 hours at 60 volts at 4ºC. The membrane was immersed in blocking buffer (1x Tris Buffered Saline (TBS) pH 7.6 with 5% non-fat dry milk and 0.02 % Na Azide) for one hour with gentle agitation at room temperature. Membrane was incubated with primary antibody overnight at 4 °C on rotator. Primary antibody was diluted in blocking buffer. Specific antibodies used are listed in Table 2.2. Phospho p44/42 MAPK (ERK1/2) antibody was diluted in 1X TBS with 5% BSA (Sigma #A9647) instead of non-fat dry milk. After the primary antibody incubation, the blot was washed once with 1x TBS, twice with 1x TBS-T (1x TBS with 0.1% Tween-20), and twice again with 1x TBS (each wash was for 5 min). Now the nitrocellulose membrane was ready to be incubated with secondary

antibody by gentle shaking at room temperature for 1 ½-2 hours. Secondary antibody was diluted 1:25000 in 1x TBS with 5% non-fat dry milk or BSA containing no sodium azide. Washes were repeated once again for 5 minutes each: once with 1X TBS, twice with 1X TBS-T, and twice with 1X TBS. The membrane was exposed to Luminata Forte Western HRP Substrate (Millipore # WBLUF0100) reagent for chemiluminescent detection, and subjected to Amersham Hyperfilm ECL (GE Healthcare) exposure to visualize membrane-protein complexes. ImageJ software was used to quantify protein expression using ERK-1 intensity to correct for loading.

Table 2.2- Western Blotting Antibodies

Primary Antibodies	Dilutions
ERK 1 (Santa Cruz Biotechnology Cat# sc-94)	1 : 2000
p20K (601-Y) (Bedard et al., 1987)	1 : 1500
CHOP (AB-1 Tulip)	1 : 1500
C/EBPβ (AB3-B5) (Gagliardi et al., 2001)	1 : 2000
Phospho- p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (Cell Signaling Cat# 4370)	1 : 2000
Secondary Antibody	
Anti-rabbit IgG HRP linked (Cell signaling Cat# 7074)	1 : 25000

2.7 Co-Immunoprecipitation

Cells were collected in 1 ml of 1 X PBS, and pelleted by centrifugation. Cell pellet was lysed and resuspended in 1 ml of TNE buffer (50mM Tris HCl pH 7.6, 150mM NaCl, 1% Nonident P-40, 2mM EDTA pH 8, EGTA pH 8.5, with protease /phosphatase inhibitor cocktail). Samples were allowed to sit on ice for 20 minutes to ensure full lysis in TNE buffer. Samples were then centrifuged for 10 minutes at 13000 rpm at 4ºC to remove insoluble debris. To prepare protein samples, Bradford Assay was used to determine protein concentration. 500µg of proteins from the original lysate were used per co-immunoprecipitation. Samples were incubated overnight on a rotator at 4 °C with corresponding antibodies *i.e.* 2 µL of AB3 pre-immune Antibody (control) or 2µL of C/EBPβ AB3 antibody.

The following day, 40µl of blocked Protein G Sepharose Beads (GE Healthcare 17-0618-01) were added to each sample, and incubated for 1.5 hours on a rotator platform at 4ºC. Samples were pulse centrifuged for 20 seconds, and supernatant was carefully aspirated. Bead-Antibody mixture was subjected to five washes (5 minutes each) with 1 ml of TNE buffer on a rotator at 4ºC. With each wash samples were pulse centrifuged for 20 seconds to collect the bead-antibody complex, and the supernatant was discarded. After all washes were completed, 60 µL of SDS sample buffer was added to each sample. Samples were resuspended, quickly vortexed, and boiled for 3 minutes to elute protein complexes. Lastly, samples were centrifuged at 13000 rpm for 15 minutes at 4 °C, and the supernatant was transferred to a new microcentrifuge tube. At this point samples were stored at -80°C for later use for Western blotting analysis. The same protocol as previously described in Section 2.6 was followed for Western blotting analysis. However, instead of using rabbit secondary antibody for detection, Clean blot IP detection reagent (Thermo Scientific #21230) was used at a 1:500 dilution in 1 X TBS with 5% non-fat dry milk.

2.8 Reverse Transcriptase-quantitative PCR

2.8.1 RNA extraction by the Trizol Method

CEF cells were collected in 1 ml of cold 1 X PBS, and pelleted gently by centrifugation (6500 rpm at 4°C for 3 minutes). RNA was extracted from cycling or contact inhibited CEF in different conditions (normoxia, hypoxia, or ER stress) using Trizol reagent protocol from the manufacturer (Invitrogen). Trizol reagent (1ml) was added to each sample and mixed vigorously by pipetting. Samples were allowed to sit for 5 minutes at room temperature for complete cell lysis. 200µl of chloroform was added to each sample, quickly vortexed, and incubated at room temperature (3 minutes). Then samples were centrifuged at 13000 rpm for 15 minutes at 4 °C.

After centrifugation, the clear top aqueous layer was collected carefully without disturbing the organic bottom layer into a separate microcentrifuge tube. To precipitate the RNA from solution, 500 µl of isopropyl alcohol was added to each sample, vortexed, and incubated at room temperature for 10 minutes. Once again, samples were centrifuged for 10 minutes. After centrifugation, the precipitated RNA pellet became visible and the supernatant was discarded. The pellet was washed twice with 1ml of 75% cold ethanol (DEPC treated), followed by 5 minute centrifugation at maximum speed in a microfuge. After supernatant was carefully removed, the RNA pellet was air dried for 5-10 minutes, and then resuspended in 20µl of 0.1% DEPC treated ddH_20. The RNA concentration was determined using an Ultrospec 2100 pro UV/Visible Spectrophotometer at 260nm.

2.8.2 DNase I treatment and PCR

RNA (1 µg) was treated with DNase I (Thermo Scientific #EN0521) and 10X Reaction Buffer with $MgCl_2$ (Thermo Scientific). DNase treated total RNA was reverse transcribed to cDNA using Protoscript AMV First Synthesis Kit (New England Biolabs #E6550S). Prior to quantitative PCR, the primers and cDNA were first checked by performing a PCR reaction using GoTaq Green PCR mix (Promega #M712B). Each PCR reaction contained 12.5 µl of GoTaq Mix, 1-2 µl of cDNA template, 1 mM forward and reverse primers, and nuclease free ddH_20 to a final volume of 25 µl. PCR amplification is described in Table 2.3. Following amplification, PCR samples were run on a 1.2% agarose gel with ethidium bromide and visualized on a UV transilluminator.

Table 2.3- PCR Amplification Protocol

	Temperature	Time
Denaturation	95°C	2 minutes
X 30	95°C 60°C 72°C	20 sec 20 sec 20 sec
Extension	72°C 4°C	2 minutes

2.8.3 Quantitative PCR Protocol

PerfeCTa SYBR Green FastMix low ROX (Quanta Biosciences#14058) cocktail was used to prepare samples for real-time quantitative PCR analysis. Samples were prepared in 96-well PCR plates (Thermo Scientific #AB0600). A master mixture containing all necessary ingredients such as nuclease free water, 1mM of forward and reverse primers, and 1-2 µl of cDNA template was added to each sample for a total volume of 20µl. Specific primers used are described in Table 2.4. Stratagene MX3000P Real-time PCR machine was used to carry out qPCR reaction. The $2^{-\Delta\Delta CT}$ method was used to quantify amplification levels (Livak and Schmittgen, 2001). In this method, data is shown as relative change in gene expression normalized to an endogenous reference gene, GAPDH in this case, and relative to a calibrator, also referred to as the untreated control (Livak and Schmittgen, 2001). In hypoxia experiments, cycling cells in normoxia were used as calibrator or relative control for comparison in gene expression analysis. In ER stress experiments, DMSO treated cells were used as relative control. Statistical calculations represent three independent RNA preparations.

Table 2.4- Primers used in qPCR reaction

Protein Coded	Forward Primer (5'-3')	Reverse Primer (5'-3')
p20K	GCC CAG CCA GGA GGA ATG CA	AGC AGC CTC GAG CTT TGG CA
CHOP	GTC CTT GTA GGG CAG CAC AC	GGG GAC GTT GAG ACA GCA AT
GADPH	GTC GGA GTC AAC GGA TTT GGC CG	ATG GCC ACC ACT TGG ACT TTG CC

Chapter 3: Results

3.1. To examine if different oxygen concentrations in hypoxia induce a change in p20K expression

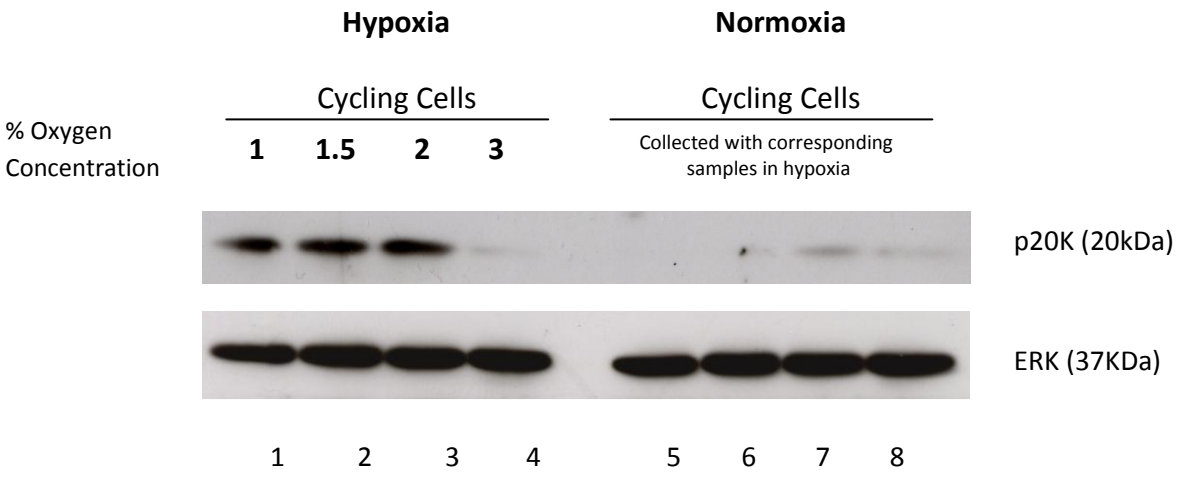
Previous studies have established that p20K is a GAS gene highly induced in conditions of hypoxia (2%O_2) or contact inhibition (Fielding, MSc. Thesis, 2011; Kim et al., 1999). It was therefore important to determine the conditions of hypoxia (1%, 1.5%, 2%, and 3% O_2) leading to p20K induction. Thus, cycling CEFs were incubated in various hypoxic conditions or normoxia (21% O_2) for 24 hours. At the end of the 24 hour incubation periods, cycling CEF samples were collected, quantified with Bradford assay, and examined by western blotting. Results show high levels of p20K expression in all hypoxic conditions except at 3% O_2 levels and normoxia (Figure 3.1A). This further confirms that hypoxia (2% O_2 or less) is required to induce expression of p20K in cycling CEF.

Confluent CEF cultures show a significantly elevated level of p20K expression in both normoxia and hypoxia conditions. However, this time it was of interest to determine if levels of p20K or CHOP, a negative regulator of p20K expression, would fluctuate in contact inhibited cells incubated at different oxygen concentrations. Thus contact inhibited CEF were incubated in hypoxia (1%, 1.5%, 2%, 2.5% and 3% O_2) or normoxia (21% O_2) for 24 hours. Figures 3.1B and 3.1D represent the quantification of protein expression in results in 3.1A and 3.1C. As shown in Figure 3.1C Western blotting analysis revealed a lack of CHOP expression in contact inhibited CEF in both normoxia and hypoxia conditions (Lanes 1-10). CHOP levels are only detected in cycling CEF in the absence of p20K expression or in response to ER stress (Lanes 11-12; Figure 3.1C and Figure 3.6A). The lack of CHOP in contact inhibited CEFs demonstrates that hypoxia (2% O_2) does not activate CHOP expression and therefore does not generate ER stress in these conditions. In contrast, p20K is actively expressed in all contact inhibited cells

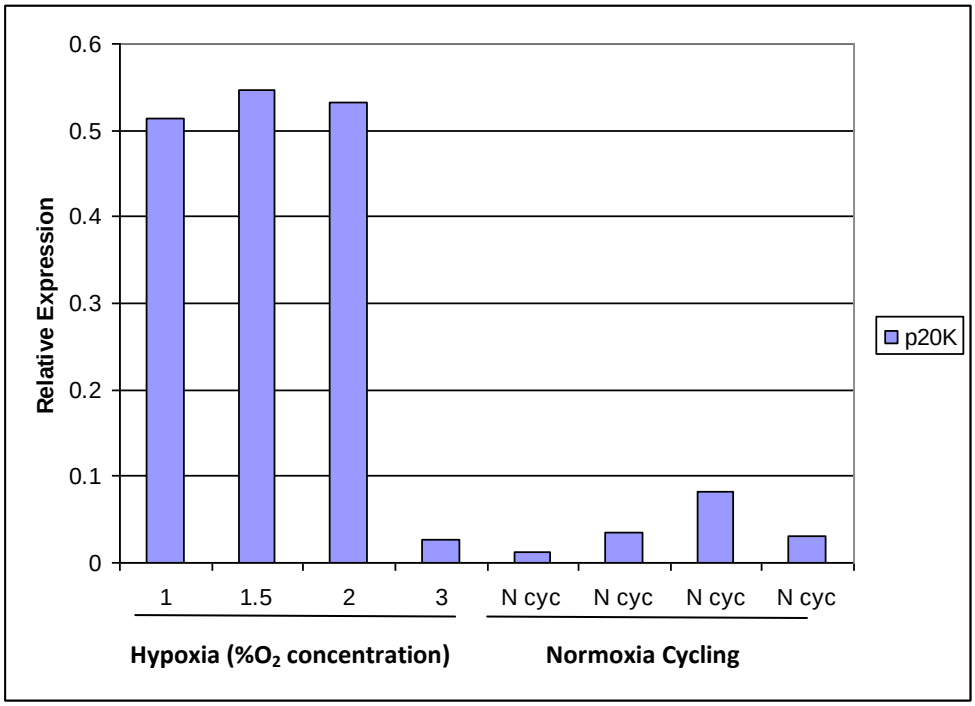
incubated in normoxic or hypoxic conditions. However as shown in Figure 3.1C, p20K levels are undetectable in normoxia cycling cells, where CHOP expression is observed.

Figure 3.1: Induction of p20K in different oxygen concentrations. Cycling or contact inhibited CEFs were incubated in a hypoxic chamber set to different oxygen concentrations for 24 hours. Expression of ERK-1 was used as loading control **A.** Western blotting analysis of p20K expression from cycling CEF after 24hr incubation in different hypoxia conditions (1%, 1.5%, 2%, or 3% O_2) or normoxia. (21% O_2). Results show high levels of p20K expression in all hypoxic conditions except at 3% O_2 levels or normoxia cycling**. B.** Quantification of protein expression from Western blot Figure 3.2A. Levels of p20K are corrected to the levels of ERK **C.** Western blotting analysis of expression of p20K and CHOP in contact inhibited CEF in response to different oxygen concentrations in hypoxia (1%, 1.5%, 2%, 2.5%, or 3% O_2.) vs. normoxia. CHOP levels are only detected in cycling CEF in the absence of p20K expression (Lanes 11 & 12). p20K is actively expressed in all contact inhibited CEF incubated in normoxic or hypoxic conditions. **D.** Quantification of protein expression from Western blot Figure 3.2C. Levels of CHOP and p20K are corrected to the levels of ERK

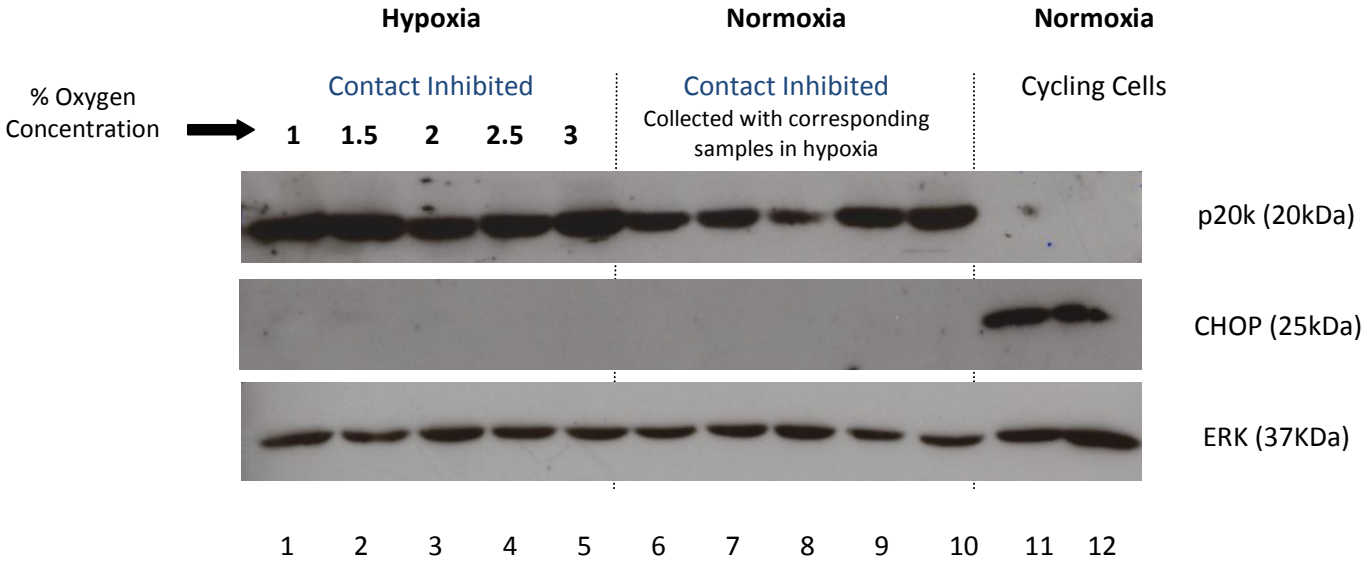
A.


Hypoxia
Normoxia
Cycling Cells
Cycling Cells
% Oxygen Concentration
1 1.5 2 3
Collected with corresponding samples in hypoxia
p20K (20kDa)
ERK (37KDa)
1 2 3 4 5 6 7 8

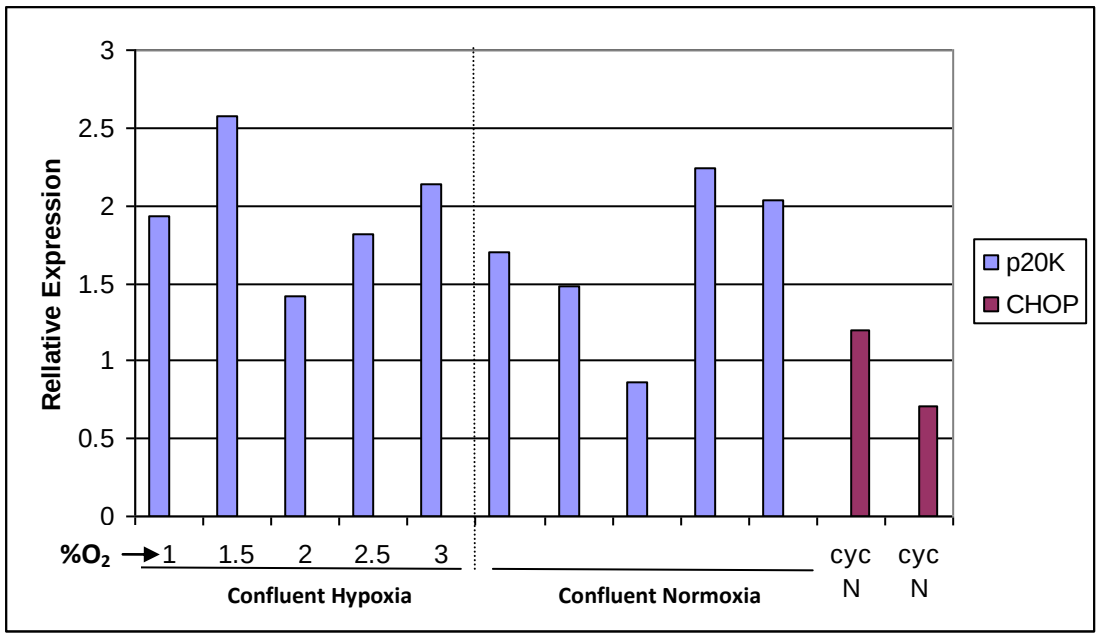
B.


Relative Expression
0.6
0.5
0.4
0.3
0.2
0.1
0
1 1.5 2 3 N cyc N cyc N cyc N cyc
p20K
Hypoxia (%O2 concentration)
Normoxia Cycling

C.


Hypoxia
Normoxia
Normoxia
Contact Inhibited
Contact Inhibited
Collected with corresponding samples in hypoxia
Cycling Cells
% Oxygen Concentration
1 1.5 2 2.5 3
p20k (20kDa)
CHOP (25kDa)
ERK (37KDa)
1 2 3 4 5 6 7 8 9 10 11 12

D.


Rellative Expression
3
2.5
2
1.5
1
0.5
0
%O2 1 1.5 2 2.5 3
cyc N
cyc N
Confluent Hypoxia
Confluent Normoxia
p20K
CHOP

3.2 Kinetics of CHOP expression

Despite a basal level of expression in cycling cells (Figure 3.1C), CHOP is generally involved in promoting cellular apoptosis in conditions of ER stress (Wang and Ron, 1996); therefore it was important to determine the effect of CHOP down-regulation on cell proliferation in normoxia. In these experiments, CHOP levels were down-regulated by transfecting CEF cells via calcium phosphate method with a retrovirus vector expressing a shRNAi 354-CHOP-10 that inhibits CHOP expression. In addition, CEFs were also transfected with a corresponding empty vector RCAS(A) RNAi as a control. After transfection, the cells were passaged four times to allow complete infection of the CEF population. Attenuating CHOP expression by shRNAi reduced CHOP protein level in CEF as determined by Western Blotting analysis (Figure 3.2A). Additionally, proliferation assays were performed to determine the effect of CHOP down-regulation on cell proliferation.

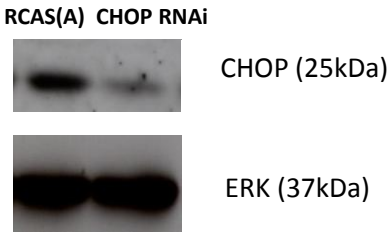
CEF were plated in 24-well dishes and the number of cells was determined using a Beckman Coulter Particle Counter. CEF were counted every day for a period of 7 consecutive days, and every other day after day 7. Results demonstrate that CHOP down-regulation enhances CEF proliferation rate (Figure 3.2B). CEF transfected with shRNAi 354-CHOP-10 display a significantly higher rate of proliferation than in control RCAS(A) cell line between days 1, 2, 3, 5, and 6. Cell count average between cell lines during these particular days is statistically significant as determined by a t-test analysis ($p < 0.001$, $p < 0.01$, or $p < 0.05$) (Figure 3.2B). Proliferation curves show that 354-CHOP-10 cells nearly tripled in numbers by Day 4 in comparison to control (Figure 3.2B). Thus diminishing CHOP expression provides CEF with accelerated proliferation. It is important to note that at day 14 both cell lines experienced a higher rate of cell loss. Previous studies by former lab technician, Sam Yan, showed that CHOP

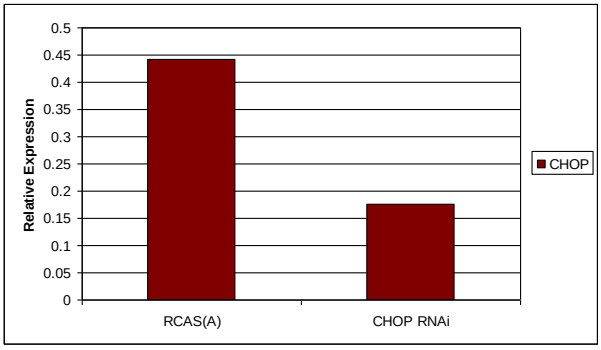
down-regulation by 354-CHOP-10 shRNA enhances CEF survival in conditions of starvation (Data not shown). Therefore, it is likely that the level of CHOP inhibition in my studies was not sufficiently high to observe this effect. However, attenuating CHOP expression proved to have a proliferation advantage.

A separate set of experiments characterized the expression of CHOP in normoxia as CEF became contact inhibited. In addition, proliferation assays were also performed using a CHOP knockdown by shRNA (354-CHOP-10) and RCAS(A) as control. The 354-CHOP-10 and RCAS(A) cell lines were seeded in 60mm dishes in normoxia conditions for counting, and corresponding cell lysates were also collected every day for a period of 4 consecutive days for Western blotting analysis. After cells were seeded, CEFs were counted and lysates were collected until both cell lines became contact inhibited by day 4. Western blotting analysis indicated that CEF with CHOP down-regulation exhibit elevated levels of p20K compared to RCAS(A) cells (control) (Lane 4 and 8; Figure 3.3A). This indicates that abrogating CHOP expression induced an early induction of p20K. As levels of p20K increased by day 4, a decline in CHOP levels was also noted in both cell lines. Interestingly, although both cell lines did not have a significant difference in cell numbers by day 3, results show that by down-regulating CHOP expression, p20K is induced prematurely. Both cell types proliferated at similar rates until day 4, where 354-CHOP-10 continued to accumulate while control cells became contact inhibited (Figure 3.3C & D). The cell count difference at Day 4 is statistically significant in an unpaired t-test ($p<0.05$). This data suggests that down-regulation of CHOP promotes CEF proliferation, and an earlier induction of p20K expression as cells become confluent.

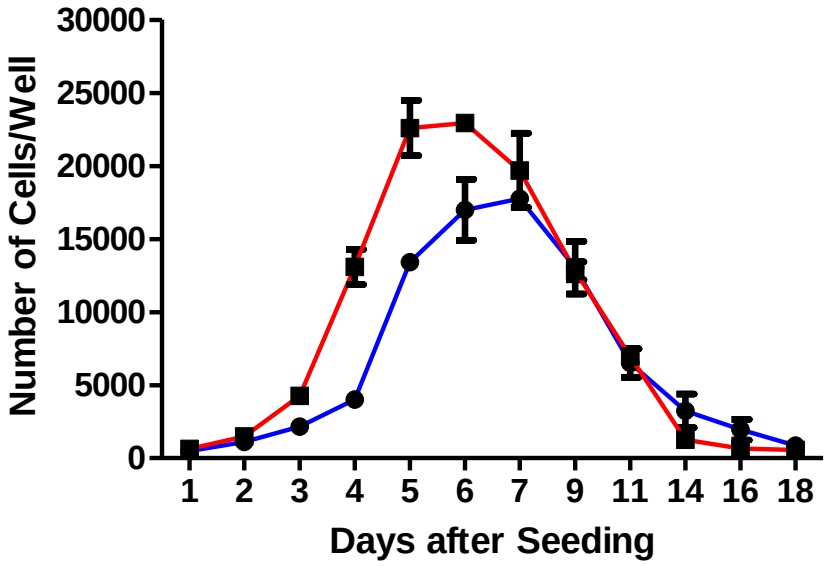
Figure 3.2: Kinetics of CHOP expression from cycling to starvation to cell death. CEF were transfected with RCAS(A) RNAi (control vector) and RNAi CHOP-10 # 354 to down-regulate CHOP expression. CEF were seeded in 24 well plates, and counted for 7 consecutive days, and every other day after day 7 without media replenishment. Cell counts were performed in quadruple, by counting four wells at each time point. **A.** Western blotting analysis of CHOP and ERK protein levels of cycling CEF in normoxia. Western blotting analysis shows the down-regulation of CHOP with RNAi CHOP-10 # 354 versus the control RCAS(A) RNAi. Protein expression from Western blot was quantified. Levels of CHOP are corrected to the levels of ERK **B.** Proliferation Assays of RNAi CHOP-10 # 354 and RCAS(A) cell lines . Results show that CEF with down-regulation of CHOP expression proliferated at a faster rate than control. Error bars at each time point represent standard error. Proliferation rate between cell lines was significantly different in a separate t-test. An asterisk * indicates that the cell count average between cell lines is significantly different in *t-test* (***$p < 0.001$, **$p < 0.01$, or *$p < 0.05$).

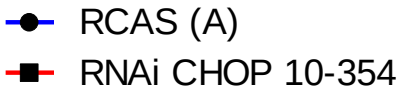
A.





B.

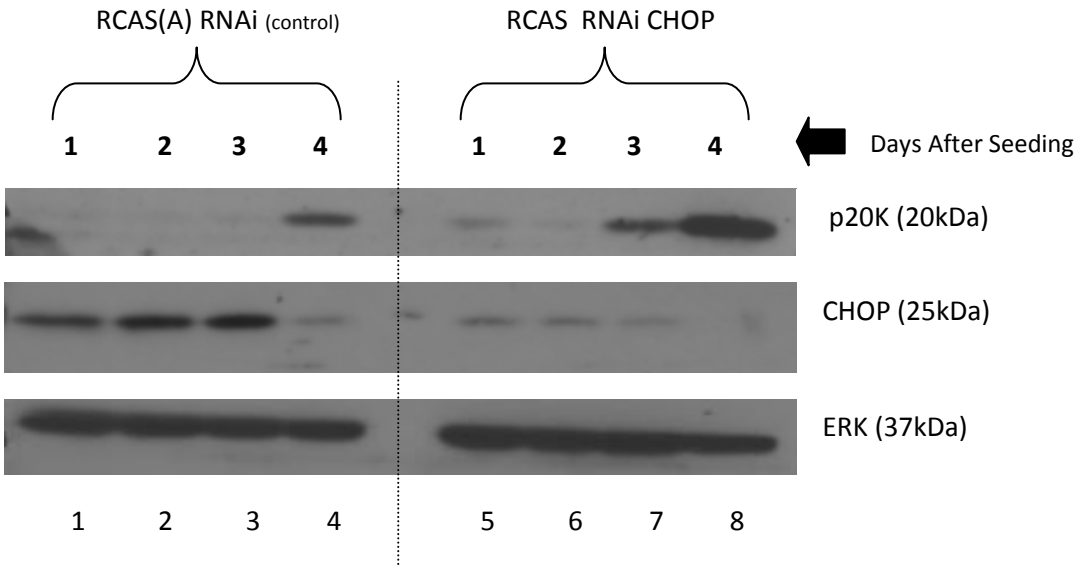




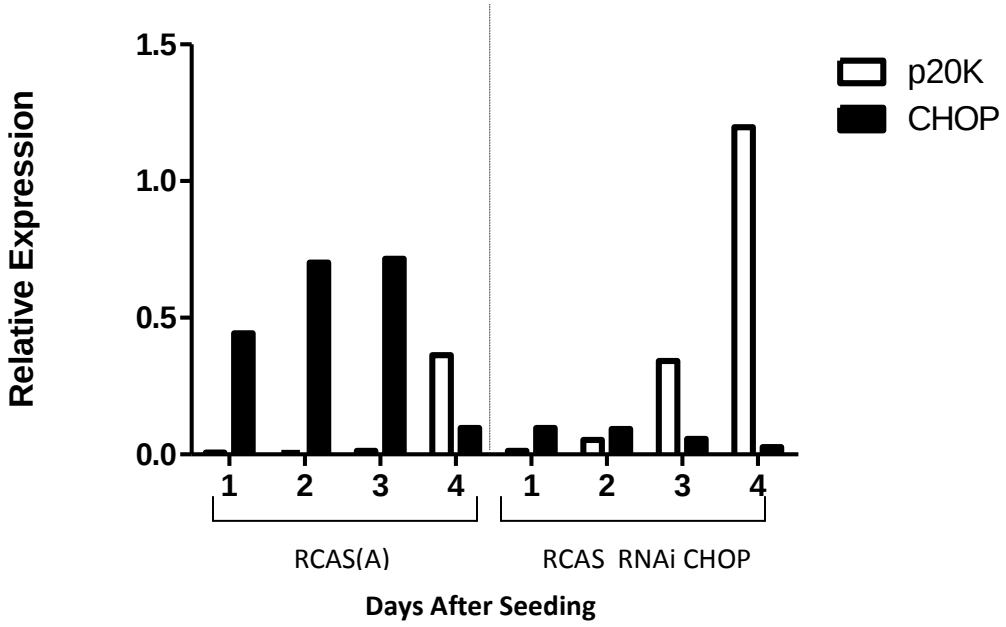
Statistical Significance via t-test Analysis	
Days 2-4	$p < 0.001$ ***
Days 1 & 6	$p < 0.01$ **
Days 5, 14, 16	$p < 0.05$ *

Figure 3.3: Kinetics of CHOP expression in response to contact inhibition. Cell lines were seeded in 60mm dishes in normoxic conditions. Cell lysates from these cells were collected every day for a period of 4 days for Western blotting analysis. CEF numbers were also determined with a Beckman Coulter Particle Counter for a period of 4 days. Cell counts were performed in triplicates **A.** Western blotting analysis of CEF down-regulated for CHOP expression (#354-CHOP-10) and RCAS(A) (control vector). Western blotting analysis showed that cells that were down-regulated for CHOP, display a rise of p20K expression by day 3, and a robust level of expression by day 4 relative to control **B.** Quantification of protein expression from Western blot in Figure 3.3A. Levels of CHOP and p20K are corrected to the levels of ERK**. C. & D.** Bar graph and proliferation curve of cell count average of CEF transfected with RCAS(A) RNAi (control vector) and RNAi CHOP-10 # 354. Error bars at each time point were calculated to indicate standard error of triplicate counts. 354-CHOP-10 cells showed a significant increase in cell proliferation at day 4 relative to control. The * indicates that the comparison between cell count difference at Day 4 is statistically significant in an unpaired t-test ($p<0.05$).

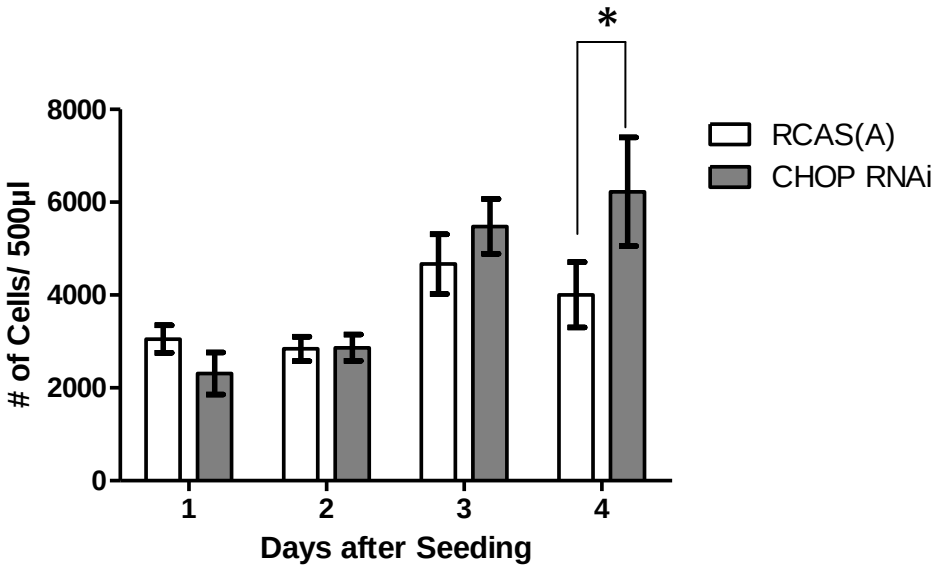
A.


RCAS(A) RNAi (control)
RCAS RNAi CHOP
1 2 3 4
1 2 3 4
Days After Seeding
p20K (20kDa)
CHOP (25kDa)
ERK (37kDa)
1 2 3 4 5 6 7 8

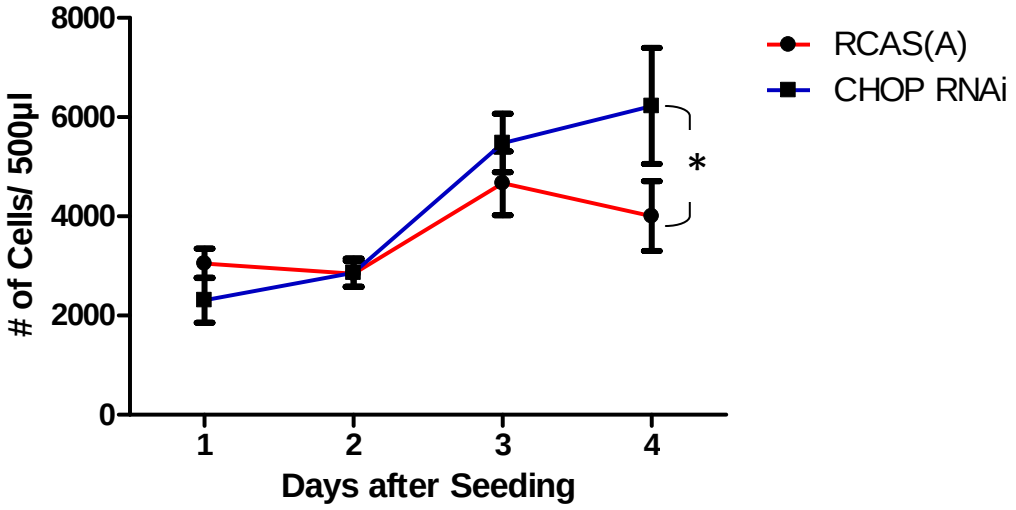
B.


Relative Expression
1.5
1.0
0.5
0.0
p20K
CHOP
1 2 3 4 1 2 3 4
RCAS(A)
RCAS RNAi CHOP
Days After Seeding

C.


*
8000
6000
4000
2000
0
# of Cells/ 500µl
1
2
3
4
Days after Seeding
RCAS(A)
CHOP RNAi

D.


8000
6000
4000
2000
0
# of Cells/ 500µl
1
2
3
4
Days after Seeding
*
RCAS(A)
CHOP RNAi

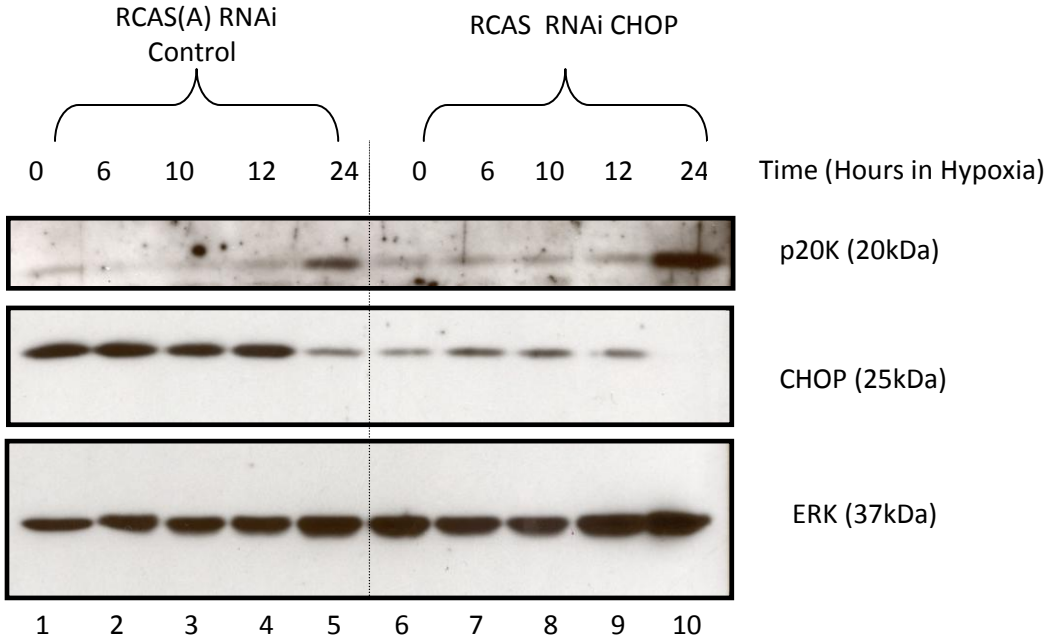
3.3 Effect of CHOP down-regulation on p20K expression in hypoxia

Preliminary experiments showed that repression of CHOP at 24 hours in hypoxia leads to p20K induction in cycling CEF (Fielding, MSc. Thesis, 2011). In the course of the following experiments, my objective was to study how the expression of p20K in hypoxia would be affected by the down-regulation of CHOP by shRNAi. Therefore, CEFs were transfected with the previously described retrovirus vector 354-CHOP-10 and RCAS (A) (Control) to down-regulate CHOP expression (Materials and Methods 2.4.2). Transfected cycling CEF were used to conduct a time course experiment by incubating CEF in hypoxic (2% O_2) conditions for 24 hours. Cell lysates were collected at different time intervals at 0, 6, 10, 12, and 24 hours in hypoxia. Western blotting analysis was used to examine the levels of expression of p20K, CHOP, and ERK in these conditions. The results indicated that CHOP down-regulation induced increased levels of p20K expression in cycling CEF in hypoxia at the 24 hour time point (Lane 10; Figure 3.4A). Although RCAS(A) cells also showed an increase in p20K levels at 24 hours, the levels of p20K in 354-CHOP-10 CEF were elevated albeit modestly by the down-regulation of CHOP (Lane 5 and 10; Figure 3.4A). In addition, at the 24 hour time point, cells transfected with 354-CHOP-10 showed complete repression of CHOP, parallel with a high induction of p20K. Also, the low levels of p20K at 0, 6, 10, 12 hours are slightly more prominent in 354-CHOP-10 cells relative to RCAS(A) cells, but not comparable to the robust increase at 24 hours in both cell lines (Figure 3.4B). The initial hypothesis was that by down-regulating CHOP, p20K would be super-induced at earlier time points in hypoxia. However, the lack of super induction of p20K earlier on might indicate that reducing CHOP levels is not sufficient to induce p20K. Thus, an additional signal/pathway may be controlling p20K in these conditions.

Additional experiments showed that CHOP and p20K expression display an inverse relationship as cells become denser in hypoxia (Figure 3.5A). Cycling CEF transfected with RCAS(A) (control vector) were incubated in hypoxic (2% O2) conditions for 24 hours. Cell lysates were also collected at 0, 6, 10, 12, and 24 hours. These cells became very dense and were contact inhibited by 24 hours in hypoxia. Western blotting analysis showed the inverse relationship between CHOP and p20K, therefore as CHOP levels decreased, p20K expression was induced (Figure 3.5). Accordingly, the 24 hour time point shows a drastic decrease in CHOP levels, and a significant induction of p20K (Lane 5; Figure 3.5). These studies suggest that loss of CHOP levels promotes the induction of p20K at high cell density.

Figure 3.4. Effect of CHOP down-regulation on p20K expression at different time points in hypoxia. CEFs were transfected with retroviral vector 354-CHOP-10 to inhibit CHOP expression and RCAS (A) (Control). Transfected cycling CEF were incubated in hypoxic (2% O_2) conditions for 24 hours. Cell lysates were collected at 0, 6, 10, 12, and 24 hours in hypoxia. **A.** Western blotting analysis of expression of p20K, CHOP, and ERK protein levels. Lack of super induction of p20K earlier than 24 hours in hypoxia indicates that reducing CHOP levels is not sufficient to induce p20K expression. Thus, an additional signal/pathway may be controlling p20K in these conditions. **B.** Quantification of protein expression from Western blot in Figure 3.4A. Levels of CHOP and p20K are corrected to the levels of ERK. The intensity of CHOP and p20K in RCAS(A) cells was normalized to 1 at 0 hour.

A.



B.

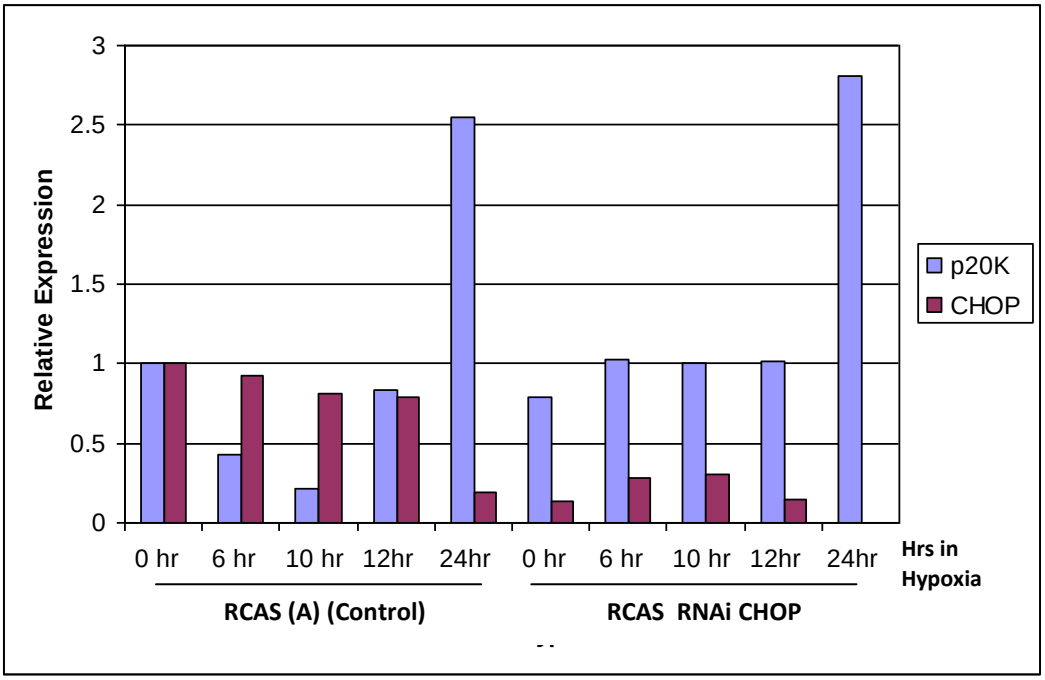
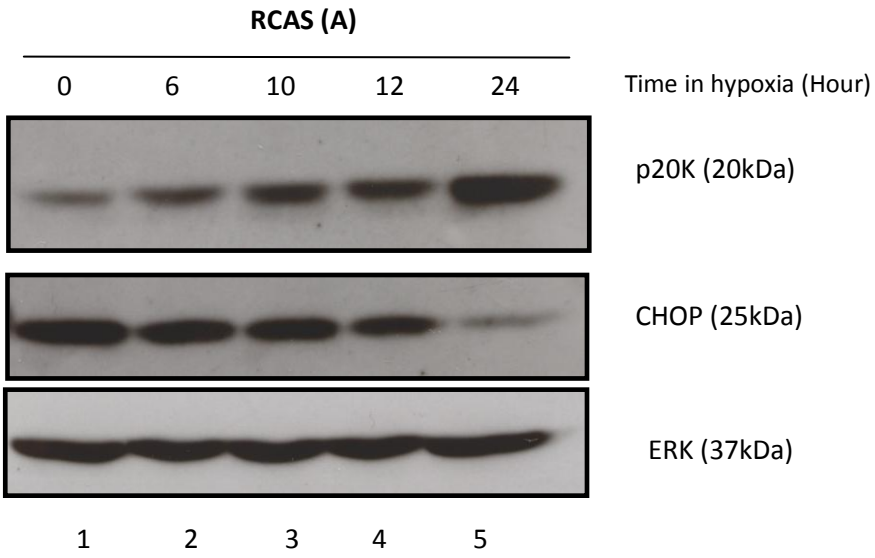
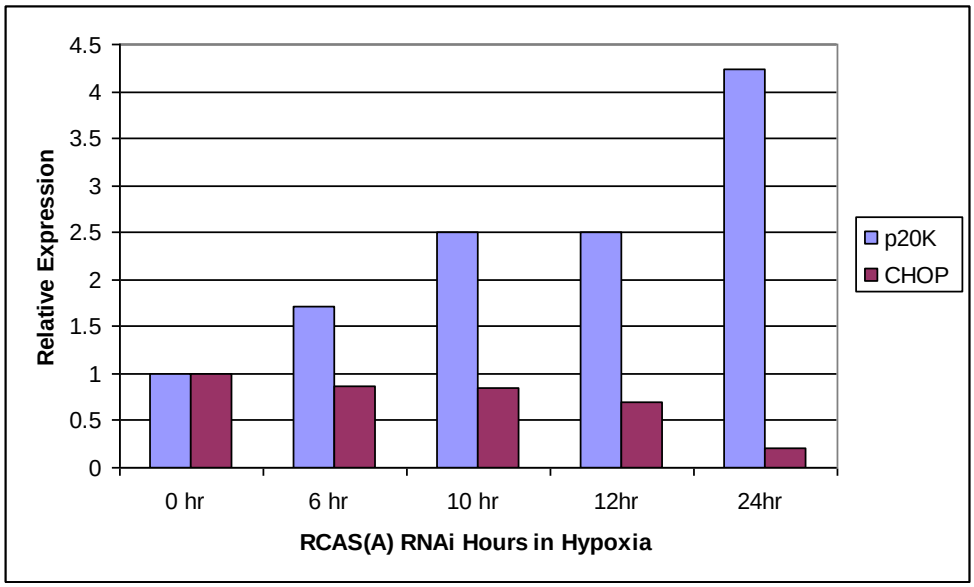


Figure 3.5: Inverse relationship between CHOP and p20K as cells reach high cell density. A. Western blotting analysis of *cycling* to *contact inhibited* CEF transfected with RCAS(A) (control vector) incubated in hypoxia (2% Oxygen) for 24 hours. Cell lysates were collected at 0, 6, 10, 12, and 24 hours in hypoxia. Western blotting analysis of p20K, CHOP, and ERK shows the inverse relationship between CHOP and p20K expression as cells become contact inhibited in hypoxia at 24 hours. **B.** Quantification of protein expression from Western blotting in Figure 3.5A. Levels of CHOP and p20K are corrected to the levels of ERK. The intensity of CHOP and p20K was normalized to 1 at 0 hour.

A.



B.



3.4 Effects of CHOP over-expression on p20K induction during ER stress

CHOP is a transcription factor associated with the ER stress response (Ron and Habener, 1992). Many conditions can induce ER stress such as protein folding disturbance, interference with calcium movement across the ER membrane and nutrient deprivation (Zinszner et al., 1998). The two main ER stress inducer drugs used in these experiments were thapsigargin and tunicamycin. Tunicamycin is an N-linked glycosylation inhibitor that interferes with protein folding in the ER (Marciniak et al., 2004). Thansigargin (Tg) is an inhibitor of the Sacroplasmic/ER Ca^{2+}-ATPase (SERCA) pump, which decreases the rate of Ca^{2+} ER influx, leading to an ER Ca^{2+} reduction (Gupta et al., 2010; Moore et al., 2011). Therefore, these ER stress inducing agents were used in experiments to investigate the effect of CHOP over-expression on p20K induction.

In these experiments, confluent cells were treated with 1μg/ml tunicamycin, 1μg/ml thapsigargin, or 0.1% DMSO (Diluent control), to trigger CHOP expression. Once cells became contact inhibited, media would be changed 24 hours prior to drug treatment to avoid any nutrient depletion bias. Cell lysates were collected at 12, 24, and 36 hours post treatments with DMSO, tunicamycin or thapsigargin. Western blotting analysis indicated that ER stress inducing drugs lead to elevated levels of CHOP expression and the repression of p20K (Figure 3.6A). CHOP was rapidly induced in response to tunicamycin or thapsigargin, as elevated protein levels were evident within 12 hrs post treatment (Lane 2 & 3; Figure 3.6A). Thapsigargin induced higher CHOP expression and stronger repression of p20K than tunicamycin (Lane 3 and 6; Figure 3.6A). p20K was only observed in contact inhibited cells treated with DMSO (Control) (Lanes 4

and 7; Figure 3.6A). This supports the notion that CHOP is acting as a negative regulator of p20K expression during ER stress.

3.4.2 To study the interaction between C/EBPβ and CHOP in presence of ER Stress by Co-Immunoprecipitation (Co-IP)

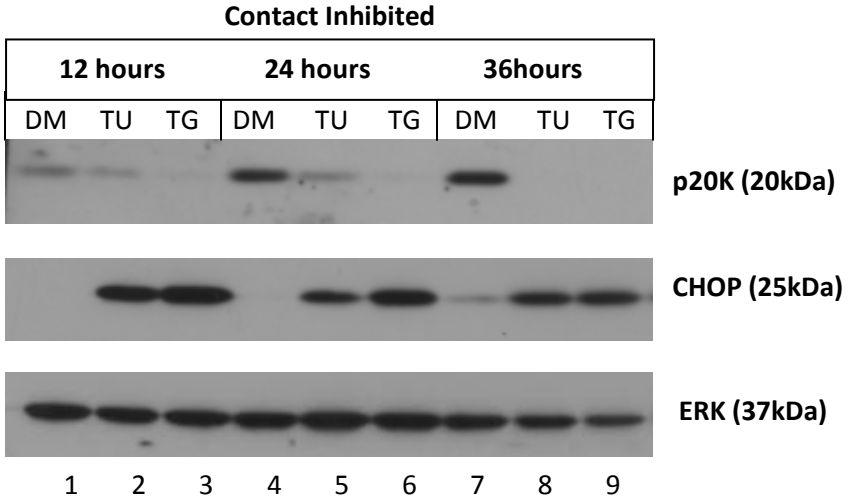
Research by Kim et al. (1999) described that C/EBPβ is responsible for the activation of p20K in quiescent CEF. It is hypothesized that CHOP induction will promote the formation of inactive CHOP-C/EBPβ heterodimers inhibiting p20K expression. Other studies have shown that CHOP can act as a negative regulator of C/EBP family members, by forming heterodimers with members of the C/EBP family, inhibiting their ability to bind to consensus DNA target sequences (Ron and Habener, 1992; Wei et al., 2007). Therefore, these results suggest that CHOP heterodimerizes with C/EBPβ, hindering C/EBPβ ability to bind to the p20K QRU promoter, and resulting in the inhibition of p20K during ER stress.

Co-Immunoprecipitation experiments were used to study C/EBPβ and CHOP interaction. Since CHOP is highly inducible by ER stress and starvation, CHOP levels were stimulated by treating contact inhibited CEF with 1μg/ml tunicamycin, 1μg/ml thapsigargin, or 0.1% DMSO (control). Once again, when the cells reached contact inhibition, the medium was changed 24 hours prior to drug treatment to avoid any nutrient depletion effects. CHOP is also induced by nutrient deprivation; therefore lysates from starving (6 days) CEF were also collected. Alternatively, lysates from cycling CEF in normoxia were also analyzed since basal levels of CHOP expression is typically observed in CEF in these conditions. The results indicated that increased levels of CHOP co-immunoprecipitated with C/EBPβ in tunicamycin and thapsigargin treated cells, suggesting that C/EBPβ –CHOP heterodimers accumulated during ER stress (Figure 3.7; Lanes 9 and 10). In addition, there was also a C/EBPβ and CHOP association

during periods of starvation and, to a lesser extent, in normoxia cycling CEF (Figure 3.7; Lanes 6 and 7). These results support our initial hypothesis, that accumulation of inactive C/EBPβ–CHOP heterodimers is the mechanism responsible for repressing p20K in conditions of ER stress and starvation.

Figure 3.6. Effect of CHOP over-expression on p20K induction during ER stress. *Contact inhibited* CEF were treated with either 0.1% DMSO (DM) (Control), 1µg/ml tunicamycin (TU), or 1µg/ml thapsigargin (TG) to examine the effect of CHOP expression on p20K **A.** Western blotting analysis of p20K, CHOP, and ERK in contact inhibited CEF lysates collected at 12, 24, and 36 hours post treatment with DM, TU, or TG. As shown by Western blotting, ER stress inducing drugs (TU & TG) elevated levels of CHOP, resulting in repression of p20K. In addition, thapsigargin treated CEF showed a stronger inhibition of p20K after 12 hours post treatment than tunicamycin treated CEF. **B.** Quantification of protein expression from Western blot shown in Figure 3.6A. Levels of CHOP and p20K are corrected to the levels of ERK.

A.



B.

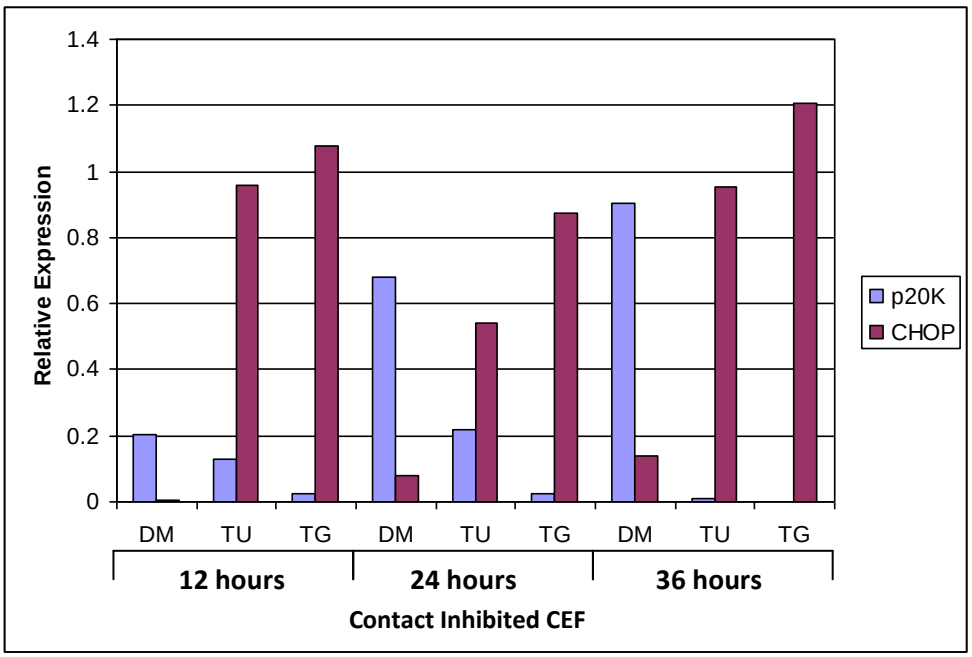
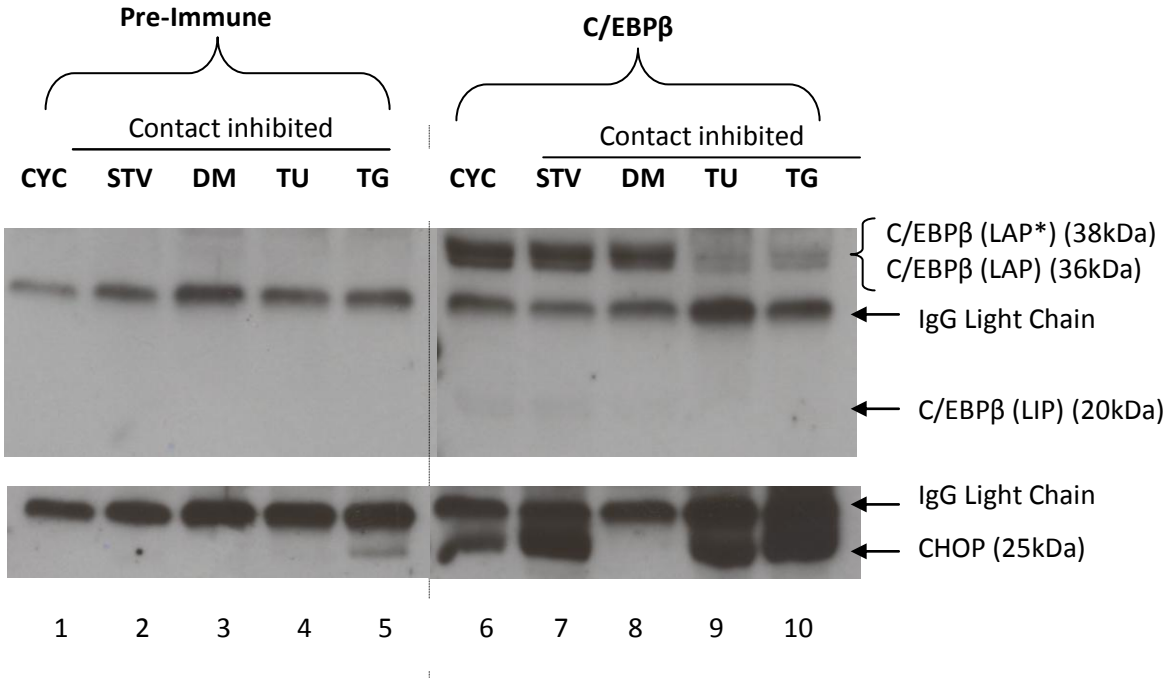


Figure 3.7. **Analysis of C/EBPβ and CHOP interaction by Co-Immunoprecipitation.** *Contact inhibited* CEF were treated with either 0.1% DMSO (DM) (Control), 1µg/ml tunicamycin (TU), or 1µg/ml thapsigargin (TG) for 24 hours. In addition, starving (STV) (6 days) and cycling (CYC) CEFs in normoxia were also used to determine if there was a CHOP-C/EBPβ interaction in these conditions. Cell lysates were subjected to immunoprecipitation using C/EBPβ polyclonal antibody or Pre-Immune serum as control. CHOP protein was detected from immunoprecipitates by Western blotting. CHOP co-immunoprecipitated with C/EBPβ in starving CEF and cycling normoxia, as well as TU & TG treated cells. The only condition where there was a lack of CHOP- C/EBPβ interaction was in contact inhibited CEF treated with 0.1% DMSO (control), corresponding to conditions where p20K is expressed (Lane 8).



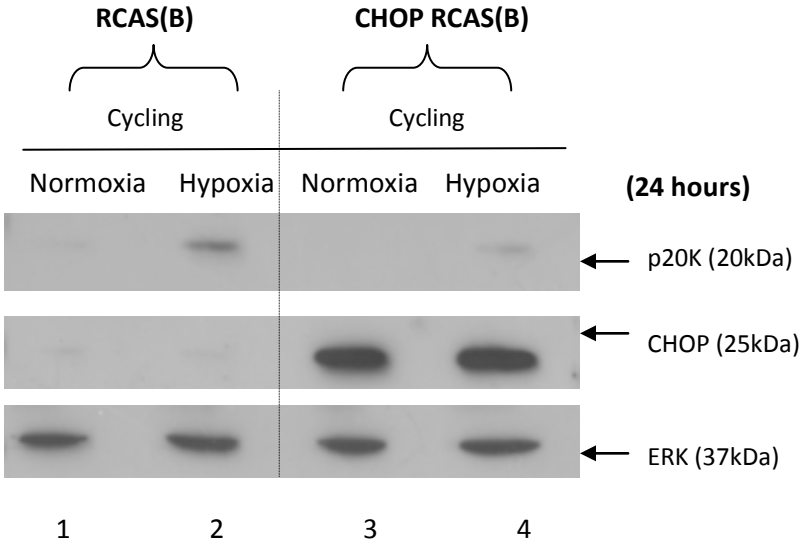
CYC- Cycling
STV- Starving
DM- Cells treated with 0.1% DMSO (Control)
TU- Cells treated with tunicamycin
TG- Cells treated with thapsigargin

3.5 Effect of CHOP over expression by the RCASB system in hypoxia

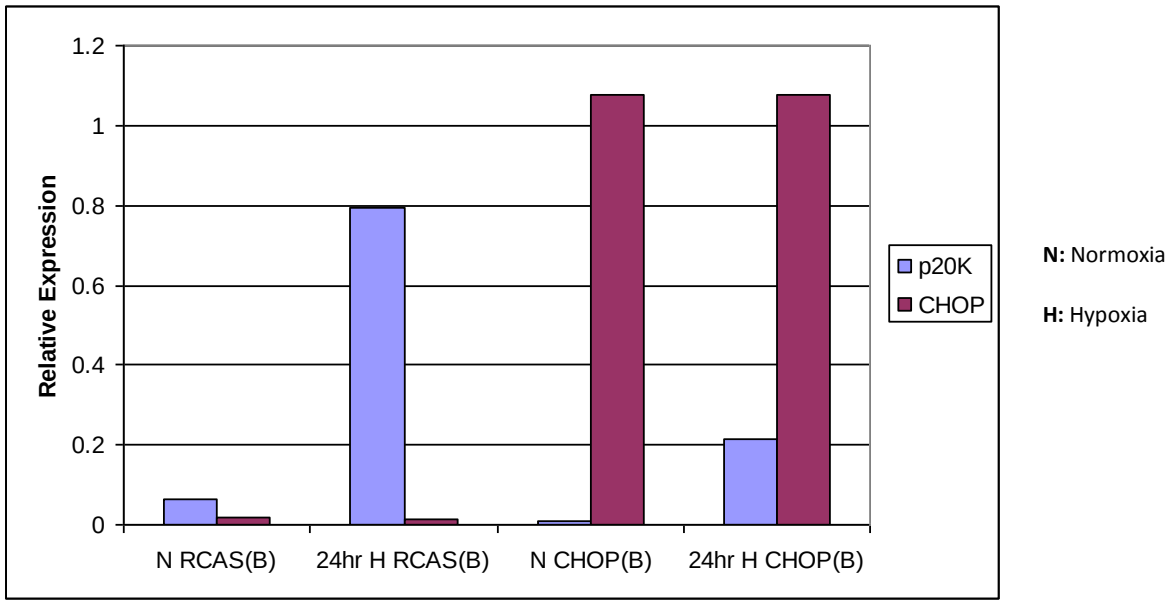
In previous experiments, treating CEF with thapsigargin or tunicamycin showed that the induction of CHOP during ER stress is associated with the inhibition of p20K. To confirm if the levels of CHOP affect p20K expression in hypoxia, CEF were transfected with a retrovirus expressing RCAS(B) (control vector) or CHOP RCAS(B) vector to obtain CHOP over-expression. After transfection, CEFs were passaged three times to allow complete infection of the CEF population. Transfected cycling CEFs were placed in normoxic (21% O_2) or hypoxic (2% O_2) conditions for 24 hours. Cell lysates were collected and levels of p20K, CHOP, and ERK-1 were analyzed by Western blotting. Western blot results demonstrate that subconfluent CEF over-expressing CHOP had reduced levels of p20K expression in comparison to RCAS(B) cells at 24 hours in hypoxia (Figure 3.8). These results indicate that CHOP levels have a significant impact on p20K induction.

Figure 3.8: Effect of CHOP over- expression by the RCASB system in hypoxia. CEF were transfected with RCAS(B) (control vector) and CHOP RCAS(B) to obtain CHOP over-expression. Transfected cycling CEFs were incubated in normoxic (21% O_2) or hypoxic (2% O_2) conditions for 24 hours. **A.** Western blotting analysis of p20K, CHOP, and ERK-1 protein levels of subconfluent CEF lysates collected after 24 hour incubation in normoxia or hypoxia. Results show that constitutive over-expression of CHOP in transfected CEF is associated with the down-regulation of p20K in hypoxia (Lane 4). **B.** Quantification of protein expression from Western blot shown in Figure 3.8A. Levels of CHOP and p20K are corrected to the levels of ERK.

A.



B.



3.6 Gene validation of p20K and CHOP mRNA expression in normoxia, hypoxia, and ER stress conditions by quantitative RT-PCR

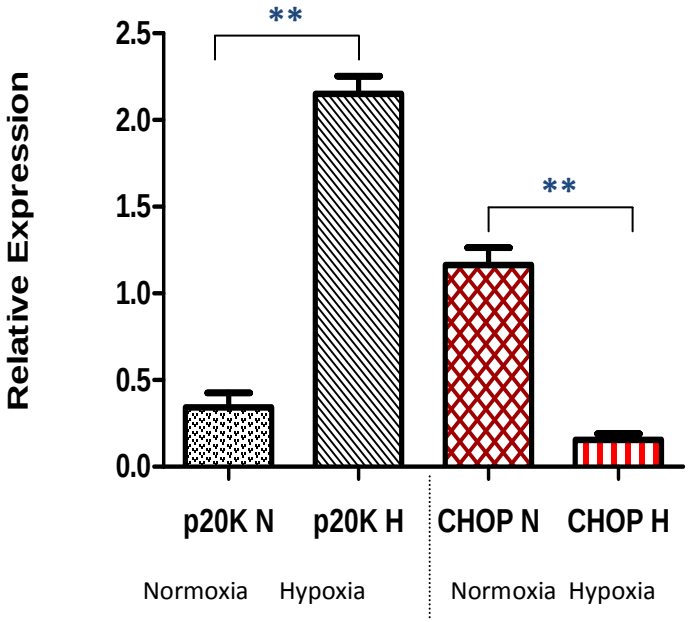
Accurate and sensitive methods have been developed for the quantification of mRNA by quantitative reverse transcription (RT) polymerase chain reaction (PCR). Previous experiments with Western blotting analysis were used to quantify CHOP protein levels in various conditions. Here, RT-qPCR was used to confirm that the expression of CHOP in CEF during ER stress, normoxia, hypoxia and contact inhibition is regulated at the mRNA level.

Total RNA was extracted from CEF by the Trizol Method (Invitrogen). Total RNA (1 μg) was reverse transcribed to cDNA with Protoscript AMV First Synthesis Kit (New England, Biolabs). CHOP and p20K mRNA levels from subconfluent or contact inhibited CEF incubated in conditions of normoxia or hypoxia were assessed by RT-qPCR and normalized to *GAPDH* expression. RT-qPCR confirmed a down-regulation of CHOP mRNA levels in subconfluent or contact inhibited CEF in hypoxia (24 hours), as well as contact inhibited CEF incubated in normoxia (Figure 3.9). In contrast, RT-qPCR analysis demonstrated an opposite pattern for p20K gene expression, since there was an up-regulation of p20K mRNA in hypoxia or contact inhibited CEF. This further confirms the inverse relationship between p20K and CHOP expression that was previously observed by Western blotting (Figure 3.5). Results from a one-way ANOVA with Bonferroni post hoc test on RT-qPCR data demonstrated a significant difference ($p < 0.001$) in CHOP or p20K expression between subconfluent or contact inhibited CEF in hypoxia or normoxia (Figure 3.9). A separate analysis on p20K hypoxia subconfluent vs. hypoxia contact inhibition showed significant differences in transcript levels in an unpaired *t-test* analysis ($p < 0.001$) (Figure 3.9). RT-qPCR confirmed that CHOP mRNA levels in hypoxia (24 hours) or contact inhibition were reduced, which are conditions where p20K is highly expressed.

Additional RT-qPCR experiments showed that both ER stressors (tunicamycin and thapsigargin) triggered an increased expression of CHOP mRNA levels and decreased levels of p20K transcripts in response to either of these agents. These experiments were conducted by treating contact inhibited CEF with either 0.1% DMSO (control), 1μg/ml tunicamycin or 1μg/ml thapsigargin for 24 hours. As previously mentioned, when CEF became contact inhibited, the medium was replenished 24 hours prior to drug treatment to avoid any nutrient depletion effects. Total RNA was extracted from treated CEF cells 24 hour post drug treatments and subjected to RT-qPCR analysis. Indeed, a robust induction in CHOP mRNA and protein levels was observed after treatment with tunicamycin or thapsigargin. Additionally, CHOP and p20K continue to display the same inverse relationship during ER stress (Figure 3.10). As shown by RT-qPCR and Western blotting analysis, both tunicamycin and thapsigargin promote a high induction of CHOP but antagonize p20K expression, by the formation of inactive CHOP-C/EBPβ heterodimers (Figures 3.10 & 3.6)

Figure 3.9. **Gene validation of p20K and CHOP mRNA expression.** p20K and CHOP mRNA levels were assessed by quantitative RT-PCR and normalized to *GAPDH* expression. Results are expressed as fold change between cycling (cyc) or contact inhibited (CI) CEF cultured in 24hrs hypoxia (2% O_2) or normoxia (21% O_2). Error bars represent the average+SD of three independent experiments. An *** indicates that the value is significantly different ($p < 0.001$) by one-way ANOVA with Bonferroni post hoc test versus the control (cycling normoxia). An ** indicates the value is significantly different ($p < 0.001$) in a separate *t-test* analysis. **A.** Represents relative expression of p20K and CHOP mRNAs in cycling CEF incubated in normoxia or hypoxia for 24 hours. **B**. Represents the comparison of relative expression levels of p20K and CHOP mRNAs in CEF in conditions of Normoxia cycling/contact inhibited vs. Hypoxia subconfluent/contact inhibited. RT-qPCR confirms the inverse relationship between p20K and CHOP gene expression.

A.



B.

Subconfluent

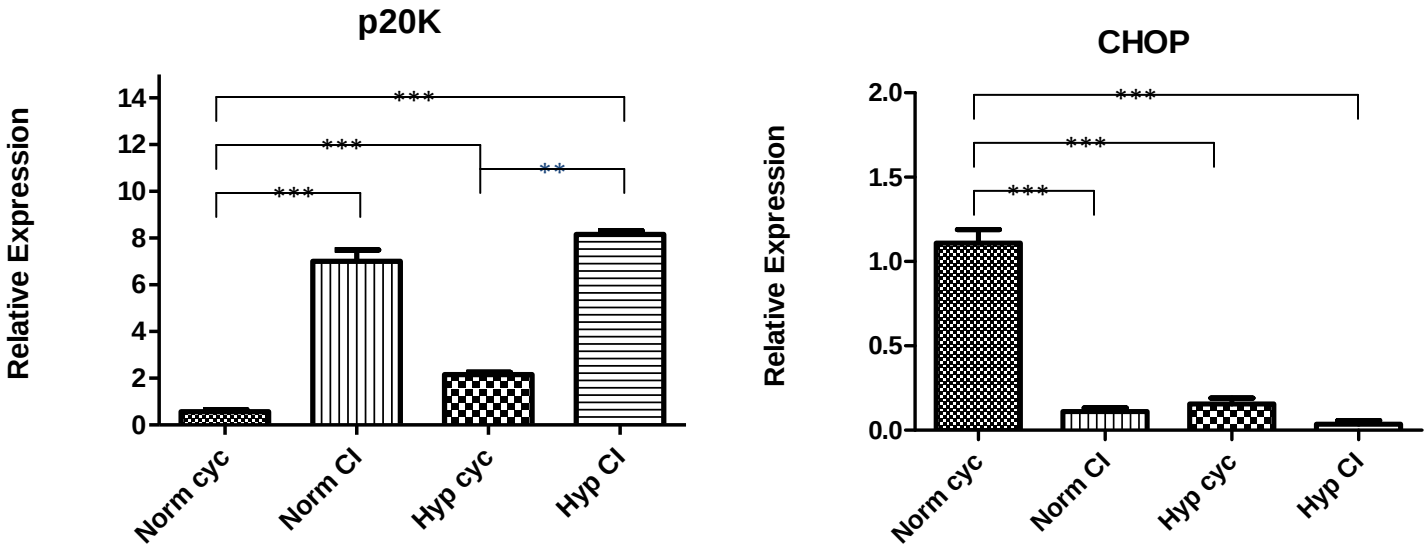
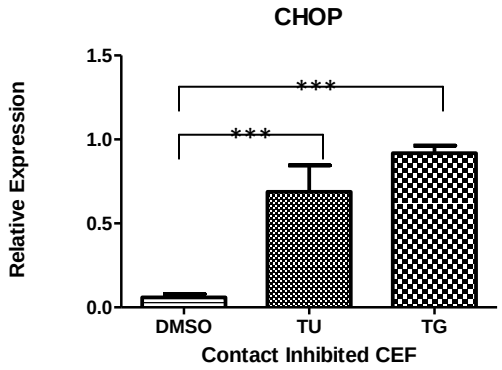
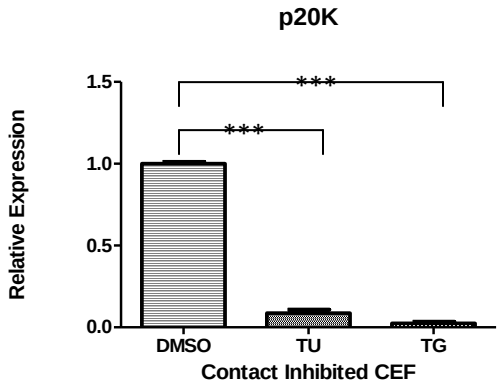


Figure 3.10. **Gene validation of p20K and CHOP mRNA expression during ER stress**. CHOP and p20K mRNA levels were assessed by quantitative RT-PCR and normalized to *GAPDH* expression. Contact inhibited CEF cells were treated for 24 hours with 0.1% DMSO (Control), tunicamycin, or thapsigargin to induce expression of CHOP by ER stress. Error bars represent the average+SD of three independent experiments. An *asterisk* indicates that the value is significantly different ($p< 0.001$) from the control (DMSO treated) by one-way ANOVA with Bonferroni post hoc test. **A.** Represents relative expression of CHOP mRNA in contact inhibited CEF treated with either with DMSO (Control), tunicamycin, or thapsigargin for 24 hours. **B.** Represents relative expression of p20K mRNA in contact inhibited CEF treated with either with DMSO (Control), tunicamycin, or thapsigargin for 24 hours. Results show that both tunicamycin and thapsigargin promote high induction of CHOP mRNA levels but antagonize p20K mRNA expression.

A.



B.



3.7 Effect of DMOG treatment on p20K induction

Earlier studies by previous MSc. Students R. Ghosh (2009) and B. Fielding (2011) showed that p20K expression can also be induced by dimethyloxalylglycine (DMOG). DMOG is a prolyl-hydroxylase inhibitor that can stabilize Hypoxia Inducible Factor (HIF) under normoxic conditions mimicking a hypoxia response (Barnucz et al., 2013).

HIF is regulated by post-translational modification by hydroxylation of specific prolyl and asparaginyl residues in HIF-α subunits (Elvidge et al., 2006). These modifications are catalyzed by members of the 2-oxoglutarate (2-OG) dioxygenase family which comprises of the PHD family (PHD1, PHD2, and PHD3) and FIH-1 (Elvidge et al., 2006). During high oxygen conditions, HIF post-modification mechanisms are activated which mediate HIF binding to the Von Hippel–Lindau (VHL) E3 ubiquitin ligase complex, promoting its proteasomal degradation (Fraisl et al., 2009). However, DMOG results in the inhibition of PHDs and FIH-1 under normoxic conditions, which leads to HIF stabilization, blocking its degradation (Barnucz et al., 2013; Lando et al., 2002). Since DMOG mimics a hypoxia response, it was of interest to study p20K induction in these conditions.

To test the effect of DMOG on p20K induction at different time points, a concentration of 1mM of Dimethyloxaloylglycine (DMOG) was added to the media of cycling CEFs. CEF lysates from DMOG treated and untreated (control) cells were collected at 24 and 36 hours post-treatment. Prior to DMOG treatment, cells were split 1:4 one day before, and left overnight to recover. Levels of p20K, CHOP, Phospho-specific ERK and ERK-1 were analyzed by Western blotting analysis. In CEF, the inhibition of prolyl-hydroxylases by DMOG caused a significant induction of p20K expression at 24 and 36 hours (Lanes 2 and 4; Figure 3.11). It is important to note that at 36 hours both DMOG treated and untreated cells were very dense and contact

inhibited. Thus, at 36 hours, untreated cells also exhibited p20K expression; however DMOG treated cells displayed a more robust expression of p20K. Interestingly, DMOG treatment induced a modest increase in CHOP levels, which is contrary to what has been observed in CEF placed in hypoxia (2% O_2). This indicates a significant difference between DMOG treatment and hypoxia (2% O_2), since hypoxia reduced the levels of CHOP in subconfluent CEF (Figures 3.4 & 3.5). At 36 hours, CHOP levels were virtually undetectable in contact inhibited untreated CEF in comparison to DMOG treated cells. In DMOG treated CEF, CHOP levels remain despite the robust amount of p20K expression at that time point.

3.7.1 Effect of DMOG treatment on levels of phospho-ERK expression

Research by previous MSc. student M. Athar (2011) demonstrated that ERK-2 is a transcriptional repressor of p20K. ERK 1/2 are part of the mitogen-activated protein kinase (MAPK) pathway. This pathway is deemed important in all eukaryotes because of its various roles in regulating proliferation and differentiation (Kolch, 2000). ERK 1/2 is activated through phosphorylation on its threonine and tyrosine residues by MAPK/ ERK kinase (MEK) (Kolch, 2000). The phosphorylation of ERK 1/2 is important for its activation and nuclear translocation (Lenormand et al., 1998). Additionally, activated ERK-2 can block access to transcription factors when it binds to a DNA consensus sequence (G/CAAAG/CN G/CAAAG/C) that closely resembles the QRU sequence (GAAGGAGAAAG) overlapping the two C/EBPβ binding sites of the QRU (Hu et al., 2009; Athar, MSc. Thesis, 2011).

My project also focused in studying the effect of DMOG and thapsigargin on levels of phospho-ERK expression. A phospho-site specific p44/42 MAPK (ERK1/2) antibody was used to detect phosphorylated threonine and tyrosine specific ERK 1/2 sites. Studies by M. Athar demonstrated through ChIP experiments that during activation of p20K, ERK-2 does not bind to

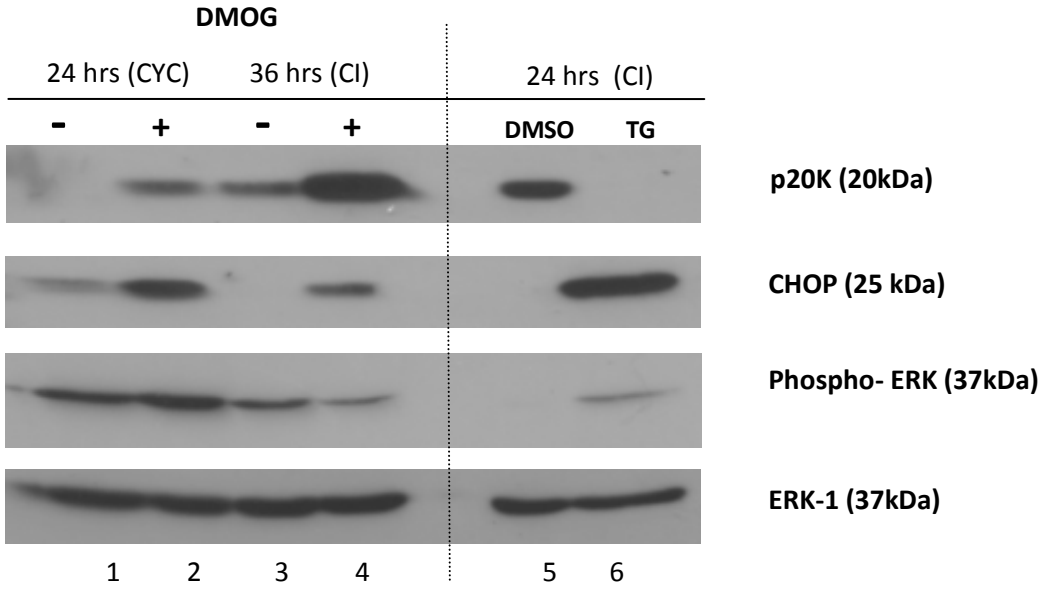
the QRU promoter (MSc Thesis, 2011). In addition, recent studies by undergraduate student M. Fox showed that phospho-specific ERK expression levels in CEF decreased at 24 hours in hypoxia (Unpublished Results, 2013). Therefore, these findings suggest that levels of activated ERK-2 decrease during hypoxia and in conditions of growth arrest corresponding to the induction of p20K. In these conditions, ERK-2 is unable to bind to the QRU promoter to hinder p20K expression (M. Athar, MSc Thesis, 2011). In DMOG experiments at later time points, CEF displayed a decrease in phospho-ERK levels, while CHOP levels were modestly induced post DMOG treatment (Lane 4; Figure 3.11). However at 24 hours post-treatment, there is a small increase of activated ERK levels. At 36 hours, as DMOG treated cells became density arrested, both CHOP and phospho-ERK levels decreased relative to 24 hour post DMOG treatment (Lane 4; Figure 3.11). These results are preliminary and need to be repeated as results in Figure 3.12 show a different pattern of phospho-ERK expression at 24 hours. However, the modest up-regulation of CHOP remains consistent in all DMOG experiments. In light of these recent observations, this data suggests that DMOG might be responsible for p20K induction via a separate mechanism independent from CHOP repression which needs to be investigated further.

Additional experiments were also conducted to determine the effect of thapsigargin on phospho-ERK levels during ER stress. For these experiments, CEF were treated with thapsigargin, which induces higher levels of ER stress and CHOP than tunicamycin (Figure 3.6) (Bertolotti et al. 2000). Thus, contact inhibited CEF were treated with 1μg/ml thapsigargin (24 hours) and 0.1% DMSO as a control. After 24 hours post thapsigargin treatment, CEF lysates were collected, and analyzed by Western blotting. Earlier experiments as previously described (Figure 3.6 & 3.10) have shown that thapsigargin induces high CHOP expression inhibiting p20K induction. Subsequent experiments demonstrated that thapsigargin treated CEF display a

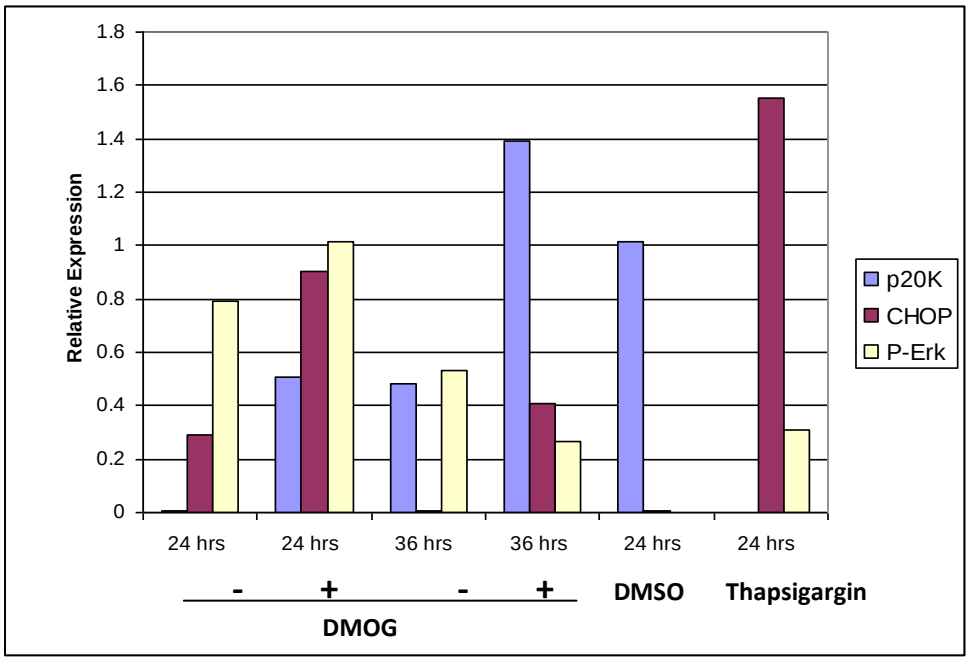
significant reduction of phospho-ERK levels, while CHOP levels are elevated (Lane 6; Figure 3.11). In contact inhibited CEF treated with DMSO (control), both CHOP and phospho-ERK levels are virtually undetectable, corresponding with elevated p20K expression (Lane 5; Figure 3.11). This data demonstrates that during a high ER stress response, activated ERK does not appear to play a major role in p20K inhibition, but CHOP induction is the main factor repressing p20K in these conditions.

Figure 3.11: Induction of p20K by DMOG. Cycling CEFs were treated with 1mM dimethyloxaloglycine (DMOG) for 24 and 36 hours. In addition, contact inhibited CEF were treated with 1µg/ml thapsigargin and 0.1% DMSO as a control. Thapsigargin and DMSO treated CEF lysates were collected 24 hours post treatment. DMOG treated (24 & 36 hours) and untreated CEF lysates were also collected. **A.** Western blotting analysis of CHOP, p20K, and phospho-ERK. Thapsigargin treated CEF display a significant reduction of phospho-ERK levels, while CHOP levels are elevated. At later time points, DMOG treated CEF have a decrease in phospho-ERK levels, while at the same time CHOP levels are modestly induced. **B.** Quantification of protein expression from Western blot in Figure 3.11A. Levels of CHOP, p20K, and Phospho-specific ERK are corrected to the levels of ERK-1.

A.



B.



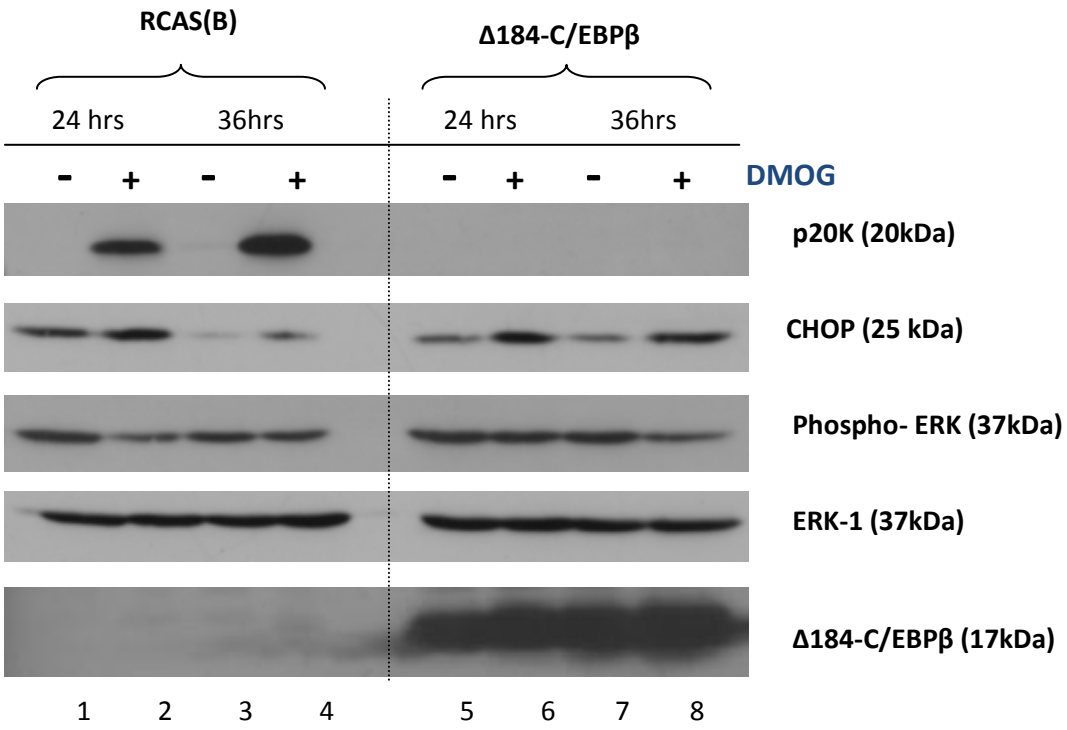
3.8 To examine the effect of DMOG treatment on CEF expressing the Δ184-C/EBPβ mutant

C/EBPβ is crucial for p20K induction in conditions of contact inhibition and hypoxia (Fielding, MSc. Thesis, 2011; Kim et al., 1999). It was also important to determine if the induction of p20K by DMOG is C/EBPβ dependent. CEF were transfected with a retrovirus vector expressing a dominant negative mutant of C/EBPβ (Δ184-C/EBPβ) or RCAS(B) (control vector) and treated with DMOG for 24 and 36 hours. The dominant negative mutant of C/EBPβ (Δ184-C/EBPβ) is missing the first 183 amino acids, lacking the TAD domain (Gagliardi et al., 2001). Therefore, this form of C/EBPβ has a dominant negative effect that represses C/EBPβ. DMOG treated and untreated samples were collected and analyzed via Western blotting analysis. Results confirmed that DMOG induction of p20K is C/EBPβ dependent as Δ184-C/EBPβ abolished p20K expression despite of DMOG treatment (24 & 36 hrs) (Lanes 6 &8; Figure 3.12). In support of the results observed in Figure 3.11, CHOP levels also increased in DMOG treated cells for both Δ184-C/EBPβ and RCAS(B) cell lines. Phospho-specific ERK levels decreased in DMOG treated cells at both 24 and 36 hours post treatment.

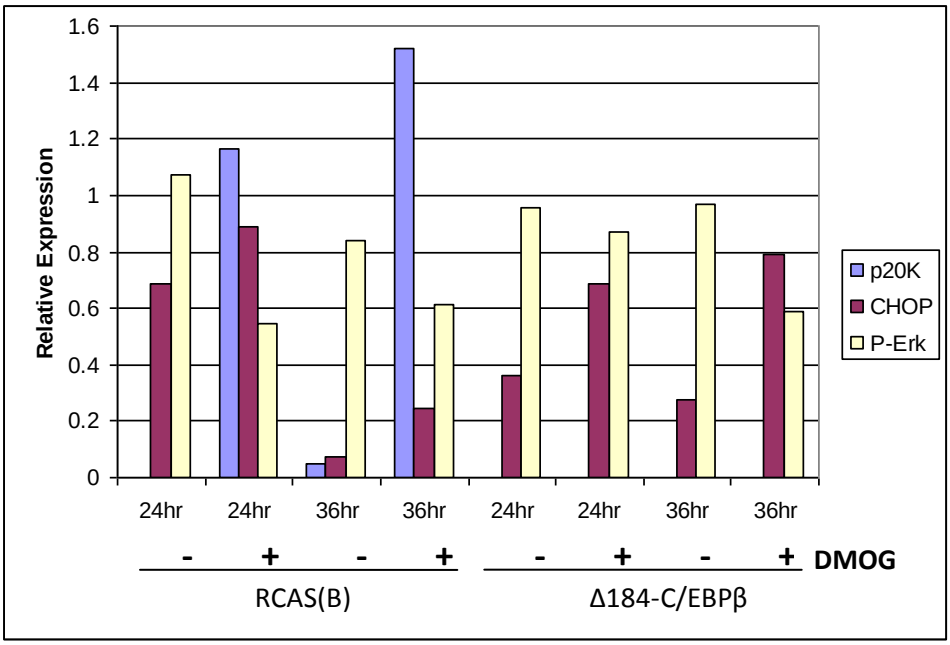
To summarize, these experiments provided evidence that DMOG induces high expression of p20K at 24 and 36 hours post treatment. It was confirmed that its induction of p20K is C/EBPβ dependent. In addition, in conditions where p20K is strongly induced such as 24 hours hypoxia and contact inhibition, CHOP levels are usually attenuated, however the opposite is observed in DMOG treated cells. In addition, at later time points phospho-ERK levels are reduced, while CHOP levels remain detectable. Thus, DMOG provides a unique system in studying the roles of p20K inhibitors, CHOP and activated ERK. However, these experiments remain to be repeated and, in particular, to determine the effects of DMOG on the levels of activated ERK and the interaction of phospho-ERK-2 on the QRU.

Figure 3.12: DMOG induction of p20K is C/EBPβ dependent. CEF were transfected with RCAS(B) (Empty Vector) and C/EBPβ dominant negative mutant (Δ184-C/EBPβ). Cycling CEFs were treated with 1mM dimethyloxaloglycine (DMOG) for 24 & 36 hours. **A.** Western blotting analysis of CHOP, p20K, and Phospho-specific ERK. DMOG induction of p20K is C/EBPβ dependent as Δ184-C/EBPβ mutants abolished p20K expression despite of DMOG treatment (24 & 36 hrs) (Lanes 6 &8). **B.** Quantification of protein expression from Western blot in Figure 3.12A. Levels of CHOP, p20K, and Phospho-specific ERK are corrected to the level of ERK-1.

A.



B.



CHAPTER 4: Discussion

4.1 Quiescence and Apoptosis

Quiescence is a reversible, non-dividing state that is distinct from other non-dividing cell states, such as senescence, apoptosis, and terminal differentiation (Coller et al., 2006). Certain genes regulated in quiescence serve to suppress entry into these irreversible cell states (Coller et al., 2006). However, there is little known about the network of gene regulation during quiescence (Liu et al., 2007). For that reason, more studies are needed to understand the underlying mechanisms by which cells enter the quiescent state.

Studies have identified quiescent human fibroblasts to be more resistant to apoptosis in response MG132, a proteasome inhibitor (Legesse-Miller et al., 2012). Other studies, treated quiescent transformed CEF with anti-inflammatory agents that induced apoptosis, leading to the repression of p20K (Lu et al., 1995). Studies by Gentili et al. (2005) proposed that p20K is acting as a pro-survival protein by maintaining cell viability in chondrocytes, myoblast, and cardiomyocytes. In their studies chicken embryos had increased rate of cellular apoptosis when injected with p20K antibodies during early development (Gentili et al., 2005). This is in agreement with studies by Di Marco et al. (2003) which also observed a pro-survival role of p20K in chondrocytes, when inhibiting p20K expression with a DNA vector; in their studies inhibition of p20K also increased cellular apoptosis.

Several other studies have implicated CHOP as an inhibitor of pro-survival gene expression to initiate apoptosis mechanisms. Studies by McCullough et al. (2001) examined the influence of CHOP expression on Bcl-2 repression in MEFs. Other studies have shown CHOP negatively regulating autophagy mechanisms during ER stress (Shin et al., 2010). Autophagy is a homeostatic process that degrades cellular organelles to preserve energy levels during prolonged

periods of starvation (Maiuri et al., 2010). Bcl-2 gene expression or autophagy promotes cell survival by protecting cells from ER stress mechanisms (Cullough et al., 2001; Shin et al., 2010). However, during constitutive CHOP expression, Bcl-2 gene expression or autophagy mechanisms are repressed to promote cellular apoptosis (Cullough et al., 2001; Shin et al., 2010). Although it is unknown how CHOP represses Bcl-2, McCullough et al. (2001) proposed that CHOP may inhibit Bcl-2 expression indirectly by negatively regulating another transcription factor possibly from the C/EBP family. This proposed mechanism is similar to the way CHOP indirectly inhibits p20K GAS gene expression during ER stress. CHOP influences p20K expression through the complex formation with C/EBPβ. The link between CHOP and p20K is unclear, as CHOP is induced in response to ER stress, and p20K is expressed in growth arrest conditions. However, a mechanism repressing a possible pro-survival factor, such as p20K, is certainly relevant to the induction of apoptosis in response to excessive stress. Thus, prolonged ER stress can trigger cell death mechanisms that lead to CHOP induction, and repression of p20K.

4.2 CHOP induction represses p20K activity

My studies show that the ER stress transcription factor CHOP expression decreases during growth arrest conditions, favorable for the induction of quiescent specific protein p20K. The sharp decrease of CHOP expression in density arrested or proliferative CEF in hypoxia (24 hours) is associated with p20K induction. CHOP mRNA and protein levels decrease while p20K is induced in these conditions. Kinetic experiments where p20K and CHOP expression was assessed at different time points provided evidence for the inverse relationship between these two genes. Additionally, CHOP over-expression experiments also established a p20K increased repression mediated through C/EBPβ.

To confirm that CHOP repressed p20K, CHOP levels were stimulated in various conditions. As shown in Figure 3.8 and preliminary studies by B. Fielding (MSc. Thesis, 2011), the forced expression of CHOP by RCASB system attenuated p20K expression in hypoxic subconfluent CEF. CHOP expression triggered by two ER stressors (tunicamycin and thapsigargin), also proved to repress p20K activity. In these conditions, protein levels and mRNA levels of p20K are repressed, while CHOP levels are strongly induced as observed in Western blotting and RT-qPCR analysis (Figures 3.6 & 3.10). Interestingly, thapsigargin stimulated higher CHOP levels, and stronger p20K repression than tunicamycin (Figures 3.6 & 3.10). This has also been documented in other studies, where thapsigargin is preferentially chosen over tunicamycin because of its faster and more sensitive method of stimulating ER stress (Fritsch et al., 2007; Marciniak et al., 2004). Induction of CHOP by tunicamycin or thapsigargin has been observed in several mammalian cell lines such as murine skeletal muscle myoblasts, human islet cells, primary rat cortical neuronal cells, and mouse embryonic fibroblasts (MEF) to study ER stress responses associated with CHOP activation (Chung et al., 2011; Deldicque et al., 2011; Lai et al., 2008). This also proved to be a useful method in studying the effect of CHOP on p20K induction in CEF.

4.3 Mechanism by which CHOP interferes with p20K induction

CHOP is distinct from other C/EBP family members because it is unable to form homodimers capable of binding to DNA (Ron and Habener, 1992). As a hetorodimer with C/EBPβ in particular, CHOP can bind and activate transcription from an alternative C/EBP binding site, but not from the canonical C/EBP element (Ubeda et al., 1996). Therefore, CHOP often acts as an inhibitor of C/EBPβ, sequestering it away from its normal targets. For instance, the formation

of inactive heterodimers between C/EBPβ and CHOP delay C/EBPβ mediated adipocyte differentiation (Tang and Lane, 2000).

C/EBPβ is an essential regulator of p20K GAS gene activation in density arrested CEF or during hypoxia (Fielding, MSc. Thesis, 2011; S. Kim et al. 1999). C/EBPβ binds to two distinct domains within the Quiescent-Responsive Unit (QRU) promoter region to activate p20K expression during contact inhibition. C/EBPβ binding to the QRU was also deemed important for the transcriptional regulation of p20K during hypoxia. In current studies, co-immunoprecipitation experiments were used to test for a direct interaction between C/EBPβ and CHOP in CEF during ER stress, starvation, or in proliferative CEF in normoxia. C/EBPβ-CHOP complex formation was observed in all three conditions, except for control conditions (contact inhibited CEF treated with 0.1% DMSO) (Figure 3.7). As CHOP protein levels accumulate during ER stress or starvation, CHOP and C/EBPβ form heterodimers. Thus, CHOP induces a relative decline in C/EBPβ homodimers that can bind to the QRU promoter to activate p20K expression. The ratio of CHOP and C/EBPβ proteins determines whether p20K transcription will be activated. For this reason, in conditions where CHOP is over-expressed, CHOP functions as a negative modulator of p20K (Figures 3.6 - 3.8). CHOP stimulation prevents C/EBPβ dependent p20K activation (Figure 4.1).

Basal levels of CHOP expression always remain detectable in actively dividing CEF in normoxia. The mechanism responsible for CHOP expression in these conditions is not well characterized. This is in accordance with studies by Fornace et al. (1989) that also showed that CHOP is expressed at low levels in proliferating Chinese Hamster Ovarian (CHO) cells. In my studies, co-immunoprecipitation assays provided evidence for the modest formation of C/EBPβ-CHOP complexes in actively dividing CEF in normoxia. These data suggest that a low amount of

inactive C/EBPβ-CHOP heterodimers, relative to ER stress conditions, is also present in normoxia cycling CEF. This can be contributing to the inhibitory mechanism of p20K expression in these conditions. In hypoxia conditions, experiments by B. Fielding (MSc. Thesis, 2011) provided evidence that less CHOP co-immunoprecipitates with C/EBPβ in hypoxic subconfluent CEF. Additionally, CHOP does not associate with C/EBPβ in contact inhibited CEF treated with DMSO (control) (Lane 8; Figure 3.7). In these conditions, CHOP mRNA and protein levels are also reduced considerably relative to p20K expression (Figures 3.4, 3.5, & 3.9B). This demonstrates that in absence of C/EBPβ-CHOP heterodimers, p20K is actively induced in conditions of contact inhibition or hypoxia.

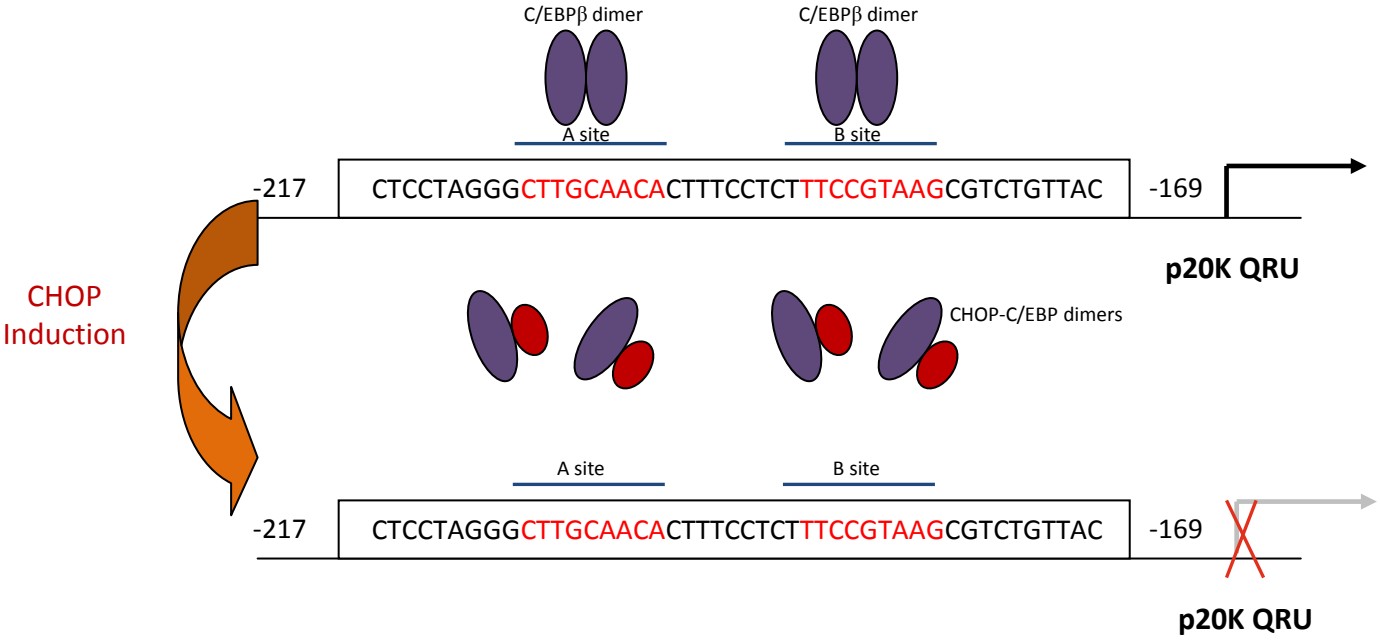


Figure 4.1. Inactive C/EBPβ-CHOP heterodimers fail to activate p20K expression. C/EBPβ contains a consensus recognition site 5'-T[TG]NNGNAA[TG]-3' that is important for the transcriptional activation of its target genes. The QRU promoter region of p20K contains two C/EBPβ binding sites (Sites A and B). The binding of C/EBPβ to these two sites results in the transcriptional activation of p20K. CHOP over-expression by ER stress leads to the formation of inactive C/EBPβ-CHOP heterodimers. Therefore, CHOP sequesters C/EBPβ preventing its binding to the QRU, repressing p20K activity.

4.4 Down-regulating CHOP expression does not promote p20K expression at earlier time points in hypoxia

Based on previous experiments, CHOP induction decreases p20K expression. To investigate the role of CHOP down-regulation on p20K induction, its expression was attenuated by RNA interference (Figures 3.2-3.4). CHOP protein levels were notably reduced in CEF expressing a retrovirus vector shRNA (354-CHOP-10). Decreased CHOP expression had two major outcomes; increased cell proliferation and survival relative to control, and higher levels of p20K induction in subconfluent CEF at later time points in hypoxia or as cells reached contact inhibition (Chapter 3, Figures 3.2-3.4). Prior to the recent discovery of p20K expression of subconfluent CEF in hypoxia, expression of p20K had only been primarily observed in contact inhibited CEFs (B. Fielding, MSc. Thesis, 2011). However, preliminary data also indicates that CEF proliferation is reduced in conditions of hypoxia (2% O_2), which is likely contributing to p20K expression in these conditions (W. Xie & M. Fox-Chen, Unpublished results, 2013). These experiments focused on determining whether down-regulating CHOP expression would restore p20K levels at earlier time points in hypoxic subconfluent CEF. However, this was not the case, with Fig. 3.4A demonstrating that down-regulating CHOP does not induce a robust expression of p20K until 24 hours in hypoxia. The absence of p20K expression at earlier time points in subconfluent CEF in hypoxia suggests that an additional mechanism is repressing p20K in these conditions.

4.5 ERK-2 is an inhibitor of p20K in proliferating CEF

Attenuating CHOP expression was insufficient to stimulate p20K induction in subconfluent CEF at earlier time points in hypoxia. The role of ERK-2 as a transcriptional repressor was first analyzed in GATE-elements, when binding of ERK-2 to a consensus DNA sequence within GATE promoter blocked transcriptional activity of GATE-driven genes (Hu et al., 2009).

Studies by M. Athar (MSc. Thesis, 2011) demonstrated the role of ERK-2 as transcriptional repressor of p20K in proliferative CEF. ERK-2 can bind to a consensus DNA sequence (GAAGGAGAAAG) of the QRU, overlapping the two C/EBPβ binding domains (Figure 4.2). Binding of ERK-2 to QRU, inhibits p20K by hindering C/EBPβ interaction. ChIP experiments confirmed the DNA-binding activity of ERK-2 to QRU in proliferative CEF in normoxia (M. Athar, MSc. Thesis, 2011). Since the phosphorylation of ERK 1/2 is required for its activation and nuclear translocation, a phospho-site specific ERK antibody was used to assess the level of ERK activation in CEF (Lenormand et al., 1998). Preliminary experiments where p20K and activated ERK expression was assessed at 36 hours after treatment with DMOG (hypoxia mimetic), suggested that phospho-ERK levels are modestly down-regulated, while p20K is strongly induced (Figures 3.11 & 3.12). It is likely that DMOG is responsible for p20K induction by decreasing the activated ERK levels, however more studies are needed to identify and characterize the mechanism in which DMOG is controlling p20K and activated ERK levels. In other experiments by undergraduate student M. Fox-Chen, Western blotting analysis showed that phospho-ERK levels are down-regulated after 24 hours in hypoxia, while levels of p20K increased (Unpublished Results, 2013). Additionally, contact inhibited CEF treated with DMSO (control) showed complete repression of phospho-ERK levels, while p20K was highly induced (Figure 3.12). These studies demonstrate that both CHOP and ERK-2 play different repressive roles on p20K to regulate its expression in conditions of ER stress and activate proliferation, respectively.

4.6 Induction of p20K by DMOG

The PHD family (PHD1, PHD2, and PHD3) and FIH-1 are responsible for promoting HIF-1α proteasomal degradation at high oxygen levels (Elvidge et al., 2006). However, DMOG is a

hypoxia mimetic that inhibits both PHD family members and FIH-1 in normoxia (Köditz et al., 2007; Lando et al., 2002). Thus, DMOG exerts its actions by stabilizing HIF-1α, and preventing its degradation mimicking a hypoxia response in normoxic conditions. These data demonstrate that p20K is positively up-regulated by hypoxia-mimetic chemical DMOG. Treating CEF with 1mM of DMOG induced robust p20K expression at 24 and 36 hours post treatment. Figure 3.12 also confirms that the induction of p20K by DMOG was C/EBPβ dependent. In these experiments, CEF expressing a dominant negative C/EBPβ mutant (RCASBP-Δ184-C/EBPβ) blocked induction of p20K in DMOG treated CEF. These data suggest that p20K expression is mediated by C/EBPβ binding to the QRU during DMOG treatment. Finally, treating CEF with DMOG also induced a modest up-regulation of CHOP levels. Thus, DMOG is unique because it provides a method that strongly induces p20K, while stimulating CHOP expression. Whether or not DMOG also limits ERK activation and CEF proliferation remains to be determined.

Studies by Köditz et al. (2007) discovered an alternative induction of ATF-4 after treating HeLa cells with DMOG. ATF-4 is a transcription factor involved in the unfolding protein response (UPR) during ER stress (Harding et al., 2003). Interestingly, ATF-4 is also known to regulate CHOP expression (Fawcett et al., 1999). ATF-4 activates CHOP by its direct binding to its promoter following a stress response (Fawcett et al., 1999). However, the Köditz et al. studies did not investigate CHOP expression during DMOG treatment. In these studies, they discovered a direct inhibition of ATF-4 levels by PHD3. Therefore PHD inhibition by DMOG results in expression of ATF-4 (Köditz et al., 2007). A transient expression of ATF-4 was also noticed in HeLa cells incubated in severe hypoxia (0.2-1%O_2) that disappeared shortly within 24 hours (Köditz et al., 2007). Paradoxically, only PHD2 and PHD3 are induced in hypoxia as an auto regulatory mechanism, which becomes functional during re-oxygenation conditions to rapidly

degrade HIF-1 (Stiehl et al., 2006). Therefore, ATF-4 down-regulation in hypoxia can be a result of induction of PHD2 and PHD3 (Köditz et al., 2007; Stiehl et al., 2006).

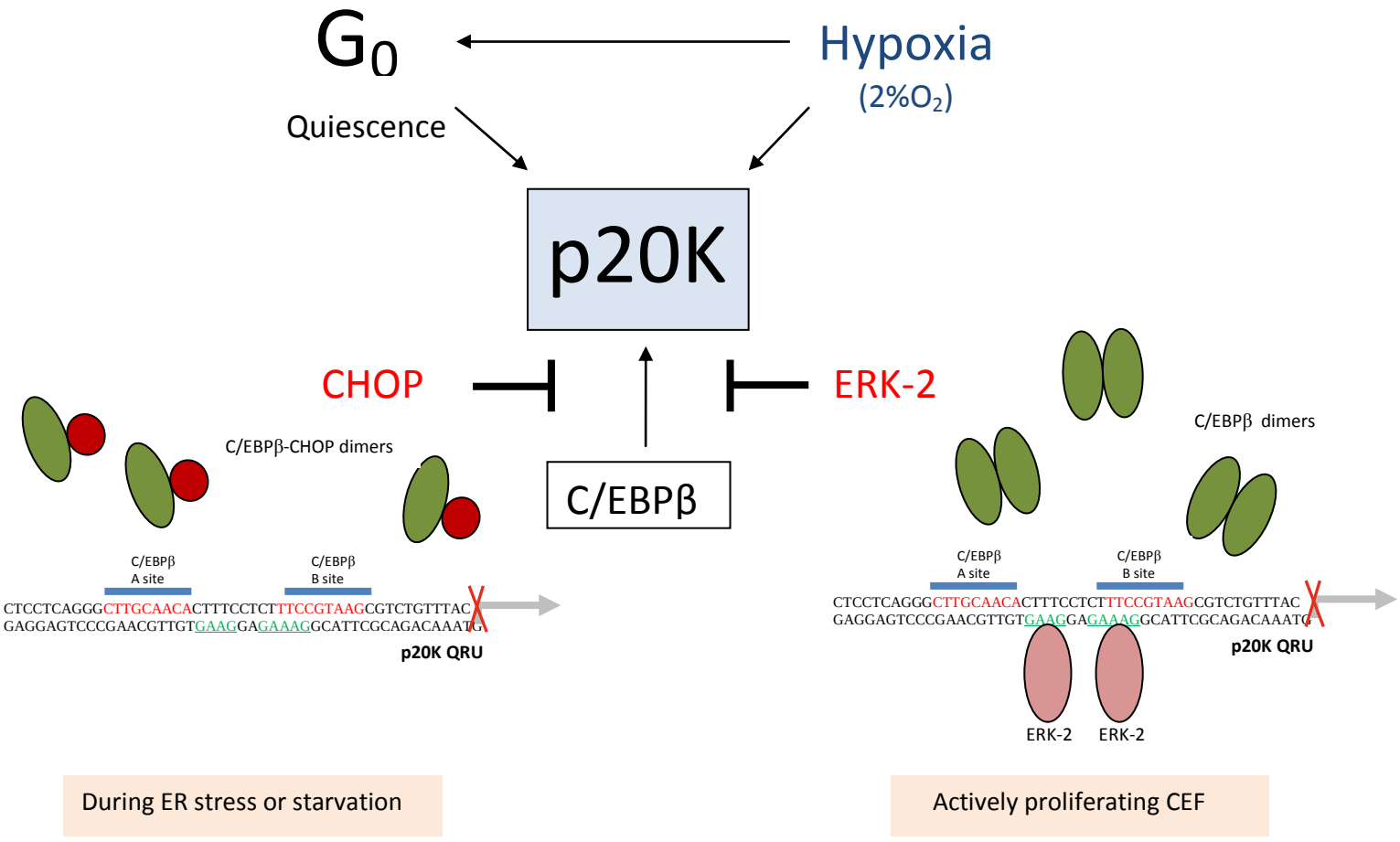


Figure 4.2- Repression of p20K by ERK-2 and CHOP mediated through C/EBPβ. C/EBPβ dimers bind to two distinct domains within in the QRU promoter to activate p20K during contact inhibition or subconfluent CEF in hypoxia (24 hours) (1-2% 0_2). However the formation of C/EBPβ-CHOP heterodimers during starvation or ER stress inhibits p20K activity. C/EBPβ-CHOP heterodimers sequester C/EBPβ, which results in a decline of C/EBPβ dimers available to activate p20K QRU promoter. Another inhibitor of p20K in proliferative CEF is ERK-2. ERK-2 exerts its repressive role on p20K by binding to a consensus DNA sequence (GAAGGAGAAAG) of the QRU, overlapping the two C/EBPβ binding domains, hindering C/EBPβ binding to the QRU. Preliminary data indicate that CEF proliferation is reduced in conditions of hypoxia (2%O_2) (W. Xie & M. Fox-Chen, Unpublished results, 2013).

4.7 Future Experiments:

4.7.1 C/EBPβ recruitment to the QRU during hypoxia and contact inhibition by ChIP

The QRU is a 48 base pair promoter region that has been well characterized in the regulation of p20K (Kim et al., 1999). During activation of p20K in contact inhibition or hypoxia two functional domains (Regions A and B) within the QRU required for C/EBPβ were identified by transient expression assays (Kim et al., 1999; Fielding, MSc. Thesis, 2011). Additionally, research by Kim et al. (1999) confirmed the binding of C/EBPβ to these two DNA consensus sites within the p20K QRU promoter by electrophoretic mobility shift assays (EMSA). With more advances in the field, future experiments can validate these binding sites by chromatin immunoprecipitation (ChIP) assays. ChIP assays would be beneficial to provide *in vivo* evidence of C/EBPβ binding to p20K QRU promoter region in growth arrest conditions.

4.7.2 ERK-2 recruitment to QRU post DMOG treatment

A series of ChIP experiments provided evidence for the role of ERK-2 as a transcriptional repressor of p20K QRU in cycling CEF (MSc. Thesis, M. Athar, 2011). During hypoxia or contact inhibition where p20K is predominantly expressed, there is no observed binding of ERK-2 to QRU. Preliminary experiments provided evidence that treating CEF with DMOG, a hypoxia mimetic chemical, provided a unique method in analyzing the roles of p20K inhibitors, CHOP and activated ERK. However, it is important to characterize the mechanism or additional signal/pathway through which DMOG is controlling p20K induction. Follow up studies will require analyzing the effect of DMOG on levels of phospho-ERK and ERK-2 binding to QRU by Western blotting and ChIP analysis. It is important to identify and characterize ERK-2 binding to QRU to determine how it modulates p20K expression in DMOG treated CEF.

4.7.3 Down-regulating CHOP expression by shRNAi and inhibiting ERK1/2 with MEK1/2 inhibitor

As shown in these experiment and studies by M. Athar (MSc. Thesis, 2011), CHOP and ERK-2 have been characterized as inhibitors of p20K expression mediated through C/EBPβ. Surprisingly, CEF expressing a retrovirus vector shRNAi (354-CHOP-10) to down-regulate CHOP levels did not restore p20K levels at earlier time points in subconfluent CEF in hypoxia. Therefore it is hypothesized that ERK-2 is working in conjunction with CHOP in a separate mechanism to repress p20K in cycling CEF. Recent attempts to abrogate ERK-2 expression by RNA interference were not successful possibly because ERK-2 regulates many cellular pathways involved in proliferation, differentiation, and survival (Kolch, 2000). However, treating CEF with a MEK1/2 inhibitor PD0325901 resulted in reduced expression levels of phospho-ERK1/2 (p-ERK1/2) in CEF. Preliminary experiments were performed by undergraduate student Laura Mantella by treating CEF with PD0325901 MEK1/2 inhibitor, which resulted in early expression of p20K in cycling CEF in hypoxia. Future studies can aim to down-regulate CHOP expression by RNAi and inhibit activated ERK expression with PD0325901. Concomitantly to achieve maximum induction of p20K, it would be insightful to characterize p20K expression in the absence CHOP following MEK inhibition.

4.7.4 Transient expression Assays to examine specific regions of QRU promoter in hypoxia

QRU contains two C/EBPβ binding sites, Region A and B (Kim et al, 1999). Previous studies by Kim et al. (1999) showed that a mutation to either of the two C/EBPβ binding sites of the QRU disrupted p20K expression in contact inhibited and serum deficient cells. In addition, Kim et al. (1999) also determined that region A had a stronger activity in quiescent cells in comparison to region B. The QRU is also crucial for induction of p20K in hypoxia; however the specific region responsible for this induction is not known (B. Fielding, MSc. Thesis, 2011).

Thus, it would be beneficial to examine further if a mutation to either the A or B site or both sites affects activation of the p20K promoter in hypoxic conditions. Also it would be informative to determine which C/EBPβ binding site(s) is more responsive during hypoxia. Transient expression assays can be used to examine the activity of the functional domains within the QRU in response to hypoxia.

4.8 Conclusion

The purpose of this study was to investigate the role of the ER stress induced transcription factor CHOP in regulating p20K Gas gene expression. As demonstrated by RT-qPCR and Western blotting analysis, both CHOP and p20K mRNA and protein levels display an inverse relationship. During ER stress or starvation, p20K is negatively regulated by the expression of the transcription factor CHOP. My studies showed that thapsigargin and tunicamycin ER stress inducible agents inhibit p20K expression by stimulating an increase in CHOP levels, culminating in a decline in C/EBPβ-mediated p20K activation. These experiments established an important mechanistic link between CHOP and quiescence specific protein p20K mediated through C/EBPβ. In conclusion, these studies contributed to the understanding of how the versatile roles of C/EBPβ can control gene expression and cell fate.

References

Agarwal, M.L., Agarwal, a, Taylor, W.R., and Stark, G.R. (1995). p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. Proceedings of the National Academy of Sciences of the United States of America *92*, 8493–8497.

Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T., and Kishimoto, T. (1990). A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. The EMBO Journal *9*, 1897–1906.

Alberini, C.M. (2006). CCAAT Enhancer Binding Proteins in the Nervous System: Their Role in Development, Differentiation, Long-Term Synaptic Plasticity, and Memory. In Transcription Factors in the Nervous System, (Wiley-VCH Verlag GmbH & Co. KGaA), pp. 243–258.

Athar, M. (2011). Characterization of ERK-2 as a Transcriptional Repressor of Growth Arrest Specific Genes. MSc. Thesis, Unpublished Results. McMaster University.

Barnucz, E., Veres, G., Hegedus, P., Klein, S., Zoller, R., Radovits, T., Korkmaz, S., Horkay, F., Merkely, B., Karck, M., et al. (2013). Prolyl-hydroxylase inhibition preserves endothelial cell function in a rat model of vascular ischemia reperfusion injury. The Journal of Pharmacology and Experimental Therapeutics *345*, 25–31.

Batchvarova, N., Wang, X., and Ron, D. (1995). Inhibition of adipogenesis by the stress-induced protein CHOP (Gaddl53). The EMBO Journal *14*, 4654–4661.

Bedard, P. a, Yannoni, Y., Simmons, D.L., and Erikson, R.L. (1989). Rapid repression of quiescence-specific gene expression by epidermal growth factor, insulin, and pp60v-src. Molecular and Cellular Biology *9*, 1371–1375.

Bedard, P.A., Balk, S.D., Gunther, H.S., Morisi, A., and Erikson, R.L. (1987). Repression of quiescence-specific polypeptides in chicken heart mesenchymal cells transformed by Rous sarcoma virus. Molecular and Cellular Biology *7*, 1450–1458.

Bedolla, D.E., Kenig, S., Mitri, E., Ferraris, P., Marcello, A., Grenci, G., and Vaccari, L. (2013). Determination of cell cycle phases in live B16 melanoma cells using IRMS. Analyst *138*, 4015–4021.

Black, A.R., and Black, J.D. (2012). Protein kinase C signaling and cell cycle regulation. Frontiers in Immunology *3*, 423.

Brown, J.M., and Wilson, W.R. (2004). Exploiting tumour hypoxia in cancer treatment. Nat Rev Cancer *4*, 437–447.

Calkhoven, C.F., Müller, C., and Leutz, a (2000). Translational control of C/EBPalpha and C/EBPbeta isoform expression. Genes & Development *14*, 1920–1932.

Cancedda, F.D., Dozin, B., Rossi, F., Molina, F., Cancedda, R., Negri, a, and Ronchi, S. (1990). The Ch21 protein, developmentally regulated in chick embryo, belongs to the superfamily of lipophilic molecule carrier proteins. The Journal of Biological Chemistry *265*, 19060–19064.

Carlson, S.G., Fawcett, T.W., Bartlett, J.D., Bernier, M., and Holbrook, N.J. (1993). Regulation of the C/EBP-related gene gadd153 by glucose deprivation. Molecular and Cellular Biology *13*, 4736–4744.

Chen, B.-R., Cheng, H.-H., Lin, W.-C., Wang, K.-H., Liou, J.-Y., Chen, P.-F., and Wu, K.K. (2012). Quiescent fibroblasts are more active in mounting robust inflammatory responses than proliferative fibroblasts. PloS One *7*, e49232.

Chi, J.-T., Wang, Z., Nuyten, D.S.A., Rodriguez, E.H., Schaner, M.E., Salim, A., Wang, Y., Kristensen, G.B., Helland, Å., Børresen-Dale, A.-L., et al. (2006). Gene Expression Programs in Response to Hypoxia: Cell Type Specificity and Prognostic Significance in Human Cancers. PLoS Med *3*, e47.

Chim, C.S., Fung, T.K., Wong, K.F., Lau, J.S., Law, M., and Liang, R. (2006). Methylation of INK4 and CIP/KIP families of cyclin-dependent kinase inhibitor in chronic lymphocytic leukaemia in Chinese patients. Journal of Clinical Pathology *59*, 921–926.

Chiribau, C.-B., Gaccioli, F., Huang, C.C., Yuan, C.L., and Hatzoglou, M. (2010). Molecular symbiosis of CHOP and C/EBP beta isoform LIP contributes to endoplasmic reticulum stress-induced apoptosis. Molecular and Cellular Biology *30*, 3722–3731.

Cho, S., and Hwang, E.S. (2012). Status of mTOR activity may phenotypically differentiate senescence and quiescence. Molecules and Cells *33*, 597–604.

Chung, H., Chung, H.-Y., Bae, C.W., Kim, C.-J., and Park, S. (2011). Ghrelin suppresses tunicamycin- or thapsigargin-triggered endoplasmic reticulum stress-mediated apoptosis in primary cultured rat cortical neuronal cells. Endocrine Journal *58*, 409–420.

Ciccarelli, C., Philipson, L., and Sorrentino, V. (1990). Regulation of expression of growth arrest-specific genes in mouse fibroblasts. Molecular and Cellular Biology *10*, 1525–1529.

Cobrinik, D. (2005). Pocket proteins and cell cycle control. Oncogene *24*, 2796–2809.

Collado, M., Blasco, M.A., and Serrano, M. (2007). Cellular senescence in cancer and aging. Cell *130*, 223–233.

Coller, H.A., Sang, L., and Roberts, J.M. (2006). A new description of cellular quiescence. PLoS Biology *4*, e83.

Cortés-Canteli, M., Pignatelli, M., Santos, A., and Perez-Castillo, A. (2002). CCAAT/enhancer-binding protein beta plays a regulatory role in differentiation and apoptosis of neuroblastoma cells. The Journal of Biological Chemistry *277*, 5460–5467.

Cullough, K.D.M.C., Martindale, J.L., Klotz, L., Aw, T., and Holbrook, N.J. (2001). Gadd153 Sensitizes Cells to Endoplasmic Reticulum Stress by Down-Regulating Bcl2 and Perturbing the Cellular Redox State. Molecular and Cellular Biology *21*, 1249–1259.

Darlington, G.J., Ross, S.E., and MacDougald, O.A. (1998). The Role of C/EBP Genes in Adipocyte Differentiation. Journal of Biological Chemistry *273*, 30057–30060.

Dean, M., Fojo, T., and Bates, S. (2005). Tumour stem cells and drug resistance. Nature Reviews. Cancer *5*, 275–284.

Degagné, É., Turgeon, N., Moore-Gagné, J., Asselin, C., and Gendron, F.-P. (2012). P2Y2 receptor expression is regulated by C/EBPβ during inflammation in intestinal epithelial cells. FEBS Journal *279*, 2957–2965.

Deldicque, L., Bertrand, L., Patton, A., Francaux, M., and Baar, K. (2011). ER stress induces anabolic resistance in muscle cells through PKB-induced blockade of mTORC1. PloS One *6*, e20993.

Descalzi Cancedda, F., Malpeli, M., Gentili, C., Di Marzo, V., Bet, P., Carlevaro, M., Cermelli, S., and Cancedda, R. (1996). The Developmentally Regulated Avian Ch21 Lipocalin Is an Extracellular Fatty Acid-binding Protein. Journal of Biological Chemistry *271*, 20163–20169.

Descalzi Cancedda, F., Dozin, B., Zerega, B., Cermelli, S., Gentili, C., and Cancedda, R. (2002). Ex-FABP, extracellular fatty acid binding protein, is a stress lipocalin expressed during chicken embryo development. Molecular and Cellular Biochemistry *239*, 221–225.

Descombes, P., Chojkier, M., Lichtsteiner, S., Falvey, E., and Schibler, U. (1990). LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. Genes & Development *4*, 1541–1551.

Didon, L., Barton, J.L., Roos, A.B., Gaschler, G.J., Bauer, C.M.T., Berg, T., Stämpfli, M.R., and Nord, M. (2011). Lung Epithelial CCAAT/Enhancer-binding Protein-β Is Necessary for the Integrity of Inflammatory Responses to Cigarette Smoke. American Journal of Respiratory and Critical Care Medicine *184*, 233–242.

Dulic, V., Stein, G.H., Far, D.F., and Reed, S.I. (1998). Nuclear Accumulation of p21 Cip1 at the Onset of Mitosis : a Role at the G 2 / M-Phase Transition. Molecular and Cellular Biology *18*, 546–557.

Eaton, E.M., Hanlon, M., Bundy, L., and Sealy, L. (2001). Characterization of C/EBPβ isoforms in normal versus neoplastic mammary epithelial cells. Journal of Cellular Physiology *189*, 91–105.

Elvidge, G.P., Glenny, L., Appelhoff, R.J., Ratcliffe, P.J., Ragoussis, J., and Gleadle, J.M. (2006). Concordant regulation of gene expression by hypoxia and 2-oxoglutarate-dependent dioxygenase inhibition: the role of HIF-1alpha, HIF-2alpha, and other pathways. The Journal of Biological Chemistry *281*, 15215–15226.

Ercan, C., Vermeulen, J.F., Hoefnagel, L., Bult, P., Van der Groep, P., Van der Wall, E., and Van Diest, P.J. (2012). HIF-1alpha and NOTCH signaling in ductal and lobular carcinomas of the breast. Cellular Oncology (Dordrecht) *35*, 435–442.

Erdmann, J. (2005). Cancer's big sleep: senescence may be potential target for cancer therapies. Journal of the National Cancer Institute *97*, 89–91.

Fan, H., Liu, Z., Shimada, M., Sterneck, E., Johnson, P.F., Hedrick, S.M., and Richards, J.S. (2010). NIH Public Access. Science *324*, 938–941.

Fawcett, T.W., Eastman, H.B., Martindale, J.L., and Holbrook, N.J. (1996). Physical and functional association between GADD153 and CCAAT/enhancer-binding protein beta during cellular stress. The Journal of Biological Chemistry *271*, 14285–14289.

Fawcett, T.W., Martindale, J.L., Guyton, K.Z., Hai, T., and Holbrook, N.J. (1999). Complexes containing activating transcription factor (ATF)/cAMP-responsive-element-binding protein (CREB) interact with the CCAAT/enhancer-binding protein (C/EBP)-ATF composite site to regulate Gadd153 expression during the stress response. . Biochemical Journal *339*, 135–141.

Ferrini, J.-B., Rodrigues, E., Dulic, V., Pichard-Garcia, L., Fabre, J.-M., Blanc, P., and Maurel, P. (2001). Expression and DNA-binding activity of C/EBPα and C/EBPβ in human liver and differentiated primary hepatocytes. Journal of Hepatology *35*, 170–177.

Fielding, B. (2011). Regulation of Growth Arrest Specific (GAS) protein p20K in Hypoxia Chicken Embryonic Fibroblasts. Master Thesis, Unpublished results. McMaster University.

Fleming, J. V, Fontanier, N., Harries, D.N., and Rees, W.D. (1997). The growth arrest genes gas5, gas6, and CHOP-10 (gadd153) are expressed in the mouse preimplantation embryo. Molecular Reproduction and Development *48*, 310–316.

Foijer, F., and Te Riele, H. (2006). Restriction beyond the restriction point: mitogen requirement for G2 passage. Cell Division *1*, 8.

Fornace, A.J., Nebert, D.W., Hollander, M.C., Luethy, J.D., Papathanasiou, M., Fargnoli, J., and Holbrook, N.J. (1989). Mammalian genes coordinately regulated by growth arrest signals and DNA-damaging agents. Molecular and Cellular Biology *9*, 4196–4203.

Fox-Chen, M. (2013). Undergraduate Honors Thesis. Unpublished Results. McMaster University.

Fraisl, P., Aragones, J., and Carmeliet, P. (2009). Inhibition of oxygen sensors as a therapeutic strategy for ischaemic and inflammatory disease. Nature reviews.Drug Discovery *8*, 139–152.

Friedman, A.D. (1996). GADD153/CHOP, a DNA damage-inducible protein, reduced CAAT/enhancer binding protein activities and increased apoptosis in 32D c13 myeloid cells. Cancer Research *56*, 3250–3256.

Fritsch, R.M., Schneider, G., Saur, D., Scheibel, M., and Schmid, R.M. (2007). Translational repression of MCL-1 couples stress-induced eIF2 alpha phosphorylation to mitochondrial apoptosis initiation. The Journal of Biological Chemistry *282*, 22551–22562.

Gagliardi, M., Maynard, S., Bojovic, B., and Bédard, P. a (2001). The constitutive activation of the CEF-4/9E3 chemokine gene depends on C/EBPbeta in v-src transformed chicken embryo fibroblasts. Oncogene *20*, 2301–2313.

Gagliardi, M., Maynard, S., Miyake, T., Rodrigues, N., Tjew, S.L., Cabannes, E., and Bedard, P.A. (2003). Opposing roles of C/EBPbeta and AP-1 in the control of fibroblast proliferation and growth arrest-specific gene expression. The Journal of Biological Chemistry *278*, 43846–43854.

Gao, X., Tangney, M., and Background, A.B. (2011). A Multiscale Model for Hypoxia-induced Avascular Tumor Growth. *5*, 53–58.

Gardner, L.B., Li, Q., Park, M.S., Flanagan, W.M., Semenza, G.L., and Dang, C. V (2001). Hypoxia inhibits G1/S transition through regulation of p27 expression. The Journal of Biological Chemistry *276*, 7919–7926.

Gelman, I.H., and Sudol, M. (2010). Introduction: hanafusa memorial issue, part 2. Genes & Cancer *1*, 1163.

Gentili, C., Tutolo, G., Zerega, B., Di Marco, E., Cancedda, R., and Cancedda, F.D. (2005). Acute phase lipocalin Ex-FABP is involved in heart development and cell survival. Journal of Cellular Physiology *202*, 683–689.

Gery, S., Park, D.J., Vuong, P.T., Chih, D.Y., Lemp, N., and Koeffler, H.P. (2004). Retinoic acid regulates C/EBP homologous protein expression (CHOP), which negatively regulates myeloid target genes. Blood *104*, 3911–3917.

Ghosh, R. (2009). Characterizing the Role of CCAAT/ Enhancer Binding Protein β on Quiescence-Specific Gene Expression in Chicken Embryo Fibroblasts. Master Thesis, Unpublished Results. McMaster University.

Gos, M., Miloszewska, J., Swoboda, P., Trembacz, H., Skierski, J., and Janik, P. (2005). Cellular quiescence induced by contact inhibition or serum withdrawal in C3H10T1/2 cells. Cell Proliferation *38*, 107–116.

Graham, S.M., Jørgensen, H.G., Allan, E., Pearson, C., Alcorn, M.J., Richmond, L., and Holyoake, T.L. (2002). Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. Blood *99*, 319–325.

Graña, X., and Reddy, E. (1995). Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs) Title. Oncogene *11*, 211–219.

Gray, J. V, Petsko, G.A., Johnston, G.C., Ringe, D., Singer, R.A., and Werner-Washburne, M. (2004). “Sleeping beauty”: quiescence in Saccharomyces cerevisiae. Microbiology and Molecular Biology Reviews : MMBR *68*, 187–206.

Gupta, S., Cuffe, L., Szegezdi, E., Logue, S.E., Neary, C., Healy, S., and Samali, A. (2010). Mechanisms of ER Stress-Mediated Mitochondrial Membrane Permeabilization. International Journal of Cell Biology *2010*, 170215.

Gustafsson, M. V, Zheng, X., Pereira, T., Gradin, K., Jin, S., Lundkvist, J., Ruas, J.L., Poellinger, L., Lendahl, U., and Bondesson, M. (2005). Hypoxia requires notch signaling to maintain the undifferentiated cell state. Developmental Cell *9*, 617–628.

Guyton, K.Z., Xu, Q., and Holbrook, N.J. (1996). stress : role of AP-1 element. *554*, 547–554.

Harding, H.P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000). Regulated Translation Initiation Controls Stress-Induced Gene Expression in Mammalian Cells. Molecular Cell *6*, 1099–1108.

Harding, H.P., Zhang, Y., Zeng, H., Novoa, I., Lu, P.D., Calfon, M., Sadri, N., Yun, C., Popko, B., Paules, R., et al. (2003). An Integrated Stress Response Regulates Amino Acid Metabolism and Resistance to Oxidative Stress. Molecular Cell *11*, 619–633.

Hattori, T., Ohoka, N., Hayashi, H., and Onozaki, K. (2003). C/EBP homologous protein (CHOP) up-regulates IL-6 transcription by trapping negative regulating NF-IL6 isoform. FEBS Letters *541*, 33–39.

Hawke, T.J., and Garry, D.J. (2001). Myogenic satellite cells: physiology to molecular biology. Journal of Applied Physiology (Bethesda, Md. : 1985) *91*, 534–551.

Hess, J., Angel, P., and Schorpp-Kistner, M. (2004). AP-1 subunits: quarrel and harmony among siblings. Journal of Cell Science *117*, 5965–5973.

Hu, S., Xie, Z., Onishi, A., Yu, X., Jiang, L., Lin, J., Rho, H., Woodard, C., Wang, H., Jeong, J.-S., et al. (2009). Profiling the human protein-DNA interactome reveals ERK2 as a transcriptional repressor of interferon signaling. Cell *139*, 610–622.

Hughes, S.H. (2004). The RCAS vector system. Folia Biologica *50*, 107–119.

Hwang, H.C., and Clurman, B.E. (2005). Cyclin E in normal and neoplastic cell cycles. Oncogene *24*, 2776–2786.

Jackman, J., Alamo, I., and Fornace, A.J. (1994). Genotoxic Stress Confers Preferential and Coordinate Messenger RNA Stability on the Five gadd Genes Genotoxic Stress Confers Preferential and Coordinate Messenger RNA Stability on the Five gadd Genes. Cancer Research 5656–5662.

Jousse, C., Bruhat, a, Carraro, V., Urano, F., Ferrara, M., Ron, D., and Fafournoux, P. (2001). Inhibition of CHOP translation by a peptide encoded by an open reading frame localized in the chop 5'UTR. Nucleic Acids Research *29*, 4341–4351.

Jover, R., Bort, R., and Jose, M. (2002). Down-regulation of human CYP3A4 by the inflammatory signal interleukin-6 : molecular mechanism and transcription factors involved 1. The FASEB Journal *16*, 1799–1801.

Kim, S., Mao, P.L., Gagliardi, M., and Bedard, P.A. (1999). C/EBPbeta (NF-M) is essential for activation of the p20K lipocalin gene in growth-arrested chicken embryo fibroblasts. Molecular and Cellular Biology *19*, 5718–5731.

Köditz, J., Nesper, J., Wottawa, M., Stiehl, D.P., Camenisch, G., Franke, C., Myllyharju, J., Wenger, R.H., and Katschinski, D.M. (2007). Oxygen-dependent ATF-4 stability is mediated by the PHD3 oxygen sensor. Blood *110*, 3610–3617.

Kolch, W. (2000). Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. . Biochemical Journal *351*, 289–305.

Komarova, N.L., and Wodarz, D. (2007). Stochastic modeling of cellular colonies with quiescence: an application to drug resistance in cancer. Theoretical Population Biology *72*, 523–538.

Kowenz-Leutz, E., and Leutz, a (1999). A C/EBP beta isoform recruits the SWI/SNF complex to activate myeloid genes. Molecular Cell *4*, 735–743.

Küppers, M., Ittrich, C., Faust, D., and Dietrich, C. (2010). The transcriptional programme of contact-inhibition. Journal of Cellular Biochemistry *110*, 1234–1243.

Lai, E., Bikopoulos, G., Wheeler, M.B., Rozakis-Adcock, M., and Volchuk, A. (2008). Differential activation of ER stress and apoptosis in response to chronically elevated free fatty acids in pancreatic beta-cells. American Journal of Physiology. Endocrinology and Metabolism *294*, E540–50.

Lando, D., Peet, D.J., Gorman, J.J., Whelan, D.A., Whitelaw, M.L., and Bruick, R.K. (2002). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. 1466–1471.

Legesse-Miller, A., Raitman, I., Haley, E.M., Liao, A., Sun, L.L., Wang, D.J., Krishnan, N., Lemons, J.M.S., Suh, E.J., Johnson, E.L., et al. (2012). Quiescent fibroblasts are protected from proteasome inhibition-mediated toxicity. Molecular Biology of the Cell *23*, 3566–3581.

Lenormand, P., Brondello, J., Brunet, A., and Pouysségur, J. (1998). Growth Factor–induced p42/p44 MAPK Nuclear Translocation and Retention Requires Both MAPK Activation and Neosynthesis of Nuclear Anchoring Proteins. Cell Biology *142*, 625–633.

Liu, Y., and Kulesz-Martin, M. (2001). p53 protein at the hub of cellular DNA damage response pathways through sequence-specific and non-sequence-specific DNA binding. Carcinogenesis *22*, 851–860.

Liu, H., Adler, A.S., Segal, E., and Chang, H.Y. (2007). A transcriptional program mediating entry into cellular quiescence. PLoS Genetics *3*, e91.

Liu, W., Wen, Y., Bi, P., Lai, X., Liu, X.S., Liu, X., and Kuang, S. (2012). Hypoxia promotes satellite cell self-renewal and enhances the efficiency of myoblast transplantation. Development (Cambridge, England) *139*, 2857–2865.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif.) *25*, 402–408.

Lu, X., Xie, W., Reed, D., Bradshaw, W.S., and Simmons, D.L. (1995). Nonsteroidal antiinflammatory drugs cause apoptosis and induce cyclooxygenases in chicken embryo fibroblasts. Proceedings of the National Academy of Sciences of the United States of America *92*, 7961–7965.

Ma, Y., Brewer, J.W., Alan Diehl, J., and Hendershot, L.M. (2002). Two Distinct Stress Signaling Pathways Converge Upon the CHOP Promoter During the Mammalian Unfolded Protein Response. Journal of Molecular Biology *318*, 1351–1365.

Maiuri, M.C., Criollo, A., and Kroemer, G. (2010). Crosstalk between apoptosis and autophagy within the Beclin 1 interactome. The EMBO Journal *29*, 515–516.

Majmundar, A.J., Skuli, N., Mesquita, R.C., Kim, M.N., Yodh, A.G., Nguyen-McCarty, M., and Simon, M.C. (2012). O(2) regulates skeletal muscle progenitor differentiation through phosphatidylinositol 3-kinase/AKT signaling. Molecular and Cellular Biology *32*, 36–49.

Malumbres, M., and Barbacid, M. (2005). Mammalian cyclin-dependent kinases. Trends in Biochemical Sciences *30*, 630–641.

Mao, P.L., Beauchemin, M., and Bedard, P.A. (1993). Quiescence-dependent activation of the p20K promoter in growth-arrested chicken embryo fibroblasts. The Journal of Biological Chemistry *268*, 8131–8139.

Marciniak, S.J., Yun, C.Y., Oyadomari, S., Novoa, I., Zhang, Y., Jungreis, R., Nagata, K., Harding, H.P., and Ron, D. (2004). CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. Genes & Development *18*, 3066–3077.

Di Marco, E., Sessarego, N., Zerega, B., Cancedda, R., and Cancedda, F.D. (2003). Inhibition of cell proliferation and induction of apoptosis by ExFABP gene targeting. Journal of Cellular Physiology *196*, 464–473.

Matsuoka, S., Edwards, M.C., Bai, C., Parker, S., Zhang, P., Baldini, a, Harper, J.W., and Elledge, S.J. (1995). p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene. Genes & Development *9*, 650–662.

Maytin, E. V., Ubeda, M., Lin, J.C., and Habener, J.F. (2001). Stress-Inducible Transcription Factor CHOP/gadd153 Induces Apoptosis in Mammalian Cells via p38 Kinase-Dependent. Experimental Cell Research *267*, 193–204.

Mebratu, Y., and Tesfaigzi, Y. (2009). How ERK1/2 activation controls cell proliferation and cell death: Is subcellular localization the answer? Cell Cycle (Georgetown, Tex.) *8*, 1168–1175.

Miyake, T. (2004). Growth Arrested Specific Gene Regulation by C/EBP Beta in Chicken Embryonic Fibroblasts. MSc. Thesis, Unpublished Results. York University.

Moore, C.E., Omikorede, O., Gomez, E., Willars, G.B., and Herbert, T.P. (2011). PERK activation at low glucose concentration is mediated by SERCA pump inhibition and confers preemptive cytoprotection to pancreatic beta-cells. Molecular Endocrinology (Baltimore, Md.) *25*, 315–326.

Oyadomari, S., and Mori, M. (2004). Roles of CHOP/GADD153 in endoplasmic reticulum stress. Cell Death and Differentiation *11*, 381–389.

Pal, R., Janz, M., Galson, D.L., Gries, M., Li, S., Jöhrens, K., Anagnostopoulos, I., Dörken, B., Mapara, M.Y., Borghesi, L., et al. (2009). C/EBPbeta regulates transcription factors critical for proliferation and survival of multiple myeloma cells. Blood *114*, 3890–3898.

Park, B., Qiang, L., and Farmer, S.R. (2004). Phosphorylation of C / EBP Beta at a Consensus Extracellular Signal-Regulated Kinase / Glycogen Synthase Kinase 3 Site Is Required for the Induction of Adiponectin Gene Expression during the Differentiation of Mouse Fibroblasts into Adipocytes. Molecular and Cellular Biology *24*, 8671–8680.

Pietras, A., Von Stedingk, K., Lindgren, D., Pahlman, S., and Axelson, H. (2011). JAG2 induction in hypoxic tumor cells alters Notch signaling and enhances endothelial cell tube formation. Molecular Cancer Research : MCR *9*, 626–636.

Polioudakis, D., Bhinge, A. a, Killion, P.J., Lee, B.-K., Abell, N.S., and Iyer, V.R. (2013). A Myc-microRNA network promotes exit from quiescence by suppressing the interferon response and cell-cycle arrest genes. Nucleic Acids Research *41*, 2239–2254.

Potten, C.S., and Loeffler, M. (1990). Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. Development (Cambridge, England) *110*, 1001–1020.

Puthalakath, H., Huang, D.C.S., OReilly, L.A., King, S.M., and Strasser, A. (1999). The Proapoptotic Activity of the Bcl-2 Family Member Bim Is Regulated by Interaction with the Dynein Motor Complex. Molecular Cell *3*, 287–296.

Ramji, D.P., and Foka, P. (2002). CCAAT / enhancer-binding proteins : structure , function and regulation. Biochemistry *575*, 561–575.

Ranjan, P., and Boss, J. (2006). C/EBPβ regulates TNF induced MnSOD expression and protection against apoptosis. Apoptosis *11*, 1837–1849.

Rocco, J.W., and Sidransky, D. (2001). p16(MTS-1/CDKN2/INK4a) in Cancer Progression. Experimental Cell Research *264*, 42–55.

Ron, D., and Habener, J.F. (1992). CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. Genes & Development *6*, 439–453.

Saletta, F., Rahmanto, Y.S., Siafakas, a. R., and Richardson, D.R. (2011). Cellular Iron Depletion and the Mechanisms Involved in the Iron-dependent Regulation of the Growth Arrest and DNA Damage Family of Genes. Journal of Biological Chemistry *286*, 35396–35406.

Schmaltz, C., Hardenbergh, P.H., Wells, a, and Fisher, D.E. (1998). Regulation of proliferation-survival decisions during tumor cell hypoxia. Molecular and Cellular Biology *18*, 2845–2854.

Schneider, C., King, R.M., and Philipson, L. (1988). Genes specifically expressed at growth arrest of mammalian cells. Cell *54*, 787–793.

Sherr, C.J., and Roberts, J.M. (1995). Inhibitors of mammalian G1 cyclin-dependent kinases. Genes & Development *9*, 1149–1163.

Shin, Y.-J., Han, S.-H., Kim, D.-S., Lee, G.-H., Yoo, W.-H., Kang, Y.-M., Choi, J.-Y., Lee, Y.C., Park, S.J., Jeong, S.-K., et al. (2010). Autophagy induction and CHOP under-expression promotes survival of fibroblasts from rheumatoid arthritis patients under endoplasmic reticulum stress. Arthritis Research & Therapy *12*, R19.

Shugart, E.C., Umekt, R.M., and Constance, C.M. (1995). Arrest Points during Adipocyte. Cell Growth and Differentiation *6*, 1541–1547.

Sok, J., Wang, X.Z., Batchvarova, N., Kuroda, M., Harding, H., and Ron, D. (1999). CHOP-Dependent stress-inducible expression of a novel form of carbonic anhydrase VI. Molecular and Cellular Biology *19*, 495–504.

Stark, G., and Taylor, W. (2004). Analyzing the G2/M Checkpoint. In Checkpoint Controls and Cancer SE - 2, A. Schönthal, ed. (Humana Press), pp. 51–82.

Starostina, N.G., and Kipreos, E.T. (2012). Multiple degradation pathways regulate versatile CIP/KIP CDK inhibitors. Trends in Cell Biology *22*, 33–41.

Stiehl, D.P., Wirthner, R., Köditz, J., Spielmann, P., Camenisch, G., and Wenger, R.H. (2006). Increased prolyl 4-hydroxylase domain proteins compensate for decreased oxygen levels. Evidence for an autoregulatory oxygen-sensing system. The Journal of Biological Chemistry *281*, 23482–23491.

Su, N., and Kilberg, M.S. (2008). C/EBP homology protein (CHOP) interacts with activating transcription factor 4 (ATF4) and negatively regulates the stress-dependent induction of the asparagine synthetase gene. The Journal of Biological Chemistry *283*, 35106–35117.

Suh, E.J., Remillard, M.Y., Legesse-Miller, A., Johnson, E.L., Lemons, J.M., Chapman, T.R., Forman, J.J., Kojima, M., Silberman, E.S., and Coller, H. a (2012). A microRNA network regulates proliferative timing and extracellular matrix synthesis during cellular quiescence in fibroblasts. Genome Biology *13*, R121.

Takiguchi, M. (1998). Focus on hepatology in Japan The C / EBP family of transcription factors in the liver and other organs. International Journal of Experimental Pathology 369–391.

Tang, Q.Q., and Lane, M.D. (2000). Role of C/EBP homologous protein (CHOP-10) in the programmed activation of CCAAT/enhancer-binding protein-beta during adipogenesis. Proceedings of the National Academy of Sciences of the United States of America *97*, 12446–12450.

Tvrdik, D., Djaborkhel, R., Nagy, A., Eckschlager, T., Raska, I., and Müller, J. (2002). Cyclin D–cdk6 complex is targeted by p21WAF in growth-arrested lymphoma cells. Journal of Structural Biology *140*, 49–56.

Ubeda, M., and Habener, J.F. (2003). CHOP transcription factor phosphorylation by casein kinase 2 inhibits transcriptional activation. The Journal of Biological Chemistry *278*, 40514–40520.

Ubeda, M., Wang, X., Zinszner, H., Wu, I., Habener, J.F., and Ron, D. (1996). Stress-Induced Binding of the Transcription Factor CHOP to a Novel DNA Control Element. Molecular and Cellular Biology *16*, 1479–1489.

Ubeda, M., Vallejo, M., and Habener, J.F. (1999). CHOP enhancement of gene transcription by interactions with Jun/Fos AP-1 complex proteins. Molecular and Cellular Biology *19*, 7589–7599.

Wang, X., and Ron, D. (1996). Stress-Induced Phosphorylation and Activation of the Transcription Factor CHOP (GADD153) by p38 MAP Kinase. Science *272*, 1347–1349.

Wang, X.Z., Kuroda, M., Sok, J., Batchvarova, N., Kimmel, R., Chung, P., Zinszner, H., and Ron, D. (1998). Identification of novel stress-induced genes downstream of chop. The EMBO Journal *17*, 3619–3630.

Wei, W., Yang, H., Cao, P., Menconi, M., Chamberlain, C., Petkova, V., and Hasselgren, P.O. (2006). Degradation of C/EBPbeta in cultured myotubes is calpain-dependent. Journal of Cellular Physiology *208*, 386–398.

Wei, W., Yang, H., Menconi, M., Cao, P., Chamberlain, C.E., and Hasselgren, P.O. (2007). Treatment of cultured myotubes with the proteasome inhibitor beta-lactone increases the expression of the transcription factor C/EBPbeta. American Journal of physiology.Cell Physiology *292*, C216–26.

Wethmar, K., Smink, J.J., and Leutz, A. (2010). Upstream open reading frames: Molecular switches in (patho)physiology. BioEssays *32*, 885–893.

Williams, G.H., and Stoeber, K. (2012). The cell cycle and cancer. The Journal of Pathology *226*, 352–364.

Williams, S.C., Cantwell, C. a, and Johnson, P.F. (1991). A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. Genes & Development *5*, 1553–1567.

Zetterberg, A., Larsson O., Wiman, K. (1995). What is the restriction point? Current Opinion in Cell Biology *7*, 835–842.

Zhan, Q., Lord, K. a, Alamo, I., Hollander, M.C., Carrier, F., Ron, D., Kohn, K.W., Hoffman, B., Liebermann, D. a, and Fornace, a J. (1994). The gadd and MyD genes define a novel set of mammalian genes encoding acidic proteins that synergistically suppress cell growth. Molecular and Cellular Biology *14*, 2361–2371.

Zinszner, H., Kuroda, M., Wang, X., Batchvarova, N., Lightfoot, R.T., Remotti, H., Stevens, J.L., and Ron, D. (1998). CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes & Development *12*, 982–995.

Appendix

5.1 Microarray Analysis

Quiescence is a complex genetic program. However, little is known about the genes and pathways regulated during quiescence. Many studies have used microarray data for large scale analysis of thousands of genes to obtain a broader picture of genes that might be repressed or activated during quiescence. For instance, studies by Suh et al., (2012) examined the role of microRNA expression in contact inhibition, serum withdrawl, and proliferation in human fibroblast cells using Microarray analysis. Their analysis provided insight into different microRNAs that regulate gene expression associated with quiescence and proliferation (Suh et al., 2012). Additional Microarray analysis focused on examining downstream targets of miR-22 in proliferative and transfected quiescent fibroblasts (Polioudakis et al., 2013). In these studies, miR-22 was of particular interest because of its role in tumorigenesis (Polioudakis et al., 2013). Other studies used microarray analysis to determine which genes are responsible for maintaining viability in quiescent fibroblasts in response to a clinically approved proteasome inhibitor, MG132. Quiescent fibroblasts cells have acquired a resistance to MG132, while proliferating cells do not survive, and cells numbers rapidly decline (Legesse-Miller et al., 2012). This resistance acquired by quiescence cells to MG132 may contribute to cancer recurrence in multiple myeloma cancer (Legesse-Miller et al., 2012). Microarray data provided information on which molecular mechanisms are activated or repressed in quiescent vs. proliferative fibroblasts after MG132 treatment (Legesse-Miller et al., 2012). Studies by Chen et al. (2012) also identified 35 genes predominantly expressed in quiescent vs. proliferative cells in Human foreskin fibroblasts utilizing microarray data. In their studies they established that many genes highly expressed in quiescent fibroblasts are also involved in proinflammatory mechanisms such

as cytokines, chemokines, matrix metalloproteinases and adhesive molecules (Chen et al., 2012). Microarrray data also provided information on genes that are activated or repressed during contact inhibition induced quiescence in NIH3T3 fibroblasts (Küppers et al., 2010). In summary, microarray studies can enhance our understanding on the complex interplay between signaling pathways and underlying molecular mechanisms that contribute to quiescence.

In our studies of quiescence gene regulation have fostered an interest in conditions that generate this cellular state. Initial Microarray data generated in 2009 by former MSc. Student R. Ghosh demonstrated that RNA from contact inhibited CEF shared a similar gene expression pattern with cells in hypoxia. Thus, new studies utilizing Microarray analysis were performed to further examine gene expression changes or patterns in response to cycling normoxia, contact inhibition, or subconfluent hypoxia conditions.

5.2 **Materials and Methods**

RNA was extracted from chicken embryonic fibroblasts (CEFs) by the Trizol Method (Invitrogen), and sent to the Robarts Research Institute (London, ON) for Microarray analysis. RNA extraction procedure was described in Materials & Methods Section 2.8.1. The interest of Microarray analysis is to compare and contrast gene expression in three conditions: Normoxia cycling, subconfluent hypoxia, and contact inhibition. The computer analysis of gene expression data was performed by former PhD. Student Dr. Maslikowski. Figure 6.1 was contributed by Dr. Maslikowski using VennMaster Software.

5.3 Results

The results obtained from Microarray data provided analysis for approximately 1812 genes. In these data, 1426 genes are in common between normoxia cycling vs. contact inhibition, 713 genes between contact inhibition vs. hypoxia, and 332 genes in hypoxia vs. normoxia cycling.

Microarray data showed that 139/332 (42%) genes in hypoxia are found in contact inhibited/ normoxia cycling conditions (Figure 6.1). However, only 10% of contact inhibited/normoxia cycling genes are found in hypoxia. There were only 13 overlapping genes between all three conditions. These results are summarized in a Venn diagram in Figure 6.1 generated by Dr. Maslikowski using VennMaster Software. Additionally, many overlapping genes present in hypoxia and contact inhibition also seem to be involved in proinflammatory or wound-healing mechanisms such as IL-3, IL-17 receptor, and chemokine K203 among others (data not shown). The microarray data also provided useful information of microRNAs present in quiescent, proliferating or hypoxic CEF. Although the analysis of the microarray data is still preliminary, future studies will validate changes in gene expression levels of various genes present in hypoxia, normoxia cycling or contact inhibition by RT-qPCR.

5.4 Significance

Microarray profiling analysis can expand our current knowledge of gene expression patterns in various conditions. Our results offered a better understanding of overlapping and differentiating transcriptional events that occur during hypoxia, normoxia cycling, or contact inhibition. In addition, our results described the expression of shared gene signatures in hypoxia and contact inhibition with 713 overlapping genes between these cellular states. Future experiments can validate microarray data by RT-qPCR. In summary, deciphering mechanisms induced in quiescence by microarray profiling is important in our studies of gene regulation during this cell state.

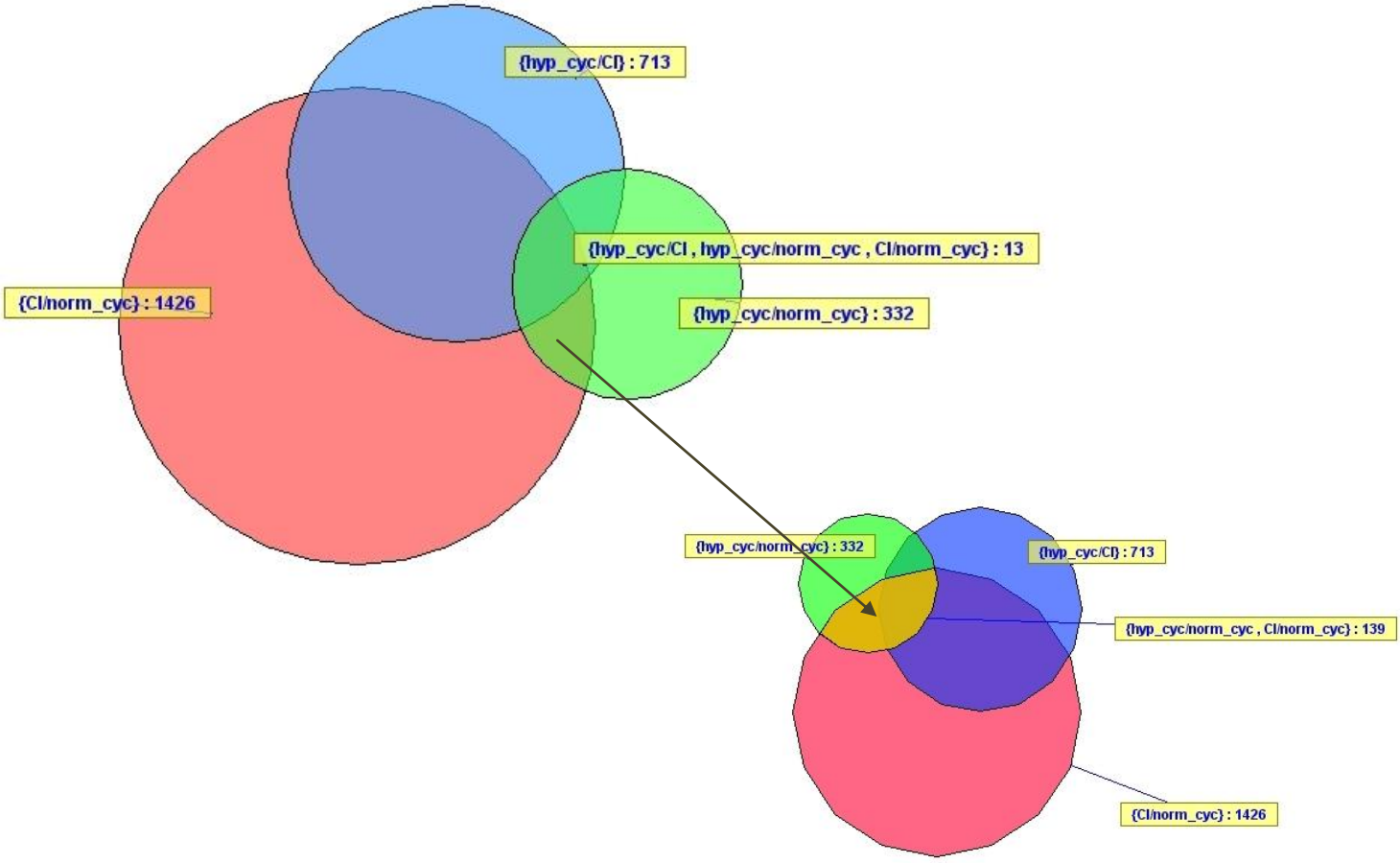


Figure 5.1. Venn diagram. Venn diagram generated by Dr. Maslikowski using VennMaster Software. This Venn diagram depicts the overlap of genes regulated during hypoxia, normoxia cycling, and contact inhibited CEF. Numbers in overlapping areas represent genes in common between each cellular state. **Red** represents contact inhibited compared to normoxia cycling (CI/norm cyc), **Blue** represents subconfluent cells in hypoxia compared to contact inhibition (Hyp cyc/CI), and **Green** represents subconfluent hypoxia compared to normoxic cycling (hyp cyc/norm cyc). The **orange** section in the small diagram represents the number of genes in hypoxia (42%) that are found in contact inhibited/ normoxia cycling conditions.

References

Chen, B.-R., Cheng, H.-H., Lin, W.-C., Wang, K.-H., Liou, J.-Y., Chen, P.-F., and Wu, K.K. (2012). Quiescent fibroblasts are more active in mounting robust inflammatory responses than proliferative fibroblasts. PloS One *7*, e49232.

Ghosh, R. (2009). Characterizing the Role of CCAAT/ Enhancer Binding Protein β on Quiescence-Specific Gene Expression in Chicken Embryo Fibroblasts. Master Thesis, Unpublished Results. McMaster University.

Küppers, M., Ittrich, C., Faust, D., and Dietrich, C. (2010). The transcriptional programme of contact-inhibition. Journal of Cellular Biochemistry *110*, 1234–1243.

Legesse-Miller, A., Raitman, I., Haley, E.M., Liao, A., Sun, L.L., Wang, D.J., Krishnan, N., Lemons, J.M.S., Suh, E.J., Johnson, E.L., et al. (2012). Quiescent fibroblasts are protected from proteasome inhibition-mediated toxicity. Molecular Biology of the Cell *23*, 3566–3581.

Polioudakis, D., Bhinge, A. a, Killion, P.J., Lee, B.-K., Abell, N.S., and Iyer, V.R. (2013). A Myc-microRNA network promotes exit from quiescence by suppressing the interferon response and cell-cycle arrest genes. Nucleic Acids Research *41*, 2239–2254.

Suh, E.J., Johnson, E.L., et al. (2012). Quiescent fibroblasts are protected from proteasome inhibition-mediated toxicity. Molecular Biology of the Cell *23*, 3566–3581.